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Research Article

Isolation and identification of different compounds from *Citrus assamensis* leaf

Mohammad Shahriar*, Mohiuddin Ahmed Bhuiyan and Md. Sohel Rana[1]

Phytochemistry Research Laboratory, Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh

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ABSTRACT

Six coumarins, one benzene, one flavone, one acridone, one limonoid, one triterpene and two phytosterol derivatives were isolated from the methanol, ethanol and chloroform leaf extracts of Citrus assamensis. Extensive spectroscopic studies, including high field ^{1}H NMR and ^{13}C NMR analyses, allowed the identification of thirteen known compounds as bergapten (1), umckalin (2), citropten (3), 4-hydroxybenzaldehyde (4), bergamottin (5), β-amyrin (6), umbeliferone (7), scopoletin (8), citrusinol (9), citracridone-III (10), limonin(11), stigmasterol (12), and β-sitosterol (13). The identity of these compounds was confirmed by comparison with published data as well as co-TLC with authentic samples.

Introduction

The *citrus* genus belongs to the large family Rutaceae which contains 130 genera in the seven subfamilies with many important fruits and essential oil producer: *citrus assamensis,* locally known as Satkora in Bangladesh, is a small tree, moderately branched and thorny plant is used as medicine by local tribes of Assam, India (Shahriar et al., 2018a). *C. assamensis* is a medicinal plant, various parts of which have been traditionally used for treating dysentery, indigestion, pimples, and intestinal worms (Das et al., 2013). Previous phytochemical studies with methanol, ethanol, and chloroform extract of leaves of *C. assamensis*provided alkaloids, phytosterols, phenol, tannin, glycoside, saponin and flavonoids.Literature survey revealed that the *C. assamensis* has *in vitro* antioxidant, antibacterial, anti-diabetic, thrombolytic membrane stabilizing activity as well as *in vivo* anti-diarrheal, anti-inflammatory, anti-tumor, anti-pyretic, anti-nociceptive, neuropharma- cological, gastro-intestinal motility activities without any acute toxicity (Shahriar et al., 2018a,b,c,d).

As part of our continuous studies on *C. assamensis,* the leaves were subjected to chemical investigation. Consequently, isolation and structure elucidation of thirteen known compounds such as bergapten (1), umckalin (2), citropten (3), 4-hydroxyben- zaldehyde (4), bergamottin (5), β-amyrin (6) umbeliferone (7), scopoletin (8), citrusinol (9), citracridone-III (10)

*Corresponding author:<shahriar@uap-bd.edu>

[1]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh

limonin (11), stigmasterol (12) and β-sitosterol (13) were done. This is the first report of the occurrence of these compounds from this plant.

Materials and Methods

Collection, identification and processing of plant samples

Leaves of *C. assamensis* were collected from Jayantapur, Sylhet, Bangladesh and the plant was taxonomically identified with the help of the National Herbarium of Bangladesh, Mirpur, Dhaka (DACB; Accession Number-38759). Leaves were sun dried for seven days. The dried leaves were then ground in coarse powder using high capacity grinding machine (Jaipan Designer Mixer Grinder, India), which was then stored in an air-tight container with necessary markings for identification and kept in a cool, dark, and dry place for further investigation.

Extraction and Isolation

Chopped-dried leaves of *C. assamensis* (1.0 kg) were immersed separately in chloroform and methanol at room temperature (12 liters, twice every 3 days) and then filtered through a cotton plug followed by a Whatman filter paper number 1. The extract was then concentrated by a rota vapor under reduced pressure. After removing the of solvents, a green-brown gum of crude chloroform (38.88 g) and the dark-brown gum of crude methanol (25.58 g) was obtained.

The chloroform extract was further dissolved in hexane to remove wax. The hexane insoluble fraction (13.50 g) was fractionated by quick column chromatography (CC) over silica gel 60H using gradient solvent of 5% acetone in hexane to acetone as eluents. Fractions with a similar characteristic on TLC were combined to afford 12 fractions. Further purification of each fraction separated by column chromatography over Sephadex™ LH-20 and elution with hexane: dichloromethane: acetone (8:1:1) and hexane: dichloromethane: acetone (7.5:1:1.5) respectively yielded four compounds (Fig. 1).

Again the methanol extract (25.58 g) was fractionated by quick column chromatography over silica gel 60H using gradient solvent of 10% acetone in hexane to acetone as eluents. Fractions with a similar characteristic on TLC were combined to obtain 14 fractions. Further purification of each fraction gave three compounds (Fig. 1) upon separation by CC over Sephadex™ LH-20.

Later chopped-dried leaves of *C. assamensis* (2.5 kg) were immersed in ethanol at room temperature (14 liters, twice every 3 days) and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was then concentrated by a rotary evaporator under reduced pressure. After removing the solvent, a dark-brown viscous ethanol extract (31.30 g) was obtained. The methanolic solution of ethanol extract (22.67 g) was fractionated by quick column chromatography over silica gel 60H using gradient solvent of 10% acetone in hexane to acetone. Fractions with a similar characteris- tic on TLC were combined to obtain 16 fractions. The fractions were further separate- ed by CC over Sephadex™ LH-20 and eluted with 50% methanol-dichloromethane to obtain six known compounds (Fig. 1).

The isolated compounds were then characterized by using ^{1}H NMR and ^{13}C NMR spectra recorded on Ultra Shield Bruker 400 and 100 NMR instrument. Deuterated chloroform ($CDCl_3$) was used as a solvent and chemical shift (δ) were reported in ppm using Tetramethylsilane (TMS) as an internal standard or residual non-deuterated solvent signal. All solvents and reagents were of the highest analytical grade.

The compounds obtained were then identified as bergapten (1), umckalin (2), citropten (3), 4-hydroxybenzaldehyde (4), bergamottin (5), β-amyrin (6) umbeliferone (7), scopoletin (8), citrusinol (9), citracridone-III (10), limonin (11), stigmasterol (12) and β-sitosterol (13).

NMR spectroscopic data of the isolated compounds

Bergapten (1): White solid mass (3.0 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 4.28 (s, 5-OCH_3), 6.28 (d, *J* = 9.9, H-3), 7.02 (d, *J* = 2.4, H-3'), 7.14 (s, H-8), 7.61 (d, *J* = 2.4, H-2'), 8.17 (d, *J* = 9.9, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C161.30 (C-2), 112.60 (C-3), 139.70 (C-4), 106.40 (C-4a), 149.70 (C-5), 115.20 (C-6), 158.40 (C-7), 98.40 (C-8), 156.50 (C-8a), 144.90 (C-2'), 105.20 (C-3'), 60.10 (C-5 OCH_3).

Umckalin (2): White solid mass (3.6 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 3.98(3H, s, 6-OCH_3), 4.03(3H, s, 5-OCH_3), 6.26(1H, d, *J*= 9.5 Hz, H-3), 6.40 (1H, s, H-8), 8.00 (1H, d, *J*=9.6Hz, H-4).

Citropten (3): Colorless crystal (2.9 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H3.88 (s, 5-OCH_3), 3.92 (s, 7-OCH_3), 6.19 (d, *J* = 9.6Hz, H-3), 6.31 (d, *J* = 2.1Hz, H-6), 6.45 (d, *J* = 2.1Hz, H-8), 8.00 (d, *J* = 9.6Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C161.30 (C-2), 111.10 (C-3), 138.70 (C-4), 104.10 (C-4a), 162.7 (C-5), 94.90 (C-6), 157.90 (C-7), 92.90 (C-8), 156.80 (C-8a), 55.90 (C-5 OCH_3), 55.80 (C-7 OCH_3).

4-Hydroxybenzaldehyde (4): White solid mass (0.9 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H6.98 (d, *J* = 8.6Hz, H-3 / H-5), 7.81 (d, *J* = 8.4Hz, H-2 / H-6), 6.98 (d, *J* = 8.6Hz, H-1'); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C130.0 (C-1), 132.5 (C-2/6), 116.1 (C-3/C-5), 161.0 (C-4), 190.7 (C-1').

Bergamottin (5): White solid mass (0.9 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H1.59 (s, H-9''), 1.68 (s, H-8''), 1.69 (s, H-10''), 2.9 (br. s, H-5''), 2.10 (br. s, H-4''), 4.95 (d, *J* = 6.9Hz, H-1''), 5.06 (br. S, H-6''), 5.52 (d, *J* = 6.7Hz, H-2''), 6.26 (d, *J* = 9.8Hz, H-3), 6.95 (d, *J* = 2.4Hz, H-3'), 7.10 (s, H-8), 7.55 (d, *J* = 2.4Hz, H-2'), 8.16 (d, *J* = 9.8Hz, H-4).

β-amyrin (6): White crystalline powder mass (14.3 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 0.83 (s, H-29), 0.91 (s, H-25), 1.11 (s, H-27), 1.32 (Ha-6, H-6), 1.48 (Ha-1, H-1), 1.52 (Hb-6, H-6), 1.55 (Ha-2, H-2), 1.67 (H-21), 1.86 (H-11), 1.94 (H-9), 3.24 (dd, *J*=4.44, 11.6 Hz, H-3), 5.25 (t, *J*=3.2 Hz, H-12) ppm.

Umbelliferone (7): White solid mass (2.8 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H6.19 (d, *J* = 9.6Hz, H-3), 6.79 (d, *J* = 2.1Hz, H-8), 6.83 (d, *J* = 8.4, 2.1Hz, H-6), 7.26 (d, *J* = 9.6Hz, H-4), 7.33 (d, *J* = 8.4Hz, H-5), 9.82 (s, 7-OH).^{13}C-NMR (100 MHz, $CDCl_3$): δ_C160.60 (C-2), 113.40 (C-3), 143.80 (C-4), 111.50 (C-4a), 128.80 (C-5), 111.60 (C-6), 161.40 (C-7), 103.10 (C-8), 155.80 (C-8a).

Scopoletin (8): Crystalline solid mass (16.4 mg);^{1}H-NMR (400 MHz, $CDCl_3$): δ_H3.92 (3H, s, C-6-OMe), 6.22 (1H, d, *J* = 9.2 Hz, H-3), 6.79 (1H, s, H-8), 7.13 (1H, s, H-5), 7.89 (1H, d, *J* = 9.2 Hz, H-4).

Citrusinol (9): Yellow solid mass (2.4 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H1.48 (s, H-12), 5.79 (d, *J* = 9.9Hz, H-10),6.26 (s, H-6), 6.73 (d, *J* = 9.9Hz, H-9), 7.06 (d, *J* = 9.0Hz, H-3', 5'), 8.22 (d, *J* = 9.0Hz, H-2', 6'), 9.13 (s, 4'-OH), 12.32 (s, 5-OH); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C145.3(C-2), 136.1(C-3), 175.6(C-4), 101.1(C-4a), 160.8(C-5), 98.8(C-6), 159.7(C-7), 103.9(C-8), 150.9(C-8a), 115.5(C-9), 127.5(C-10), 78.1(C-11), 28.4(C-1'), 122.6(C-12), 129.6(C-2', 6'), 115.7(C-3', 5'), 159.4 (C-4').

Citracridone-III (10): Yellow solid mass (0.9 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.52 (s, H-4' / H-5'), 3.83 (s, N-CH_3), 5.53 (d, *J* = 9.0Hz, H-2'), 6.18 (s, H-2), 6.70 (d, *J* = 9.0Hz, H-1'), 6.97 (d, *J* = 9.0Hz, H-7), 7.84 (d, *J* = 9.9Hz, H-8), 9.63 (s, 5-OH), 14.53 (s, 1-OH; ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 158.9 (C-1), 97.5 (C-2), 156.2 (C-3), 101.7 (C-4), 143.0 (C-4a), 129.8 (C-5), 145.2 (C-6), 118.8 (C-7), 111.7 (C-8), 113.1 (C-8a), 176.8 (C-9), 107.3 (C-9a), 132.9 (C-10a), 116.5 (C-1), 113.1 (C-2), 96.5 (C-3), 26.7 (C-4/C-5), 44.5 (N-CH_3).

Limonin (11): White solid mass (2.6 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.08 (s, H-24), 1.15 (s, H-H-26), 1.16 (s, H-18), 1.25 (s, H-25), 1.51 (m, H-12), 1.77 (m, H-11), 1.81 (m, H-12), 1.86 (m, H-11), 2.30 (dd, *J* = 15.0, 3.0Hz, H-2), 2.45 (dd, *J* = 12.0, 3.0Hz, H-5), 2.57 (dd, *J* = 9.0, 3.0Hz, H-9), 2.70 (dd, *J* = 15.0, 3.0Hz, H-2), 2.74 (dd, *J* = 12.0, 3.0Hz, H-6), 3.17 (dd, *J* = 12.0, 3.0Hz, H-6), 4.06 (s, H-15), 4.09 (br. S, H-1), 4.50 (d, *J* = 12.0Hz, H-19), 4.82 (d, *J* = 12.0Hz, H-19), 5.48 (s, H-17), 6.36 (d, *J* = 1.5Hz, H-22), 7.43 (s, H-21), 7.47 (d, *J* = 1.5Hz, H-23); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 83.9 (C-1), 41.1 (C-2), 173.3 (C-3), 82.7 (C-4), 64.8 (C-5), 42.7 (C-6), 211.4 (C-7), 55.8 (C-8), 52.6 (C-9), 50.6 (C-10) , 23.7 (C-11), 34.2 (C-12), 43.5 (C-13), 70.7 (C-14), 57.6 (C-15), 171.7 (C-16), 83.8 (C-17), 21.3 (C-18), 70.0 (C-19), 124.8 (C-20), 147.9 (C-21), 113.5 (C-22), 145.8 (C-23), 21.3 (C-24), 34.8 (C-25), 24.2 (C-26).

Stigmasterol (12): White crystal mass (2.3 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H0.66 (3H, s, H-18), 0.71 (3H, d, *J*= 7.6 Hz, H-26), 0.91 (d, *J*= 6.4 Hz, H-21), 1.02(3H, s, H-19), 3.90 (m, H-3), 4.97 (1H, m, H-22), 5.12 (1H, m, H-23), 5.37 (br. S, H-6).

β-Sitosterol (13): White waxy powder mass (0.5 mg); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 0.73 (d, 3H, H-19), 0.97 (d, 3H, H-27), 1.05 (t, 3H, H-29), 1.22 (d, 3H, H-21), 1.29 (d, 3H, H-18), 3.53 (m, H, H-3), 5.18 (m, 1H, H-23), 5.38 (S, 1H, H-6).

Results and Discussion

A total of thirteen compounds (1-13) were isolated from the leaves of *C. assamensis* by gel permeation chromatography over lipophilic Sephadex™ LH-20 followed by preparative thin layer chromatography (PTLC) using silica gel (Kiselgel F254). The structure of the isolated compounds was solved by extensive analyses of their high resolution. ^{1}H and ^{13}C NMR spectroscopic data as well as by comparison with published values. The structures are shown in Fig. 1.

The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound 1 showed an AB-type doublet of the *α*- and *β*-olefinic protons of coumarin system at δ_H 6.28 and δ_H 8.17 (*J* = 9.9 Hz), a singlet of aromatic proton H-8 at δ_H 7.14, and singlet of methoxy group at δ_H 4.28 (5-OMe). The resonances at δ_H 7.02 (*d*) and δ_H 7.61 (*d*) with a coupling constant of 2.4 Hz were assigned for the olefinic protons H-3' and

H-2' of furan ring. Compound 1 was identified as 5-methoxy-2H-furo [3,2-*g*] chromen-2-one (bergapten). The ^{13}C-NMR spectrum of compound 1 exhibited 12 carbon resonances including five methines appearing at δ_C 112.60 (C-3), 139.70 (C-4), 98.40 (C-8), 144.90 (C-2'), 105.20 (C-3'); one methoxy appearing at δ_C 60.10; one carbonyl appearing at 161.30 (C-2) as well as signal at 106.40 (C-4a), 149.70 (C-5), 115.20 (C-6), 158.40 (C-7), 156.50 (C-8a) ascribed for five quaternary carbons. Comparing with published data, the compound [Fig. 1(1)] was identified as bergapten (Chunyan et al., 2009; Aloui et al., 2015; Murakami et al., 1999).

Compound 2 was identified as 5,6-dimethoxy-7-hydroxycoumarin having characteristic signal of δ_H 6.26 (*d*, *J* = 9.5 Hz) and δ_H 8.00 (*d*, *J* = 9.6 Hz) respectively, and two methoxyl groups at δ_H 4.03 (5-OMe) and δ_H 3.98 (6-OMe). Thus, the structure of compound 2 was solved as umckalin [Fig. 1(2)], which as further supported by comparing its ^{1}H NMR spectral data with previously reported values (Meselhy, 2013).

Compound 3 was obtained as a white solid mass with a melting point of 224-226 ^{0}C. The ^{1}H-NMR spectrum showed the characteristic signal of *α*- and *β*- olefinic protons of coumarin at δ_H 8.00 and δ_H 6.19 (*d*, *J* = 9.9 Hz), meta-aromatic protons with *J* = 2.1 Hz at δ_H 6.45 (H-8) and δ_H 6.31 (H-6), and two methoxy groups at δ_H 3.88 (5-OMe) and δ_H 3.92 (7-OMe). This compound was identified as 5,7-dimethoxy-2H-chromen-2-one, which was known as citropten [Fig. 1(3)] (Murakami et al., 1999). The ^{13}C-NMR spectrum exhibited 11 carbon resonances including four methines appear at δ_C 111.10 (C-3), 138.70 (C-4), 94.90 (C-6), 92.90 (C-8); two methoxy appears at δ_C 55.90 and δ_C55.80; one carbonyl at 161.3 (C-2) as well as signal at 104.10 (C-4a), 162.70 (C-5), 157.90 (C-7) and 156.8 (C-8a) ascribed to four quaternary carbons.

The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound 4 showed characteristic signals of para-di-substituted benzene at δ_H 7.81 (d, *J* = 8.4 Hz, H-2/H-6), δ_H 6.98 (d, *J* = 8.6 Hz, H-3/H-5). One of the substituents was assigned to a formyl group and its proton resonated at δ_H 9.88 (s, CHO), and its carbonyl carbon resonated at δ_H 190.70. The other substituent was suggested to be a hydroxyl group from the resonance of an oxy-carbon signal at δ_H 161.0. It was then identified as 4-hydroxybenzaldehyde [Fig. 1(4)]. Its spectroscopic data were in agreement with previously reported data (Chan et al., 2017).

Compound 5 was obtained as a white solid mass. The ^{1}H NMR spectrum showed an AB-type doublet of *α*- and *β*-olefinic protons of coumarin at δ_H 6.26 and δ_H 8.16 (*J* = 9.8 Hz) and a singlet of aromatic proton H-8 at δ_H 7.10. The presence of the furan ring was proposed from the doublet olefinic protons with *J* = 2.4 Hz, at δ_H 7.55 (H-2') and δ_H 6.95 (H-3'). Compound 5 then was identified as 4-(3,7-dimethylocta-2,6-dienoxy) furo [3,2- g] chromen-7-one, which spectral features are in close agreement with published data for bergamottin [Fig. 1(5)] (Murakami et al., 1999).

The ^{1}H-NMR spectrum of compound 6 showed the characteristic presence of three methyl singlet's, one olefinic proton at δ_H 5.25 (t, *J*= 3.5 Hz) and an oxygenated proton at δ_H 3.24 (dd, *J*= 4.4, 11.6 Hz), all suggestive of olealane type triterpenoid. This compound was identified as 3β-hydroxylolean-12-ene (β-amyrin) and the spectral data compared well with the previously reported spectroscopic data of β-amyrin [Fig. 1(6)] (Okoye et al., 2014).

The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound **7** showed characteristic AB-type doublet of α - and β -olefinic protons at δ_H 6.19 and δ_H 7.26 (J = 9.6 Hz), indicating that it was a coumarin. The ABX-type signals of aromatic protons H-5, H-6 and H-8 at δ_H 7.33 (*d*, *J*=8.4Hz), δ_H 6.83 (*dd*, *J*=8.4,2.1Hz) and δ_H 6.79 (*d*, *J*=2.1Hz), respectively, are attributed to the tri-substituted benzene ring. This compound was identified as 7-hydroxychromen-2-one. The ^{13}C-NMR spectrum exhibited 9 carbon resonances including five methines appearing at 113.40 (C-3), 143.80 (C-4), 128.80 (C-5), 111.60 (C-6), 103.10 (C-8); one carbonyl appears at 160.60 (C-2) as well as a signal at 111.50 (C-4a), 161.40 (C-7), 155.80 (C-8a) ascribed to three quaternary carbons. Therefore, according to these findings and comparing with previously reported data (Gao et al., 2002; Bhatt et al., 2011), compound **7** was identified as umbeliferone [Fig. 1(7)].

Compound 8 was obtained as a crystalline solid mass. Its ^{1}H NMR spectrum displayed the characteristic signal of two doublets with a coupling constant of 9.2 Hz at δ_H 6.22 and δ_H 7.89, which were assigned to H-3 and H-4, respectively. The spectrum also showed a methoxy group singlet at δ_H 3.928 and an aromatic singlet at δ_H 7.13, respectively. On this basis, compound 8 was characterized as scopoletin [Fig. 1(8)] and its identity was further confirmed by comparing its ^{1}H-NMR spectral data to that of the previously reported values (Bhatt et al., 2011).

Compound 9 was obtained as a yellow solid mass with a melting point 253-254 ^{0}C. The ^{1}H NMR spectrum acquired in $CDCl_3$ showed the presence of a para-substituted B ring (δ_H 8.22, H-2'/H-6', d, J = 9.0 Hz; δ_H 7.06, H-3'/H-5', d, J = 9.0 Hz), a hydroxyl group (δ_H 9.13, 4-OH), a chelated hydroxyl group (δ_H 12.32, 5-OH), a singlet aromatic proton (δ_H 6.26, H-6) and 2,2-dimethylchromene ring (δ_H 1.48, *s*, H-12; δ_H 6.73, d, J = 9.9 Hz, H-9; δ_H 5.79, d, J = 9.9 Hz, H-10) as for compound 9. The absence of a characteristic singlet of flavones proton H-3 (δ_H 6.57) and the carbon signal of C-3 appearing at a lower field (δ_H 136.1) are in agreement with the flavanol structure. Compound 9 was identified as 4H,8H-benzo[1,2-*b*:3,4-*b'*] dipyran-4-one,3,5-dihydroxy-2-(4-hydroxyphenyl)-8,8-dimethyl-pyrano[2,3-*f*] chromen-4-one. The assignment and spectroscopic data were in agreement with those of citrusinol [(Fig. 1(9)] (Wu et al., 1993).

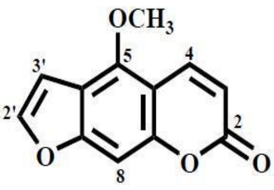
The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound 10 exhibited the characteristic signal of a chelated hydroxyl proton (1-OH) and N-methyl proton of acridone skeleton at δ_H 14.54 and δ_H 3.81, respectively. Furthermore H-6 showed correlation to oxy-carbon which resonated at δ_H 145.2 whereas H-7 correlated to oxy-carbon which resonated at δc129.8 (C-5), suggesting that δc145.2 and δc 129.8 belonged to C-6 and C-5, respectively, and the substituents at C-6 and C-5 were hydroxyl groups. A hydroxyl group that resonated at δ_H 9.63 could belong to 6-OH or 5-OH. The presence of 2,2-dimethylchromene ring was suggested from the resonance of methyl proton at δ_H 1.52(H-4'/H-5', 6H) and cis-olefinic proton (J = 9.0 Hz) at δ_H 6.70 (d, H-1') and δ_H 5.53 (H-2'). Compound 10 was then identified as 1,5,6-trihydroxy-10,3',3'-trimethyl-3,12-dihydro-3H-pyrano [2,3-c] acridin-9-one, which was known as citracridone-III and confirmed by comparison with published data [Fig. 1(10)] (Taufiq-Yap et al., 2007).

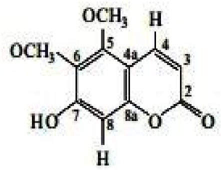
The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound 11 suggested the presence of substituted furan from a singlet of H-21 at δ_H 7.43 (s) and doublets of H-23 at δ_H 7.47 and H-22 at δ_H 6.36 associated with a coupling constant value of 1.5 Hz. It was further established that compound 11 was a limonoid with four of singlet methyl groups at δ_H 1.25 (H-25), 1.16(H-18), 1.15 (H-26) and at δ_H 1.08 (H-24). The presence of an epoxy lactone moiety of limonoid was revealed by the signals of carbonyl carbon at δc 171.7 (C-16), oxy carbon at δc 83.8 (C-17) and epoxy carbon at δc 57.6 (C-15) and δc 70.7 (C-14) together with the characteristic H-15 and H-17 singlet signal at δ4.06 and δ5.48, respectively. This compound was identified as 7,16-dioxo-7,16-dideoxylimondiol, known as limonin, and was confirmed by comparison with published data [Fig. 1(11)] (Gao et al., 2002).

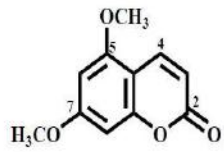
The ^{1}H-NMR spectrum of compound 12 showed the presence of two methyl singlets at δ_H 0.66, and δ_H 1.02; two methyl doublets at δ_H 0.71, and δ_H 0.91, respectively. This compound also showed protons at δ_H 4.97 (m), 5.12 (m), and 5.37 (s), suggesting the presence of three protons corresponding to that of a di-substituted and a tri-substituted olefinic bond. A multiplet at δ_H 3.90 indicated the proton corresponding to the H-3 of a sterol moiety. The assignment and spectroscopic data were in agreement with published data for stigmasterol [Fig. 1(12)] (Pierre and Moses, 2015).

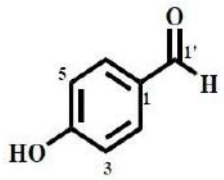
The ^{1}H NMR spectrum of compound 13 displayed that chemical shift varied between δ_H 0.73 to 5.38. This spectrum showed the presence of 4 high intensity peaks, indicating the presence of methyl doublets at δ_H 0.73, 0.97, 1.22 and 1.29, respectively. The ^{1}H NMR showed the proton of H-3 appeared as a multiplet at δ_H 3.53 and revealed the existence of signals for olefinic proton. The assignment and spectroscopic data were in agreement with those of β-sitosterol [Fig. 1 (13)] (Pierre and Moses, 2015).

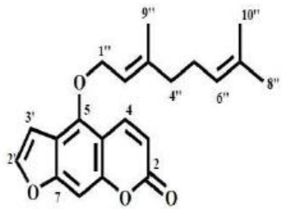
Summarizing, six cumarin derivatives (bergapten, umckalin, citropten, bergamottin, umbeliferone and scopoletin), 4-hydroxyben-zaldehyde, citrusinol, citracridone-III, limonin, β-amyrin, and two phytosterol derivatives (stigmasterol and β-sitosterol) were isolated from the leaf extracts of *C. assamensis* and their isolates are reported for the first time from any part of this plant. As this is the first attempt of any phytochemical investigation from *C. assamensis* in Bangladesh, further isolation and purification of other compounds from other plant fractions are necessary. These could yield some novel and bioactive compounds.

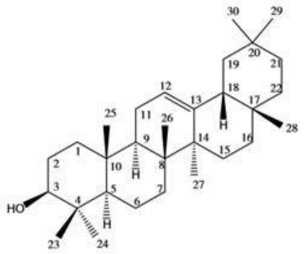

Bergapten (1)

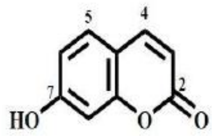

Umckalin (2)

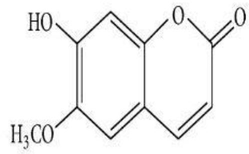

Citropten (3)

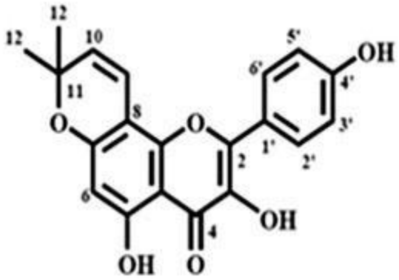

4-Hydroxybenzaldehyde (4)

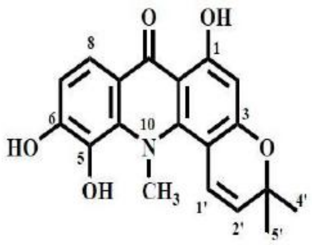

Bergamottin (5)

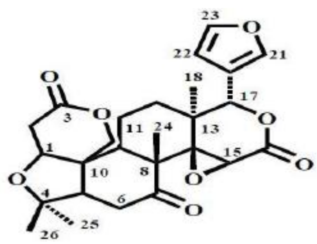

β-amyrin (6)

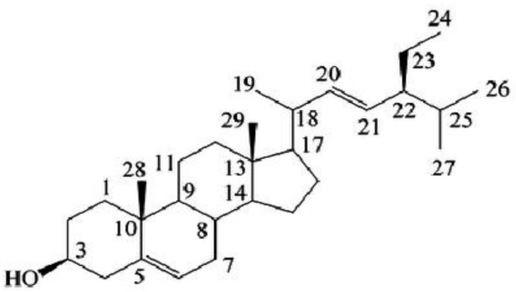

Umbelliferone (7)


Scopoletin (8)


Citrusinol (9)


Citracridone-III (10)


Limonin (11)


Stigmasterol (12)

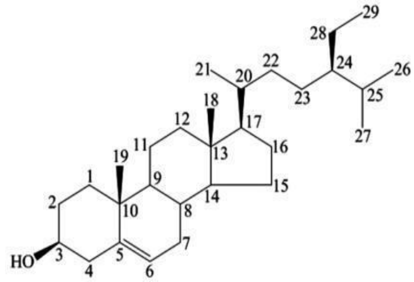

β-Sitosterol (13)

Fig. 1. Compounds isolated from the leaves of *C. assamensis.*

References

Aloui L, Kossentini M, Geffroy-Rodier C, Guillard J and Zouari S. Phytochemical investigation, isolation and characterization of coumarins from aerial parts and roots of tunisian *Pituranthos Chloranthus* (apiaceae). *Pharmacog. Commun.* 2015; 5: 237-243.

Bhatt MK, Dholwani KK and Saluja AK. Isolation and structure elucidation of scopoletin from *ipomoea reniformis* (convolvulaceae). *J. Appl. Pharm. Sci.* 2011; 1: 138-144.

Chan YY, Hwang TL, Kuo PC, Hung HY and Wu TS. Constituents of the fruits of *citrus medica* l. var. sarcodactylis and the effect of 6,7-dimethoxy-coumarin on superoxide anion formation and elastase release. *Molecules* 2017; 22: 1454.

Chunyan C, Bo S and Ping L. Isolation and purification of psoralen and bergapten from *ficus carica* leaves by high-speed counter current chromatography. *J. Liq. Chrom. Rel. Technol.* 2009; 32: 136-143.

Das AJ, Kumar R, Athar M, Rawat DS, Kumar M, Khan MA and Prakash J. Ethno-medicinal study of threatened plants of sonitpur district in assam, North East India. *Int. Res. J. Pharm.* 2013; 4:46-149.

Gao Y, Huang H, Xu H, Diao Y and Dong Z. Studies on the chemical constituents of *citrus medica* var. sarcodactylis. *J. Chin. Med. Mat.* 2002; 25: 639-640.

Meselhy KM. Study of coumarin content of *pelargonium fragrans* - willd root grown in Egypt. *Life Sci. J.* 2013; 10: 159-164.

Murakami A, Gao G, Kim OK, Omura M, Yano M, Ito C, Furukawa H, Jiwajinda S, Koshimiju K and Ohigashi H. Identification of coumarins from the fruit of *citrus hystrix* dc as inhibitors of nitric oxide generation in mouse macrophage RAW 264.7 Cells. *J. Agric. Food Chem.* 1999; 47: 333-339.

Okoye NN, Ajaghaku DL Okeke HN, Ilodigwe EE, Nworu CS and Okoye FB. Beta-amyrin and alpha-amyrin acetate isolated from the stem bark of *alstonia boonei* display profound anti-inflammatory activity. *Pharm. Biol.* 2014; 52: 1478-1486.

Pierre LL and Moses MN. Isolation and characterization of stigmasterol and β-sitosterol from *odontonema strictum* (acanthaceae). *J. Innov. Pharm. Biol. Sci.* 2015; 2: 88-95.

Shahriar M, Bhuiyan MA and Rana MS. Characterization of phytoconstituents and exploration of antioxidant and free radical scavenging activities of *Citrus assamensis* leaf. *Dhaka Univ. J. Pharm. Sci.* 2018a; 17: 29-36.

Shahriar M, Bhuiyan MA and Rana MS. Exploration of anti-diabetic, anti-diarreal, gastrointestinal motility and acute toxicity of different extracts of *Citrus assamensis* leaf. *J. Bangladesh. Aca. Sci.* 2018b; 42: 111-120.

Shahriar M, Bhuiyan MA and Rana MS. Screening of antibacterial, thrombolytic, membrane stabilizing, anti-inflammatory and antitumor activity of *Citrus assamensis* leaf extracts. *J. Sci. Res.* 2018c; 10: 195-210.

Shahriar M, Bhuiyan MA and Rana MS. Study of antinociceptive, antipyretic and neuropharma-cological activities of leaf extracts of *Citrus assamensis*. *Bangladesh. Pharm. J.* 2018d; 21: 87-95.

Taufiq-Yap YH, Peh TH, Ee GCL, Rahmani M, Sukari MA, Ali AM and Muse R. A new cytotoxic carbazole alkaloid from *Clausena excavata*. *Nat. Prod. Res.* 2007; 21: 810-813.

Wu TS, Huang SC, Lai JS, Teng CM, Ko FN and Kuoh CS. Chemical and antiplatelet aggregative investigation of the leaves of *Clausena excavate*. *Phytochem.* 1993; 32: 449-451.