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Flavones and flavone glycosides from *Halophila johnsonii*

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ABSTRACT

Halophila johnsonii Eiseman is a shallow-water marine angiosperm which contains UV-absorbing metabolites. Studies on methanol extracts of *H. johnsonii* by means of HPLC–UV, NMR, HPLC–MS resulted in isolation and identification of seven previously unknown flavone glycosides: 5,6,7,3′,4′,5′-hexahydroxyflavone-7-*O*-β-glucopyranoside (**1**), 5,6,7,3′,4′,5′-hexahydroxyflavone-7-*O*-(6″-*O*-acetyl)-β-glucopyranoside (**2**), 6-hydroxyluteolin-7-*O*-(6″-*O*-acetyl)-β-glucopyranoside (**3**), 6-hydroxyapigenin-7-*O*-(6″-*O*-acetyl)-β-glucopyranoside (**4**), 6-hydroxyapigenin-7-*O*-(6″-*O*-[E]-coumaroyl)-β-glucopiranoside (**5**), 6-hydroxyapigenin-7-*O*-(6″-*O*-[E]-caffeoyl)-β-glucopyranoside (**6**) and 6-hydroxyluteolin-7-*O*-(6″-*O*-[E]-coumaroyl)-β-glucopyranoside (**7**). Also isolated were three known flavone glycosides, 6-hydroxyluteolin 7-*O*-β-glucopyranoside (**8**), scutellarein-7-*O*-β-glucopyranoside (**9**), and spicoside (**10**), and five known flavones, pedalitin (**11**), ladanetin (**12**), luteolin (**13**), apegenin (**14**) and myricetin (**15**). Qualitative comparison of the flavonoid distribution in the leaf and rhizome-root portions of the plant was also investigated, with the aim of establishing the UV-protecting roles that flavonoids played in the sea grass.

1. Introduction

Halophila johnsonii (Hydrocharitaceae) is a threatened marine angiosperm (US Federal Register, 1998) endemic to south-eastern Florida coastal lagoons (Eiseman and McMillan, 1980). *H. johnsonii* coexists with *H. decipiens* subtidally, to depths of 3 m, but only *H. johnsonii* also occurs intertidally. Previous field studies have shown that *H. johnsonii* may have higher ultraviolet (UV) light tolerance compared to *H. decipiens* (Durako et al., 2003). Examination of acetone extracts of the leaves of both species established a strong UV absorption at 345 nm in *H. johnsonii* that was not observed in similar extracts of *H. decipiens* (Durako et al., 2003). Experimental studies found that *H. johnsonii* adjusts to varying levels of UV radiation by varying the levels of UV-absorbing compounds, while *H. decipiens* did not produce such compounds (Durako et al., 2003; Kunzelman et al., 2005).

The production of UV-absorbing compounds in submerged plants has been known for decades (Trocine et al., 1981; Dawson and Dennison, 1996; Sinha et al., 1998). Based on the observation that the deeper-water *H. decipiens* does not appear to contain photo protection compounds, whereas the shallow-water *H. johnsonii* can adjust to alterations in UV exposure (Durako et al., 2003), we reasoned that it may utilize natural sunscreens as a means of photo protection. To our knowledge, the chemical constituents of *H. johnsonii* have not been investigated before, and here we report on the isolation and structural identification of fifteen flavonoids from whole plant extracts of *H. johnsonii* that likely play a UV-protecting role in the plant. We also qualitatively compared the flavone and flavone glycoside contents in *H. johnsonii* leaves versus the below-ground rhizome and roots to provide more evidence for their UV-protecting role in this sea grass.

2. Results and discussion

HPLC analysis of methanol extracts of the whole plant of *H. johnsonii* showed the presence of a number of flavonoids, which were readily recognized by the especially characteristic strong absorption at 340–350 nm (Lu and Foo, 2000) observed by the diode array UV detector. The profile of the flavonoid components in the methanol extract of *H. johnsonii* monitored at the UV wavelength of 340 nm is shown in Fig. 1. Of these, compounds **1**–**15** were isolated and purified by means of Combiflash chromatography and HPLC methods. Spectroscopic analyses of **1**–**15** by NMR, HRMS and LC–MS analysis established that **1**–**7** are previously unknown flavone glycosides, **8**–**10** are known flavone glycosides and **11**–**15** are known flavones.

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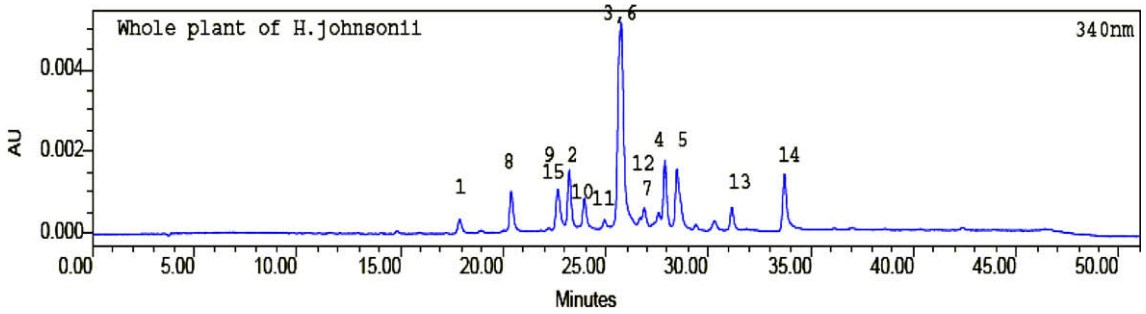
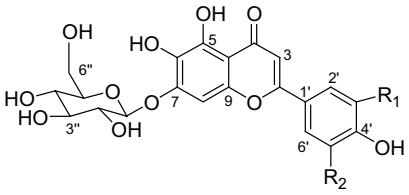
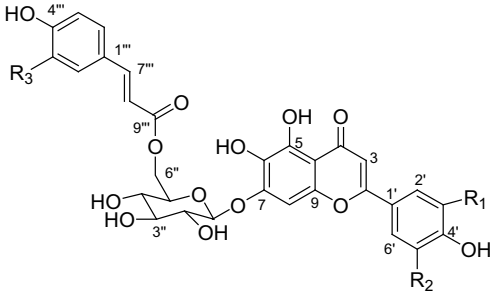


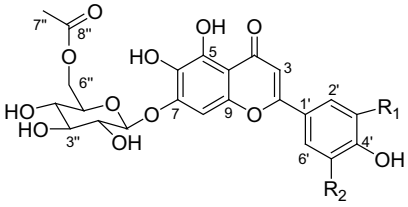
Fig. 1. The HPLC–UV chromatogram of the methanol extract of *Halophila johnsonii*.



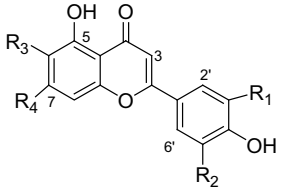
1. R_1= R_2 = OH
8. R_1=OH; R_2=H
9. R_1=R_2=H



5. R_1 = R_2 = R_3 = H
6. R_1 = R_2 = H; R_3 = OH
7. R_1=OH; R_2= R_3= H
10. R_1= R_3 = OH; R_2=H



2. R_1= R_2= OH
3. R_1 = OH; R_2 = H
4. R_1 = R_2 = H



11. R_1 = R_3= OH ; R_2 = H ; R_4 = OCH_3
12. R_1 = R_2 = H ; R_3 = OH , R_4 = OCH_3
13. R_1 = R_4 = OH ; R_2 = R_3 = H
14. R_1 = R_2 = R_3 = H ; R_4 = OH
15. R_1 = R_2 = R_3 = R_4 = OH

Compound **1** was obtained as a pale yellow solid. The UV spectral data showed three absorption bands at 222, 283 and 341 nm, indicating it might be a 6-hydroxylated flavone derivative (Harborne and Williams, 1971). The molecular formula was established as $C_{21}H_{20}O_{13}$ based on the HRMS ESI$^+$ data ([M+1]$^+$ 481.0979) and NMR data. The LC–MS ESI$^+$ showed a fragment ion at *m*/*z* 319 [M-162], confirming 1 as a glycoside composed of a hexahydroxyflavone aglycone and a sugar moiety with a molecular weight of 162 Da. The LC–MS ESI$^+$ data also showed a fragment ion at *m*/*z* 151, corresponding to a trihydroxylated C ring moiety ($C_6H_5O_3$) containing C2 and C3, in an established fragmentation pattern for flavone glycosides (Es-Safi et al., 2005). The ^{1}H NMR spectrum of 1 showed four aromatic protons appearing as three singlets at δ 6.57 (1H, *s*), 6.92 (1H, *s*) and 6.98 (2H, *s*) and their corresponding carbon signals appear at δ 103.2, 95.0 and 106.2 (2C), respectively. These four protons were readily assigned as H-3, H-8 and H-2′, 6′ in 1. Two broad ^{1}H singlets at δ 12.74 and 8.64 were not linked to any carbon resonance in the HSQC spectrum and were assigned as phenolic protons. From this combined information it was possible to deduce that the aglycone moiety contained five phenolic groups at C-5, C-6, C-3′, C-4′ and C-5′, and that the sugar moiety was connected to the flavone aglycone through an ether link at C-7. The ^{1}H NMR spectrum contained an anomeric proton at 5.03 (1H, *d*, 7.2) and another six oxymethine protons in the range δ 3.2–3.80 ppm. The sugar moiety was readily determined as a glucopyranose based on the ^{1}H–^{1}H COSY data, and by comparing the proton and carbon chemical shifts with published data (Antri et al., 2004). The coupling constant of the anomeric proton (J = 7.2 Hz), indicated the β configuration for the glucopyranose moiety. Thus the structure of **1** was unambiguously determined as 5,6,3′,4′,5′-pentahydroxyflavone-7-*O*-β-glucoside (**1**), a previously unknown flavone glycoside.

Compound **2** was isolated as a pale yellow powder, and the UV absorbance maxima at 222, 283 and 334 nm indicated a flavonoid similar to 1. The molecular formula was determined as $C_{23}H_{22}O_{14}$

by HRMS ESI$^+$ at 523.1073 [M+1]$^+$. The LC–MS ESI$^-$ gave a molecular ion at *m*/*z* 521 and the aglycone ion at *m*/*z* 317 (521–162–42), which indicated it may contain an acetyl group. Indeed, the ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** except for the appearance of an acetyl methyl signal at δ 2.01 (3H, *s*) and the downfield shift of the H-6″ protons to δ 4.38 (1H, *d*, 12.0) and δ 4.07 (1H, *dd*, 12.0, 8.0) in the sugar moiety indicated that **2** contained an *O*-acetyl group at C6″. This was confirmed by the ^{1}H–^{1}H COSY data and the $^3J_{HH}$ coupling constant of the anomeric proton at δ 5.06 (1H, *d*, *J* = 7.2 Hz) indicated a β configuration. Therefore, the structure of **2** was determined as an acetyl derivative of 1: 5,6,3′,4′,5′-pentahydroxyflavone-7-*O*-(6″-*O*-acetyl)-β-glycoside, a previously unknown flavone glycoside.

Compound **3** was assigned a molecular formula of $C_{23}H_{22}O_{13}$ based on HRMS ESI$^+$ data and the UV absorbance maxima at 220, 282 and 338 nm indicated that it may be a C-6 hydroxylated luteolin derivative (Harborne and Williams, 1971). The LC–MS ESI$^-$ spectrum of **3** showed an [M-H]$^-$ ion at *m*/*z* 505 and an aglycone ion at *m*/*z* 301 (505–162–42), indicating **3** is also a flavone glycoside containing an acetylated sugar moiety. The flavone aglycone moiety was readily deduced as 6-hydroxyluteolin by inspection of the ^{1}H NMR spectrum which contained characteristic resonances for H-3 (δ 6.67; 1H, *s*, δc 103.2 by HSQC), H-8 (δ 6.91; 1H, *s*, δc 94.6), H-2′ (δ 7.41; 1H, *s*, δc 115.1) and coupled doublets for H-6′ (δ 7.42; 1H, *d*, 6.5; δc 120.8) and H-5′ (δ 6.90; 1H, *d*, 6.6, δc 117.0). The remainder of the NMR data were consistent with the structure of **3** as 6-hydroxyluteolin-7-*O*-(6″-*O*-acetyl)-β-glucopyranose, a previously unknown flavone glycoside.

Compound **4** was obtained as a pale yellow solid, and once again the UV absorbance at 220, 282, 331 nm suggested it was a C-6 hydroxylated flavone derivative (Harborne and Williams, 1971; Albach et al., 2003). The molecular formula was determined as $C_{23}H_{22}O_{12}$ by HRMS ESI$^+$ while the LC–MS ESI$^-$ spectrum showed an [M-H]$^-$ ion at *m*/*z* 489 and an aglycone ion at *m*/*z* 285 (489–162–42) which indicated it might also be an acetyl flavone glycoside. Compared with compound **3**, the aglycone in **4** has one less oxygen and the structure was deduced as 6-hydroxyapegenin based on the characteristic ^{1}H aromatic resonances corresponding to H-3 δ 6.79 (1H, *s*; δc 103.8 by HSQC), H-8 δ 6.93 (1H, *s*; δc 95.1) and of four protons appearing as two doublets at δ 7.91 (2H, *d*, 8.0) and δ 6.92 (2H, *d*, 8.0). The 6″-*O*-acetyl glucopyranose moiety was confirmed by comparing the sugar ^{1}H and ^{13}C chemical shifts with those in **3**. The glucopyranose moiety was connected to C7 as indicated by the downfield-shifted C7 resonance at δc 152.0 (assigned by HSQC and HMBC). Thus the structure of **4** was determined as 6-hydroxyapegenin-7-*O*-(6″-acetyl)-β-glucopyranoside, a previously unknown flavone glycoside.

Compound **5** also displayed UV absorptions at 218, 230, 286 and 323 nm consistent with a flavonoid molecule. A molecular formula of $C_{30}H_{26}O_{13}$ was established by HRMS ESI$^+$ and by ^{1}H NMR, HSQC and HMBC spectroscopic data. In addition to the molecular [M+H]$^+$ ion at *m*/*z* 595, the LC–MS ESI$^+$ spectrum also showed a fragment ion at *m*/*z* 287, corresponding to loss of a tetrahydroxyflavone moiety. The ^{1}H NMR spectrum showed singlets at δ 6.72 (1H, *s*, δc 103.1 by HSQC) and δ 6.93 (1H, *s*, δc 94.4), assigned to H-3 and H-8 in the aglycone moiety. The ^{1}H NMR spectrum also showed four exchangeable singlets at δ 12.68, δ 10.50, δ 10.12 and δ 8.75, and of these the signals at δ 12.68 and δ 8.75 were assigned to C-5-OH and C-6-OH, respectively, by comparing with the corresponding data in **4**. The HMBC spectra showed four correlations from H-8 (δ 6.93) to carbons at δ 151.5, 149.1, 130.8 and 106.2, which were identified as C-7, C-9, C-5 and C-10 by comparison of similar data for compounds **1**–**4**. The ^{1}H NMR also showed two pairs of ortho-coupled aromatic protons at δ 7.91 (2H, *d*, ^3J_H = 8.0 Hz) and δ 6.91 (2H, *d*, ^3J_H = 8.0 Hz) as well as δ 7.20 (2H, *d*, ^3J_H = 8.0 Hz) and δ 6.57 (2H, *d*, ^3J_H = 8.0 Hz), consistent with two 1,4 substituted benzene rings. The first pair of coupled protons were readily assigned to H-2′, 6′ and H-3′, 5′ by the correlation of H-2′/6′ to C2 (δ 164.5) and H-3 to C2. Therefore, the flavonoid aglycone was identified as 6-hydroxyapegenin. The second pair of aromatic protons was also correlated to two aromatic carbons resonating at δ 159.9 and δ 125.1. From the chemical shift data the carbon at δ 159.9 must be bonded to oxygen, while the carbon at δ 125.1 showed additional correlations to two olefinic protons at δ 6.25 (1H, *d*, ^3J_H = 15.4 Hz) and δ 7.45 (1H, *d*, ^3J_H = 15.4 Hz). The olefinic proton at δ 6.25 showed a long-range correlation to a carbonyl carbon at δ 167.2, and taken together, these data were consistent with the presence of a coumaroyl moiety. A fragment ion at *m*/*z* 147 in the LC–MS ESI$^+$ spectrum of **5** further supported this assignment. An E configuration of the double bond in the coumaroyl moiety was indicated by the large coupling constant (^3J_H = 15.4 Hz) observed for the olefinic resonances. The sugar moiety present in **5** was indicated by an anomeric proton signal at δ 5.11 (1H, *d*, ^3J_H = 8.0 Hz), and six other oxymethine resonances in the range δ 3.30–4.50. The acylated sugar moiety was confirmed as a 6-*O*″-(E)-coumaroyl-β-glucopyranose by comparison of the chemical shifts with those in **4**, the ^{1}H–^{1}H COSY spectral data, and the downfield-shifted resonance of H-6′ (δ 4.46 and 4.22). Once again, the sugar was connected by an ether link to C-7 as indicated by the downfield shift of the C-7 resonance at δ 151.5. Thus the structure of **5** was established as a new acylated flavone glycoside, namely 6-hydroxyapegenin-7-*O*-(6″-*O*-coumaroyl)-β-glucopyranose.

The molecular formula of $C_{30}H_{26}O_{14}$ for compound **6** was determined by HRMS ESI$^+$ and contained an additional oxygen atom compared with **5**. Interestingly, in addition to the [M+H]$^+$ ion at *m*/*z* 611, the LC–MS ESI$^+$ spectrum of **6** showed the same aglycone ion at *m*/*z* 287 as that of **5**, suggesting that **6** and **5** have the same flavone aglycone and hence the additional oxygen atom in **6** must be located on the acylated sugar moiety. Since the ^{1}H NMR and COSY data for the sugar moiety in **6** and **5** were the same, the additional oxygen in **6** must be located in the coumaroyl portion of the molecule. This was supported by a fragment ion at *m*/*z* 163 in the LC–MS ESI$^+$ of **6**, which compares with the corresponding coumaroyl fragment ion at *m*/*z* 147 in **5**, and identified the acyl moiety in **6** as an E-caffeoyl group. This deduction was confirmed by assignment of the E-double bond protons at δ 6.20 (1H, *d*, ^3J_H = 15.4 Hz) and δ 7.39 (1H, *d*, ^3J_H = 15.4 Hz), and the ABX proton resonances appearing at δ 6.87 (1H, *s*, H-2‴; δc 116.7 by HSQC), δ 6.68 (1H, *d*, 8.5, H-6‴; δc 122.0) and δ 6.53 (1H, *d*, 116.9, C-5″, δc 116.9). The HMBC data also supported the presence of E-caffeoyl by providing the correlations from δ 7.39 to the carbonyl group (δ 167.2), δ 6.20 to C-1‴ (δ 122.5) and δ 6.53 to C-3‴ (δ 145.5) and δ 6.87 to C-4‴ (δ 146.5). Thus the structure of **6** was unambiguously determined as 6-hydroxyapigenin-7-*O*-(6″-*O*-caffeoyl)-β-glucopyranose, a previously unknown flavone glycoside.

Compound **7** displayed UV absorption bands at 217, 230, 286 and 330 nm similar to those observed for compounds **5** and **6**, and the HRMS ESI$^+$ data established a molecular formula of $C_{30}H_{26}O_{14}$ revealing the compound to be an isomer of **6**. In fact the ^{1}H spectrum of **7** is very similar to **6** with only small differences in the aromatic region. The LC–MS ESI$^-$ of **7** showed an aglycone ion at *m*/*z* 301 and another fragment ion at *m*/*z* 147 corresponding to a 6-acyl moiety. Comparing these data with the fragment ions observed for **6** (*m*/*z* 287 and *m*/*z* 161), it was possible to deduce that the aglycone of **7** contained an additional oxygen, and was identified as 6-hydroxy luteolin by comparison of the ^{1}H and ^{13}C NMR spectroscopic data with published data (Lu et al., 1980). An ABX system containing three aromatic protons appearing at δ 7.44 (1H, *s*, assigned as C-2′), δ 7.42 (1H, *d*, ^3J_H = 7.5 Hz, assigned as H-6‴) and δ 6.89 (1H, *d*, ^3J_H = 7.5 Hz, assigned as H-5‴) confirmed this assignment. Thus the 6″-acyl moiety in **7** must be a coumaryl

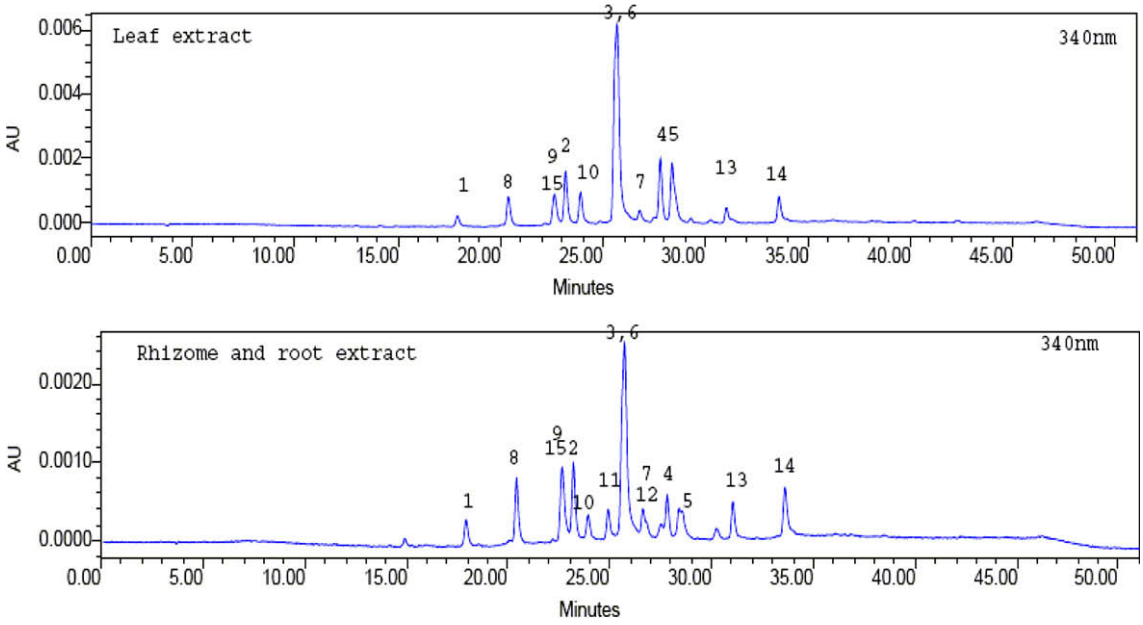


Fig. 2. HPLC–UV chromatograms of *Halophila johnsonii* leaf, rhizome and root extracts.

group instead of the caffeoyl group, as evidenced by four *ortho*-coupled aromatic protons appearing at δ 6.55 (2H, *d*, ^3J_H = 8.0 Hz, assigned as H-3‴ and H-5‴) and δ 7.22 (2H, *d*, ^3J_H = 8.0 Hz, assigned as H-2‴ and H-6‴). The E configuration of the double bond in the coumaroyl group was determined by the coupling constant of the olefinic protons at δ 6.26 (1H, *d*, ^3J_H = 15.6 Hz) and δ 7.45 (1H, *d*, ^3J_H = 15.6 Hz). Thus the structure of **7** was determined to be 6-hydroxyluteolin-7-*O*-(6″-*O*-coumaryl)-β-glucopyranose, a previously unknown acyl glycoside.

The glycosides **8**–**10** were all isolated as pale yellow powders. Their structures were determined as 6-hydroxyluteolin-7-*O*-β-glucopyranose (**8**) [$C_{21}H_{20}O_{12;}$ M-H *m*/*z* 463] (Antri et al., 2004), scutellarien-7-*O*-β-glucopyranose (**9**) [$C_{21}H_{20}O_{11}$; M-H *m*/*z* 447 (Peng et al., 2003), and spicoside (**10**) [$C_{30}H_{26}O_{15}$; M+H *m*/*z* 625] (Albach et al., 2003) on the basis of 1D and 2D NMR spectral analysis and by comparison with published data. The flavones **11**–**15** were all isolated as yellow powders. On the basis of LC–MS, 1D and 2D NMR spectroscopic analysis and comparison with published NMR data, the structures of **11**–**15** were determined as the flavonoids pedalitin (Ferraro et al., 1977), ladanetin (Arisawa et al., 1970), luteolin (Lu et al., 1980), apigenin (Fourie and Snyckers, 1984) and myricetin (Lee et al., 1998), respectively.

3. Concluding remarks

This report represents the first chemical investigation of the marine angiosperm *H. johnsonii*, and as illustrated in Fig. 1, a complex profile of flavonoids and flavonoid glycosides (**1**–**10**) and flavones (**11**–**15**) was observed in the HPLC–UV chromatogram of the methanol extract. Apart from a few very minor components, the remaining compounds in the methanol extract of *H. johnsonii* were isolated, and several new derivatives have been identified. Given the distribution of flavones in angiosperms, the discovery of flavones in extracts of *H. johnsonii* is not unreasonable, and indeed the occurrence of sulfated flavones has been reported in other *Halophila* spp. (McMillan et al., 1980). In addition, glycosylated flavones have been reported in smaller-leaved members of *Halophila* (McMillan et al., 1981). The presence of these flavonoid compounds and the fact that their concentration diminishes when transplanted to deeper-waters (Durako et al., 2003), strongly supports a photoprotectant role for these metabolites. A further striking feature is the apparent absence of flavones in the related deeper-water species *H. decipiens* which may not require such photoprotectants. The presence of this suite of flavonoid derivatives in *H. johnsonii* may provide the plant with the essential photoprotection necessary to survive in high-light shallow-water environments.

The distribution of these flavonoid compounds throughout the plant was also examined. To this end, leaves and combined rhizomes and roots were separated and extracted with methanol, and the extracts analyzed separately by HPLC–UV–MS. The various chromatograms obtained are shown in Fig. 2, and by simple comparison it can be seen that there are differences in the distribution of these metabolites. The flavonoid glycosides **3** and **6** are the most abundant flavonoid derivatives in the leaves, rhizome and roots. The glycosides **4** and **5** are the second most abundant components in the leaf, whereas in the rhizome and roots, compounds **2**, **8**, **9**, and **15** are the next most abundant components. In addition, rhizomes and roots contain more of the minor free flavones **11** and **12**, perhaps suggesting that the flavonoids are transported within the cell as glycoside derivatives.

4. Experimental

4.1. General

All solvents employed for chromatography were HPLC grade and purchased from Burdick & Jackson Company. ^{1}H NMR, ^{1}H–^{1}H COSY, HSQC and HMBC spectra were recorded in DMSO-d_6 using a 500 Hz Bruker NMR Avance spectrometer. Optical rotation data were recorded on Auto III Automatic polarimeter. UV spectra were recorded on a Molecular devices Flexstation 3 instrument coupled with Softmax pro software. IR data were recorded on a Madison CYGNUS 100 Infrared Spectrometer coupled with Winfirst software. LC–MS were recorded using an HP1100 HPLC equipped with an HP UV diode array detector and a Waters ZQ detector equipped with an electrospray ionization source. The cone voltages were set up at 40 V for negative ion mode and 55 V for positive ion mode. The LC–MS data were analyzed using the Masslynx 3.5 software. HPLC analysis and preparation was conducted on a Waters 1525

HPLC coupled with a Waters 2487 dual λ absorbance UV detector. High-resolution mass spectra were obtained using an Applied Biosystems Qstar XL spectrometer (UNCW) or on a Waters QT of Ultima spectrometer (University of Illinois at Urbana-Champaign).

4.2. Plant material

Specimens of *H. johnsonii* were collected from Jupiter Sound in southeastern Florida (26° 57′ N; 80° 04′ W) during July 2006. The plant material was identified by M.J.D. and a voucher specimen was stored in the Center for Marine Science, University of North Carolina at Wilmington.

4.3. Extraction and isolation

Fresh *H. johnsonii* (97.02 g wet weight) was blended in a Waring commercial blender with MeOH (100 mL) for five minutes. The mixture was filtered to separate the MeOH solubles and the residue was extracted another four times with MeOH (4 × 100 mL). The MeOH extracts were combined and concentrated to dryness using a rotavapor at 37 °C. The dried MeOH extract (1.35 g) was loaded onto a Combiflash C18 reversed phase column (13 g) and eluted with a gradient mobile phase of MeOH and H_2O at a flow rate of 10 mL/min. Stepwise elution with 20%, 40%, 60%, 80% and 100% MeOH in H_2O afforded fraction F1, F2, F3, F4 and F5, respectively. LC–MS analysis of F1–F5 indicated that flavonoid components were concentrated on F2, F3 and F4 only, and these were combined and separated by HPLC, using a semi-preparative Gemini 5 μ C18 column (250 × 10 mm) and a gradient mobile phase (25−80% MeOH in H_2O containing 0.02% AcOH for 60 min, then 80% MeOH in H_2O containing 0.02% AcOH for 20 min) at a flow rate of 2 ml/min. Peaks were monitored at 340 nm. Compounds **1**–**15** were isolated as pale yellow solids except **7** which was obtained as yellow crystals. The weights of compounds **1**–**15** obtained were 0.7, 1.1, 1.3, 1.4, 1.3, 1.5, 1.0, 1.4, 0.9, 0.9, 1.0, 0.7, 0.9, 1.0 and 0.7 mg, respectively.

4.4. Analytical HPLC and LC–mass analysis of the methanol extracts of the whole plant, leaf and rhizome and root of H. johnsonii

Sun-dried whole plants of *H. johnsonii* (4.78 g) were separated into leaves (1.77 g dry weight) and rhizomes and roots (3.01 g dry weight). Both fractions were extracted with MeOH using a Waring commercial blender (100 mL × 4), and in each case the residue was further extracted by MeOH in an ultrasonic bath (100 mL × 3). The extracts were combined in each case and concentrated in vacuo to yield a dried leaf extract (248.1 mg) and a rhizome-root extract (545.2 mg). A portion (1% by weight) of the rhizome-root extract (5.452 mg) and the leaf extract (2.481 mg) was removed and dissolved in MeOH–H_2O (1 mL, 6:4, v/v). An aliquot (0.5 μL) of each standard solution was taken out, mixed and an HPLC–UV chromatogram of the whole plant extract was obtained using an analytical column (Luna 5 μ C18; 250 × 4.6 mm), a gradient mobile phase (25–80% MeOH in H_2O containing 0.02% AcOH for 30 min, then 80% MeOH in H_2O containing 0.02% acetic acid for 10 min), and a flow rate of 0.5 mL/min. Peaks were monitored by UV at 340 nm. In addition, each leaf and rhizome-root extract (1 μL) was subjected to the same HPLC analysis, in order to obtain the HPLC–UV chromatogram of each one.

For LC–MS analysis, an aliquot (500 μL) of each extract was removed, dried under nitrogen, and re-dissolved in 60% MeOH/H_2O (50 μL), respectively. Additional aliquots (20 μL) of each solution were removed and combined to provide a sample of the whole plant extract for analysis. Additional samples (2 μL) of this combined extract solution as well as samples (2 μL) of the leaf and rhizome-root extract solutions were analyzed by LC–MS using an HP 1100 HPLC coupled with an HP diode array UV detector and Waters ZQ mass spectrometer (conditions as described above). The molecular and fragment ions of each peak in the chromatogram of the whole plant extract, leaf extract and rhizome-root extract were obtained by using Masslynx 3.5 software.

4.4.1. 5,6,7,3′,4′,5′-Hexahydroxyflavone-7-O-β-glucopyranoside (**1**)

Pale yellow solid (0.7 mg); $[\alpha]_D^{25}$ + 5.5 (MeOH, *c* 0.04); UV λ_{max}^{MeOH} nm (log ε): 283 (3.6) and 341 (3.5); IR ν_{max}^{neat} cm^{-1}: 3380, 2929, 1670, 1200, 1120; HRMS ESI$^+$ *m*/*z* 481.0979 [M+H]$^+$ (calc. for $C_{21}H_{21}O_{13}$, *m*/*z* 481.0982). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.57 (1H, *s*, H-3), 6.92 (1H, *s*, H-8), 6.98 (2H, *s*, H-2′, 6′), 5.03 (1H, *d*, 7.2, H-1″), 3.37 (1H, *m*, H-2″), 3.36 (1H, *m*, H-3″), 3.22 (1H, *m*, H-4″), 3.47 (1H, *m*, H-4″), 3.74 (1H, *m*, H-6″ *a*), 3.52 (1H, *m*, H-6″ *b*), 12.74 (1H, *brs*, 5-OH), 8.64 (1H, *brs*, 6-OH); ^{13}C NMR (500 MHz, DMSO-d_6, Partial, observed by HSQC) δ 103.2 (C-3), 95.0 (C-8), 106.2 (C-2′, 6′), 102.1 (C-1″), 74.1 (C-2″), 76.4 (C-3″), 70.5 (C-4″), 78.6 (C-5″), 61.2 (C-6″).

4.4.2. 5,6,7,3′,4′,5′-Hexahydroxyflavone-7-O-[6″-O-acetyl]-β-glucopyranoside (**2**)

Pale yellow solid (1.1 mg); $[\alpha]_D^{25}$ − 11.1 (MeOH, c 0.02); UV λ_{max}^{MeOH} nm (log ε): 283 (3.6) and 334 (3.7); IR ν_{max}^{neat} cm^{-1}: 3300, 2929, 1724, 1665, 1250, 1080; HRMS ESI$^+$ *m*/*z* 523.1073 [M+H]$^+$ (calc. for $C_{23}H_{23}O_{14}$, *m*/*z* 523.1088). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.59 (1H, *s*, H-3), 6.90 (1H, *s*, H-8), 6.99 (2H, *s*, H-2′, 6′), 5.05 (1H, *d*, 7.2, H-1″), 3.40 (1H, *m*, H-2″), 3.39 (1H, *m*, H-3″), 3.22 (1H, *dd*, 9.0, 6.5, H-4″), 3.75 (1H, *m*, H-5″), 4.07 (1H, *dd*, 12.0, 8.0, H-6″ *a*), 4.38 (1H, *d*, 12.0, H-6″ *b*), 2.01(3H, *s*, H-7″), 12.74 (1H, *brs*, 5-OH), 8.64 (1H, *brs*, 6-OH); ^{13}C NMR (500 MHz, DMSO-d_6, partial, observed from HSQC) δ 103.8 (C-3), 94.7 (C-8), 106.6 (C-2′, 6′), 101.5 (C-1″), 74.1 (C-2″), 76.7 (C-3″), 70.9 (C-4″), 75.1 (C-5″), 64.5 (C-6″), 22.0 (C-7″), 173.2 (C-8″).

4.4.3. 5,6,7,3′,4′-Hexahydroxyflavone-7-O-[6″-O-acetyl]-β-glucopyranoside (**3**)

Pale yellow solid (1.3 mg); $[\alpha]_D^{25}$ − 21.8 (MeOH, c 0.03); UV λ_{max}^{MeOH} nm (log ε): 282 (3.3) and 338 (3.4); IR ν_{max}^{neat} cm^{-1}: 3325, 2930, 1720, 1665, 1510, 1200, 1090; HRMS ESI$^+$ *m*/*z* 507.1120 [M+H]$^+$ (calc. For $C_{23}H_{23}O_{13}$, *m*/*z* 507.1139). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.67 (1H, *s*, H-3), 6.91 (1H, *s*, H-8), 7.41 (1H, *s*, H-2′), 6.90 (1H, *d*, 7.0, H-5′), 7.42 (1H, *d*, 7.0, H-6′), 5.02 (1H, *d*, 7.2, H-1″), 3.39 (1H, *m*, H-2″), 3.38(1H, *m*, H-3″), 3.22 (1H, *dd*, 9.0, 6.5, H-4″), 3.75 (1H, *dd*, 10.8, 9.0, H-5″), 4.08 (1H, *dd*, 12.8, 10.8, H-6″ *a*), 4.38 (1H, *d*, 12.8, H-6″ *b*). 2.01 (3H, *s*, H-7″), 12.74 (1H, *brs*, 5-OH), 8.62 (1H, *brs*, 6-OH), 9.47 and 10.08 (both 1H, *brs*, 3-OH or 4-OH); ^{13}C NMR (500 MHz, DMSO-d_6) δ 164.5(C-2), 103.2 (C-3), 182.1 (C-4), 147.0 (C-5), 130.5 (C-6), 151.8 (C-7), 94.6 (C-8), 150.8 (C-9), 105.8 (C-10), 122.0 (C-1′), 115.1 (C-2′), 146.0 (C-3′), 148.5 (C-3′), 117.0 (C-4′), 120.8 (C-5′)101.2 (C-1″), 74.0 (C-2″), 76.3 (C-3″), 70.9 (C-4″), 75.0 (C-5″), 64.1 (C-6″), 171.6 (C-8″), 21.99 (C-7″).

4.4.4. 5,6,7,4′-Tetrahydroxyflavone-7-O-[6″-O-acetyl]-β-glucopyranoside (**4**)

Pale yellow solid (1.4 mg); $[\alpha]_D^{25}$ − 13.6 (MeOH, c 0.03); UV λ_{max}^{MeOH} nm (log ε): 282 (3.3) and 331 (3.4); IR ν_{max}^{neat} cm^{-1}: 3320, 2929, 1720, 1665, 1500, 1220, 1080; HRMS ESI$^+$ *m*/*z* 491.1179 [M+H]$^+$ (calc. for $C_{23}H_{23}O_{12}$, *m*/*z* 491.1190). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.79 (1H, *s*, H-3), 6.93 (1H, *s*, H-8), 7.91 (2H, *d*, 8.0, H-2′, 6′), 6.92 (2H, *d*, 8.0, H-3′, 5′), 5.05 (1H, *d*, 7.7, H-1″), 3.40 (1H, *dd*, 9.5, 7.7, H-2″), 3.38 (1H, *dd*, 9.5, 9.0, H-3″), 3.22 (1H, *t*, 9.0, H-4″), 3.75 (1H, *dd*, 10.8, 9.0, H-5″), 4.08 (1H, *dd*, 13.8, 10.8, H-6″ *a*), 4.38 (1H, *d*, 13.8, H-6″ *b*), 2.00 (3H, *s*, H-7″), 12.72 (1H, *brs*, 5-OH); ^{13}C NMR (500 MHz, DMSO-d_6, partial assignments from HMBC) δ 164.5(C-2), 103.8 (C-3), 130.5 (C-6), 152.0 (C-7), 95.1

(C-8), 149.8 (C-9), 105.8 (C-10), 122.1 (C-1′), 130.1 (C-2′, 6′), 116.5 (C-3′, 5′), 162.0 (C-6′), 102.2 (C-1″), 74.2 (C-2″), 76.1 (C-3″), 70.5 (C-4″), 75.2 (C-5″), 64.2 (C-6″), 22.0 (C-7″), 171.2 (C-8″).

4.4.5. 5,6,7,4′-Tetrahydroxyflavone-7-O-(6″-O-[E]-coumaroyl)-β-glucopyranoside (**5**)

Pale yellow solid (1.3 mg); $[\alpha]_D^{25}$ − 19.4 (MeOH, c 0.04); UV λ_{max}^{MeOH} nm (log ε): 286 (3.6) and 323 (3.6); IR ν_{max}^{neat} cm^{-1}: 3340, 2880, 1664, 1600, 1450, 1220, 1050; HRMS ESI$^+$ *m/z* 595.1428 $[M+H]^+$ (calc. for $C_{30}H_{27}O_{13}$, *m/z* 595.1451). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.72 (1H, *s*, H-3), 6.93 (1H, *s*, H-8), 7.91 (2H, *d*, 8.0, H-2′, 6′), 6.91 (2H, *d*, 8.0, H-3′, 5′), 12.68 (1, *s*, 5-OH), 8.75 (1H, *s*, 6-OH), 10.12 (4′-OH), 5.11 (1H, *d*, 8.0, H-1″), 3.44 (1H, *dd*, 14.2, 7.7, H-2″), 3.42 (1H, *dd*, 14.2, 9.5, H-3″), 3.30 (1H, *m*, H-4″), 3.87 (1H, *t*, 7.0, H-5″), 4.24 (1H, *dd*, 14.0, 12.0, H-6″ *a*), 4.48 (1H, *d*, 14.0, H-6″ *b*), 5.59, 5.51, 5.38 and 5.11 (all 1H, *s*, OH from sugar ring), 7.20 (2H, *d*, 8.0, H-2‴, 6‴), 6.57 (2H, *d*, 8.0, H-3‴, 5‴), 7.45 (1H, *d*, 15.4, H-7‴), 6.25 (1H, *d*, 15.4, H-8‴), 10.50 (1H, *s*, 4″-OH); ^{13}C NMR (500 MHz, DMSO-d_6, partial assignments from HMBC) δ 164.5(C-2), 103.1 (C-3), 182.5 (C-4), 130.8 (C-6), 151.5 (C-7), 94.4 (C-8), 149.1 (C-9), 106.2 (C-10), 121.9 (C-1″), 129.1 (C-2′, 6′), 117.0 (C-3′, 5′), 161.5 (C-6′), 101.2 (C-1″), 74.0 (C-2″), 76.5 (C-3″), 71.2 (C-4″), 75.0 (C-5″), 64.5 (C-6″), 125.1 (C-1‴), 130.9 (C-2, 6‴), 116.4 (C-3‴, C-5‴), 159.9 (C-4‴), 146.0 (C-7‴), 114.3 (C-8‴), 167.2 (C-9‴).

4.4.6. 5,6,7,4′-Tetrahydroxyflavone-7-O-(6″-O-[E]-caffeoyl)-β-glucopyranoside (**6**)

Pale yellow solid (1.5 mg); $[\alpha]_D^{25}$ − 20.1 (MeOH, c 0.08); UV λ_{max}^{MeOH} nm (log ε): 285 (3.4) and 330 (3.5); IR ν_{max}^{neat} cm^{-1}: 3330, 2900, 1664, 1600, 1500, 1250, 1050; HRMS ESI$^+$ *m/z* 611.1404 $[M+H]^+$ (calc. for $C_{30}H_{27}O_{14}$, *m/z* 611.1401). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.73 (1H, *s*, H-3), 6.95 (1H, *s*, H-8), 7.90 (2H, *d*, 8.0, H-2′, 6′), 6.88 (2H, *d*, 8.0, H-3′, 5′), 5.12 (1H, *d*, 8.0, H-1″), 3.42 (1H, *dd*, 9.5, 8.0, H-2″), 3.39 (1H, *dd*, 12.5, 9.5, H-3″), 3.30 (1H, *dd*, 12.5, 8.5, H-4″), 3.81 (1H, *dd*, 12.0, 8.5, H-5″), 4.22 (1H, *dd*, 14.0, 12.0, H-6″ *a*), 4.46 (1H, *d*, 14.0, H-6″ *b*), 6.87 (1H, *s*, H-2‴), 6.53 (1H, *d*, 8.0, H-5‴), 6.68 (1H, *d*, 8.0, H-6‴), 7.39 (1H, *d*, 15.4, H-7‴), 6.20 (1H, *d*, 15.4, H-8‴); ^{13}C NMR (500 MHz, DMSO-d_6, partial assignments from HMBC) δ 164.5 (C-2), 103.5 (C-3), 130.2 (C-6), 151.0 (C-7), 94.6 (C-8), 148.5 (C-9), 106.3 (C-10), 120.9 (C-1′), 129.5 (C-2′, 6′), 117.0 (C-3′, 5′), 162.0 (C-6′), 102.1 (C-1″), 73.7 (C-2″), 76.8 (C-3″), 70.2 (C-4″), 74.2 (C-5″), 63.6 (C-6″), 125.5 (C-1‴), 116.7(C-2‴), 145.5 (C-3‴), 146.5 (C-4‴), 116.9 (C-5‴), 122.0 (C-6‴), 146.9 (C-7‴), 114.5 (C-8‴), 167.2 (C-9‴).

4.4.7. 5,6,7,4′,5-Pentahydroxyflavone-7-O-(6″-O-[E]-coumaroyl)-β-glucopyranoside (**7**)

Pale yellow solid (1.0 mg); $[\alpha]_D^{25}$ − 107.1 (MeOH, c 0.006); UV λ_{max}^{MeOH} nm (log ε): 286 (3.8) and 330 (3.7); IR ν_{max}^{neat} cm^{-1}: 3300, 2900, 1664, 1600, 1510, 1230, 1080; HRMS ESI$^+$ *m/z* 611.1402 $[M+H]^+$ (calc. for $C_{30}H_{27}O_{14}$, *m/z* 611.1401). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.66 (1H, *s*, H-3), 6.99 (1H, *s*, H-8), 7.44 (1H, *s*, H-2′), 6.89 (1H, *d*, 8.0, H-5′), 7.42 (1H, *d*, 8.0, H-6′), 5.12 (1H, *d*, 7.2, H-1″), 3.40 (1H, *m*, H-2″), 3.41 (1H, *m*, H-3″), 3.28 (1H, *m*, H-4″), 3.87 (1H, *m*, H-5″), 4.26 (1H, *dd*, 12.0, 8.6, H-6″ *a*), 4.42 (1H, *d*, 12.0, H-6″ *b*), 7.22 (1H, *d*, 8.0, H-2‴, 6‴), 6.55 (1H, *d*, 8.0, H-3‴, 5‴), 7.45 (1H, *d*, 15.6, H-7‴), 6.26 (1H, *d*, 15.6, H-8‴).

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