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Evolvosides C–E, flavonol-4-*O*-triglycosides from *Evolvulus alsinoides* and their anti-stress activity

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ABSTRACT

Phytochemical investigation of the *n*-butanol fraction of *Evolvulus alsinoides* (Linn.) led to the isolation of three new phenolic glycosides, evolvosides C, D and E (**1**–**3**) along with six known compounds (**4**–**9**). The structures of the compounds were elucidated on the basis of spectroscopic analysis, viz. 1D and 2D NMR experiments, chemical study, and comparison with literature data. Evolvoside C (**1**) was characterized as kaempferol 4′-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, whereas evolvosides D and E (**2**–**3**) were found to be mono and di-*O*-methyl derivatives of **1**. The new compounds (**1**–**3**) represent rare triglycoside derivatives of flavonol at C-4′. The isolated compounds (**1**–**6**) were screened for acute stress-induced biochemical changes in male Sprague–Dawley rats at a dose of 40 mg/kg body weight. Compounds **1** and **2** displayed anti-stress effects by normalizing hyperglycemia, plasma corticosterone, plasma creatine kinase, and adrenal hypertrophy. Compounds **3** and **6** were also found to be effective in normalizing most of these stress parameters, whereas compounds **4** and **5** were ineffective in normalizing most of these effects.

1. Introduction

Evolvulus (Family: Convolvulaceae) is a small genus composed of about 10–15 species widely distributed in Asian and American countries, with some of its species used medicinally.[1,2] *Evolvulus alsinoides* is one of the several well-known Ayurvedic crude drugs that have a significant place in traditional medicinal system of India due to its memory enhancing properties.[3–5] Apart from its historical indigenous use several preparations containing the plant decoction are widely commercialized as nerving tonic (Shankhpushpi) in Asia. In addition, the plant extracts have been used to treat a number of diseases including bronchitis, asthma,[2,3] brain disorders like insanity, epilepsy, nervous disability, and scrofula.[2–5] Previous work on *Evolvulus alsinoides* extracts have demonstrated antioxidant,[6] antiulcer,[7] and immunomodulatory activities.[8] However, the phytochemical studies are limited which have resulted in the identification of triacontane, pentatriacontane, scopoletin, scopolin, umbelliferon, methyl-1,2,3,4-butaneterol, β-sitosterol and the alkaloids betaine and shankpushpin.[9,10] Four unidentified alkaloids have also been described in the literature.[11] More recently Akhtar et al. reported the isolation of a new chromone, 6-hydroxy-5-nonadecanoxychroman-2-one (alsinoideschromone) along with hexatriacontane, octyl octadec-9-enoate, nonyloctadec-9-enoate, dodecanyl-octadec-9,12-dienoate, tetracosanyl hexadec-9-enoate, heptacosan-14-b-ol, stigmast-5, 22-dien-3b-ol and stigmata-5-en-3b-ol.[12]

As part of a research program on the discovery of new natural immunomodulatory agents from Ayurvedic crude drugs,[13–16] it was found that a crude ethanol extract of this plant exhibited adaptogenic and anti-amnesic properties.[13] During our recent phytochemical investigations of ethanol extract, we have reported a series of phenolic compounds including new flavonol-4′-*O*-diglycosides, evolvosides A and B[17] and two new caffeoyl derivatives, evolvoids A and B.[16] The latter metabolites were the first natural examples of the erythritol associated with phenolic substance (caffeoyl). In the course of that study, we also noted that the *n*-butanol fraction of ethanol extract contained several unidentified polar flavonoids. This paper describes the isolation and characterization of three new flavonol-4′-*O*-triglycosides, evolvosides C–E (**1**–**3**), accompanied by known compounds (**4**–**9**). The anti-stress activity of compounds **1**–**6** was evaluated in acute stress induced biochemical changes in adult male Sprague–Dawely rates.

2. Results and discussion

The dried powder of *Evolvulus alsinoides* (17 kg, whole plant) was extracted with EtOH, yielded crude extract 1.19 kg (yield

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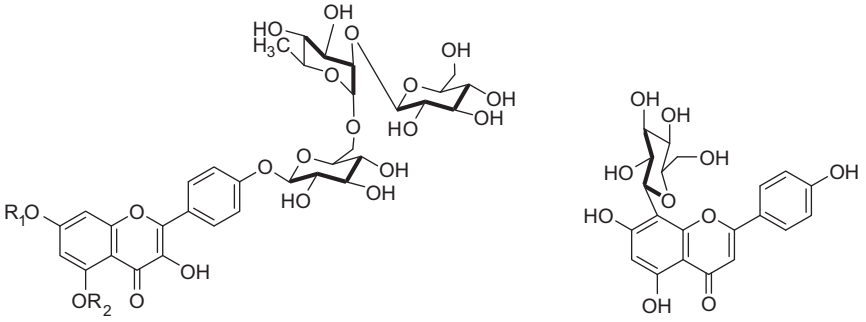
7.0%). The EtOH extract was then dissolved in water and subjected to liquid–liquid partitioning successively with $CHCl_3$ and *n*-BuOH, resulted in $CHCl_3$ (320.3 g), *n*-BuOH (260.0 g) and aqueous (511.2 g) crude residue respectively. A portion of *n*-BuOH soluble fraction (60 g) was loaded on polymeric HP-20 resin using cyclic loading method.[18] The HP-20 column was then eluted with 400 mL fractions of (1) 20% Me_2CO/H_2O (2) 40% Me_2CO/H_2O (3) 60% Me_2CO/H_2O (4) 80% Me_2CO/H_2O and finally with Me_2CO. The 20% Me_2CO/H_2O fraction (10.3 g) was then subjected to sequential CC and finally HPLC separation on a C-18 reverse phase column to yield the new flavonol-4′-*O*-triglycosides, evolvosides C–E (**1**–**3**). The other known compounds **4**–**9** were isolated from 40% and 60% Me_2CO/H_2O fractions respectively. The chemical structure of the compounds **1**–**3** were proposed on the basis of their detailed UV, IR, HRESIMS and 1D and 2D NMR spectral data analysis whereas known compounds were characterized by spectral data comparison with literature reports.

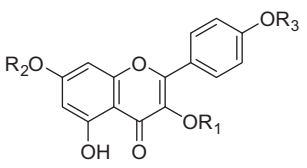
Compound **1** (Fig. 1) was isolated as light yellow hygroscopic solid, with a molecular formula of $C_{33}H_{40}O_{20}$ as determined by HRESIMS pos. spectrum (*m/z* at 779.2023 $[M+Na]^+$, calcd for 779.2011). Both the UV [λ_{max} 364 (band I) and 261 (band II) nm] and IR [ν_{max} 1667 (α,β-unsaturated carbonyl), 1590, 1461 and 1399 (aromatic rings) and 3432 (hydroxyl groups) cm^{-1}] spectra showed absorption bands characteristic of a flavonol.[19] Bathochromic shift of 11 nm in band II with sodium acetate (C-7 OH), 52 nm bathochromic shift of band I in presence of $AlCl_3$ (C-5 OH) and 21 nm bathochromic shift in band I with decreased intensity upon addition of NaOMe (C-3 OH) discovered the presence of free hydroxyl groups at C-3, 5 and 7 positions with substituted 4′-hydroxy group (no retention of peak intensity with NaOMe).[20] On acetylation (pyridine/Ac_2O) it formed duodecaacetate derivative **1a** (Supplementary data). The formation was confirmed by ESI-MS: *m/z* 1261.346 $[M+H]^+$, indicated the presence of 12 acylable hydroxyl groups in compound **1**. In addition, the FAB-MS spectrum of **1a** exhibited three diagonostic fragments due to loss of monosaccharide units sequentially at *m/z* 931 $[M+H-C_{14}H_{20}O_9]^+$, 701 $[M+H-C_{24}H_{34}O_{15}]^+$, and 413 $[M+H-C_{36}H_{50}O_{23}]^+$. The monosaccharide units were identified as D-glucose and L-rhamnose by acid hydrolysis of **1**, followed by co-TLC with authentic sample, GC analysis and optical rotation.[21] These preliminary observations indicated that **1**, could be flavonol-4′-*O*-triglycoside and was confirmed by extensive analysis of both one and two-dimensional ^1H and ^{13}C NMR spectra. The aromatic region of the ^1H NMR (Table 1) spectrum of **1** contained a characteristic resonance at δ_H 6.21 (1H, *d*, *J* = 1.6, H-6; δ_C 99.7 by HSQC) and δ_H 6.40 (1H, *d*, *J* = 1.6, H-8; δ_C 94.9 by HSQC), an assignment confirmed by long-range connectivity of H-6/C-7,8,10; H-8/C-9,10 in the HMBC spectrum (Fig. 2). Two doublets at δ_H 8.11 (2H, *d*, *J* = 8.3 Hz, H-2′, 6′); δ_C 131.1 and 7.23 (2H, d, *J* = 8.3 Hz, H-3′, 5′); δ_C 116.2 comprised the AAXX aromatic resonances, a downfield shift of H-3′/5′ at δ_H 7.23 was characteristic of 4′-*O*-substituted B-ring of a flavonoids.[17] These data confirmed that the aglycone was kaempferol (3,5,7,4′-tetrahydroxyflavone). Table 1 summarizes ^1H and ^{13}C NMR chemical shift assignments for **1**, which are similar to the literature values for known kaempferol 4′-*O*-glycosides.[17] Further evidence for the glycosidation came from the long-range correlation detected in the HMBC spectrum between the anomeric proton resonance at δ_H 5.21 (1H, *d*, *J* = 7.3 Hz; δ_C 100.5) and C-4′ of kaempferol (δ_C 161.2). Two additional resonances corresponding to anomeric protons of sugar residues appeared at δ_H 4.69 (1H, *br d*, *J* = 1.1 Hz; δ_C 101.1) and δ_H 4.62 (1H, *d*, *J* = 7.3 Hz; δ_C103.6). The remaining glycosidic proton resonances occurred between 3.14 and 3.76 ppm, with the exception of that for a rhamnose methyl doublet at δ_H 1.19 (3H, *d*, *J* = 6.1 Hz; δ_C17.7).

The ^1H and ^{13}C resonance assignments for the three sugar residues were obtained from standard 1D and 2D NMR datasets (Table 1). Anomeric configurations were determined from the magnitudes of $J_{1,2}$ coupling constant in the ^1H NMR spectrum. These data indicated that the primary sugar *O*-linked at C-4′ was a β-D-glucopyranosyl residue. The remaining anomeric proton resonances at δ_H



1: $R_1 = R_2 = H$
2: $R_1 = Me$, $R_2 = H$
3: $R_1 = R_2 = Me$

4



5: $R_1 = R_2$ =H, R_3 = β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside
6: $R_1 = R_2$ =H, R_3 = α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside
7: $R_1 = R_2$ = β-D-glucopyranoside, R_3 = H
8: R_1 = β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside, $R_2 = R_3$ = H
9: R_1 = β-D-glucopyranosyl, R_2 = α-L-rhamnopyranoside, R_3 = H

Figure 1. Chemical structures for compounds **(1–9).**

Table 1
^{13}C and ^{1}H NMR data for evolvosides C–E (**1–3**)[a]

Atom		**1**[a]		**2**[a]		**3**[a]	
		δ_C	δ ^{1}H (*J* in Hz)	δ_C	δ ^{1}H (*J* in Hz)	δ_C	δ ^{1}H (*J* in Hz)
Aglycone	2	147.4	—	147.1	—	147.2	—
	3	136.1	—	135.9	—	136.4	—
	4	176.3	—	176.9	—	175.9	—
	5	162.8	—	162.6	—	161.4	—
	6	99.7	6.21 *d* (1.6)	99.4	6.29 *d* (1.5)	99.0	6.30 *d* (1.5)
	7	165.9	—	164.8	—	164.3	—
	8	94.9	6.40 *d* (1.6)	93.9	6.49 *d* (1.4)	93.4	6.51 *d* (1.5)
	9	156.2	—	156.0	—	155.0	—
	10	104.9	—	104.7	—	104.1	—
	1	124.5	—	124.3	—	124.5	—
	2′	131.1	8.11 *d* (8.3)	132.1	8.19 *d* (8.1)	131.7	8.08 *d* (8.4)
	3′	116.2	7.23 *d* (8.3)	116.9	7.21 *d* (8.1)	116.4	7.29 *d* (8.3)
	4′	161.2	—	161.9	—	161.4	—
	5′	116.2	7.23 *d* (8.3)	116.9	7.21 *d* (8.1)	116.4	7.29 *d* (8.3)
	6′	131.1	8.11 *d* (8.3)	132.1	8.19 *d* (8.1)	131.7	8.08 *d* (8.4)
4′-*O*-β-Glc^{A}	1	100.5	5.21 *d* (7.3)	100.1	5.17 *d* (7.1)	100.4	5.09 *d* (7.0)
	2	76.1	3.64 *t* (8.5)	76.3	3.64 *t* (8.2)	76.2	3.62 *t* (8.6)
	3	77.1	3.55 *t* (8.6)	77.0	3.52 *t* (8.1)	77.1	3.51 *br d* (10.4)[c]
	4	71.6	3.34 *m*	71.4	3.32 *m*[b]	71.2	3.30 m[b]
	5	77.9	3.60 *br d* (9.0)[c]	77.6	3.58 *br d* (9.3)[c]	78.1	3.68 *br d* (9.1)[e]
	6a	68.3	3.76 *dd* (11.2,1.6)	68.0	3.70 *dd* (11.0,1.7)	68.5	3.76 *dd* (10.8,1.9)
	6b	—	3.51 *br d* (11.0)	—	3.49 *br d* (8.9)[f]	—	3.50 *br d* (10.4)[c]
6^{GlcA}-*O*-α-Rha	1‴	101.1	4.69 *d* (1.1)	101.4	4.71 *d* (1.3)	101.0	4.68 *br s*
	2‴	80.9	3.33 *m*	80.4	3.30 *m*[b]	80.5	3.31 *m*[b]
	3‴	70.1	3.41 *dd* (9.1, 3.1)	70.3	3.49 *br d* (8.9)[f]	69.9	3.42 *dd* (9.2, 3.4)
	4‴	72.9	3.20 *t* (8.9)[b]	72.6	3.23 *m*	72.5	3.18 *t* (8.7)[d]
	5‴	69.5	3.46 *dd* (9.5, 6.2)	69.7	3.49 *br d* (8.9)	69.2	3.44 *br d* (8.7)
	6‴	17.7	1.19 *br d* (6.1)	17.9	1.21 *br d* (6.7)	18.1	1.18 *br d* (5.9)
2^{Rha}-*O*-β-Glc^{B}	1⁗	103.6	4.62 *d* (7.3)	103.4	4.60 *d* (7.1)	103.2	4.64 *d* (7.3)
	2⁗	74.9	3.14 *t* (8.6)	74.9	3.16 *t* (8.3)	74.1	3.18 *t* (8.7)[d]
	3⁗	76.2	3.28 *m*	76.4	3.30 *m*[b]	76.7	3.30 *m*[b]
	4⁗	70.2	3.20 *t* (8.9)[b]	70.5	3.20 *m*[e]	70.3	3.24 *t* (8.5)
	5⁗	75.7	3.17 *m*	75.3	3.19 *m*[e]	75.2	3.18 *t* (8.7)[d]
	6⁗a	60.7	3.71 *br d* (12.2)	60.2	3.58 *br d* (9.3)[c]	60.4	3.69 *br d* (9.1)[e]
	6⁗b		3.60 *dd* (9.0)[c]		3.42 *dd* (12.2, 8.0)		3.51 *br d* (10.4)[c]
7-OCH_3				55.2	3.87 *s*	55.3	3.89 *s*
5-OCH_3						55.9	3.91 *s*
Chelated OH			13.01 *s*		13.31 *s*		

[a] Measured in DMSO-d_6 (^{1}H NMR in 400 MHz and ^{13}C NMR in 100 MHz).
[b] Overlapped within the column.

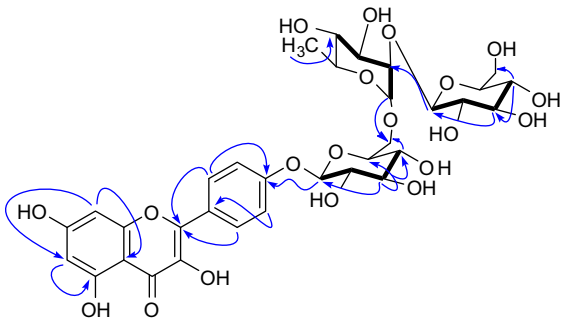


Figure 2. Selected HMBC correlations for evolvoside C (**1**).

4.69 and 4.62 were those of the α-L-rhamnopyranosyl and β-D-glucopyranosyl residues, respectively.

Assignment of the α-Rha moiety was straightforward, and characteristic multiplicities and coupling constants were extracted for all the ^{1}H resonances except H-2, which overlapped with that of moisture in DMSO-d_6 and H-4 of β-Glc^{A}. Full assignment of the β-Glc^{B} moiety was achieved using standard NMR datasets (COSY, HSQC, HMBC) alone and found that the ^{13}C resonances for C-3⁗ and C-4⁗ coincident with δ_C C-2 (Glc^{A}) and C-3‴ (Rha) respectively, and also H-6⁗ b proton was overlapped with H-5 of β-Glc^{A} (δ_H 3.60) whereas H-3 (δ_H 3.28) was overlapped with moisture. These ambiguities were resolved by using the recent H2BC (heteronuclear two-bond correlation) pulse sequence, which gives only $^{2}J_{HC}$ correlations between protons and proton-attached carbons.[22] This avoids the problems encountered in HMBC spectra of distinguishing between $^{2}J_{HC}$ and $^{3}J_{HC}$ correlations, and of 'missing' $^{2}J_{HC}$ correlations. As such, the H2BC sequence has been applied successfully to the assignment of NMR spectra of some complex carbohydrates.[23] The H2BC spectrum of **1** afforded a full set of sequential $^{2}J_{HC}$ connectivities for the Glc^{A} moiety from H-1″ to C-2, H-2 to C-1 and C-3, H-3 to C-2 and C-4, and H-4 to C-3 and C-5 (sequential $^{2}J_{HC}$ connectivities were also observed for the Glc and Rha moieties). The interglycosidic linkages defining the trisaccharide moiety of **1** were determined using HMBC data (Fig. 2). Long-range correlations from Glc^{A} H-6 to C-1‴ of Rha (δ_C 101.1), and from H-1‴ of Rha to the downfield-shifted Glc^{A} C-6 (δ_C 68.3), indicated that the primary Glc^{A} residue was 6-*O*-linked to Rha.[24] Similarly, a 2-*O*-linkage between Rha and the terminal secondary Glc^{B} residue was defined by connectivities from Rha H-2‴ to C-1⁗ of Glc^{B} (δ_C 103.6), and from H-1⁗ of Glc^{B} to the downfield-Shifted Rha C-2‴ (δ_C 80.9). Compound **1** was therefore kaempferol 4′-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyrano syl-(1→6)-β-D-glucopyranoside, a new flavonol glycolside named evolvoside C.

Compound **2** (Fig. 1) was isolated as yellow hygroscopic solid; with a molecular formula of $C_{34}H_{42}O_{20}$ as determined by HRESIMS pos. spectrum *m*/*z* 793.2206 $[M+Na]^{+}$, calcd 793.2197. The ^{1}H and ^{13}C NMR spectra clearly indicated that **2** was an analogue of **1**.

Table 2
Effect of pure compounds **1–6** and PQ on acute stress induced changes in adrenal gland weight, glucose, creatine kinase and corticosterone levels

Groups (dose mg/kg p.o.)	Adrenal gland (mg/kg wt)	Glucose (mg/dl)	CK (mg/dl)	Corticosterone (ng/ml)
NS	6.42 ± 0.41	83.5 ± 3.82	276.2 ± 15.2	232.4 ± 18.3
AS + Vehicle	10.2 ± 0.84**	154.7 ± 4.38**	1018 ± 31.43**	511.2 ± 23.2**
COMP-**1** (40)	7.11 ± 0.34*	80.17 ± 2.58*	385.3 ± 65.19*	233.3 ± 19.16*
COMP-**2** (40)	8.23 ± 0.54*	95.13 ± 2.34*	405.4 ± 69.16*	241.7 ± 17.15*
COMP-**3** (40)	Not tested	Not tested	413.7 ± 61.17*	252.6 ± 21.16*
COMP-**4** (40)	9.78 ± 0.79	92.13 ± 3.21*	821.6 ± 69.12	439.1 ± 13.11
COMP-**5** (40)	10.91 ± 0.91	113.1 ± 6.01	1078 ± 42.91	458.2 ± 29.01
COMP-**6** (40)	8.47 ± 0.63*	105.13 ± 2.71	521.4 ± 81.22*	239.2 ± 16.91*
PQ (100)	7.19 ± 0.29*	91.2 ± 3.21*	393.3 ± 31.60*	231.2 ± 11.51*

Mean ± SEM of changes in adrenal gland weight, plasma glucose, creatine kinase and corticosterone. The stress group was compared with non-stress control group and the drug treated groups were compared with acute stress group.
** p <0.001 when compared to NS control group.
* p <0.01 when compared with acute stress control group.

The only significant difference in the ^{1}H and ^{13}C NMR spectra of **2** as compared to **1** was the presence of additional methoxyl group at δ_H 3.87 (s, δ_C 55.2). Thus it appeared that compound **2** was the mono methoxy derivative of **1**. The site of O-methylation was confirmed at C-7 on the basis of shift reagent test where no bathochromic shift was observed in band II on addition of NaOAc.[20] This was further supported by three bond correlation from O–CH_3 to C-7 in HMBC spectrum on **2**. The complete assignment of the ^{1}H and ^{13}C NMR spectrum of **2** followed the procedure described for **1** (Table 1). In the corresponding HMBC spectrum, connectivities defining the site of glycosylation and interglycosidic linkages were as observed for **1**. Sugar analysis confirmed that each glycoside comprised of D-Glc^A, L-Rha and D-Glc^B residues. Thus structure of **2** was assigned as kaempferol-7-methoxy-4′-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyrano-syl-(1→6)-β-D-glucopyranoside and named as evolvoside D.

Compound **3** (Fig. 1) was isolated as light yellow hygroscopic solid; with a molecular formula of $C_{35}H_{44}O_{20}$ as determined by HRESIMS pos. spectrum *m*/*z* 807.2324 $[M+Na]^+$, calcd 807.2311. The ^{1}H and ^{13}C NMR spectra clearly indicated that **3** was an analogue of **1**. The only significant difference in the ^{1}H and ^{13}C NMR spectra of **3** as compared to **1** was the presence of additional two methoxyl groups at δ_H 3.89 (*s*, δ_C 55.2) and 3.91 (*s*, δ_C 55.9). Thus it appeared that compound **3** was the dimethoxy derivative of **1**. The site of O-methylation was shown to be at C-7 and C-5 on the basis of shift reagent test where no bathochromic shift was observed in band II and I upon addition of NaOAc and $AlCl_3$.[20] This was further supported by three bond correlations from O–CH_3 (δ_H 3.89) to C-7 and O–CH_3 (δ_H 3.91) to C-5 in HMBC spectrum on **3.** The additional support for placement of OMe at C-5 came from the absence of chelated hydroxyl hydroxyl signal in ^{1}H NMR spectrum between 11 and 13 ppm which we have observed in case of compounds **1** and **2** (Table 1).

The complete assignment of the ^{1}H and ^{13}C NMR spectra of **3** followed the procedure described for **1** (Table 1). In the corresponding HMBC spectrum, connectivities defining the site of glycosylation and interglycosidic linkages were as detected for **1**. Sugar analysis confirmed that each glycoside comprised of D-Glc^A, L-Rha and D-Glc^B residues. Thus structure of **3** was assigned as kaempferol-5,7-dimethoxy-4′-*O*-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyrano-syl-(1→6)-β-D-glucopyranoside and named as evolvoside E.

The structures of other isolated compounds were characterized as vitexin (**4**),[25] kaempferol 4′-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (**5**),[17] kaempferol 4′-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**6**),[17] kaempferol-3,7-di-*O*-β-D-glucop yranoside (**7**),[26] kaempferol 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**8**),[26] kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**9**)[27] by direct comparison of NMR data reported in the literature. The compounds **8** and **9** were isolated for the first time from this plant.

To evaluate anti-stress activity, a variety of stress situations have been employed in animals. Stress-induced effects mainly depend on duration and type of stressors.[28] Immobilization has been the ideal choice for the induction of stress responses in animals and, more specifically, for the investigation of drug effects on typical stress-related gastrointestinal, euroendocrine, and immunological pathology.[29] Hyperglycemic response during acute stress is due to the release of glucocorticoids as a result of HPA axis stimulation, to compensate for the initial demand of energy.[30] It is known that AS exposure not only stimulates and ensures the supply of glucose but also increases creatine kinase (CK) activity.[31] The CK system is important in stabilizing the ATP levels and energy metabolism of the myocardium and other skeletal muscles of rats during stress. Perturbations of CK activity during extensive stress may result in ischemia due to the nonavailability of ATP.[32] Prominent changes during stress are adrenocorticoid hypersecretion, increased plasma corticosterone, and enlarged pituitary and adrenal size.[33,34] In our previous work we have observed, the various type of stress also increased the adrenal gland weight and plasma corticosterone.[35]

Compounds **1—6** were screened for anti-stress activity in acute stress (AS) model at the dose of 40 mg/kg body weight, as shown in Table 2. A significant increase (p <0.001) in the adrenal gland weight after AS was observed when compared to non-stressed (NS) group. Compounds **1, 2, 6** and standard drug powder of the roots of *Panax quinquifolium* (PQ) were significantly (p <0.01) effective in reducing the stress induced adrenal hypertrophy. The plasma creatine kinase (CK) and glucose levels were also increased by AS significantly (p <0.001) when compared to NS control. Pretreatment with compounds **1**, **3, 6** (p <0.01) and PQ (p <0.01) were effective in reducing the AS-induced increase in CK levels and compounds **1**, **2** and **4** in reducing increased glucose levels. Similarly AS (p <0.001) exposure resulted in increased plasma corticosterone when compared to NS control. Pretreatment with compounds **1**–**3, 6** (p <0.01) and PQ (p <0.01) significantly reduced the increase in corticosterone levels.

3. Conclusion

In conclusion, present report describes the isolation and structure elucidation of the new flavonol glycosides, evolvosides C–E (**1–3**) and their anti-stress activity. The compounds **1** and **2** have shown significant (p <0.01) anti-stress activity by normalizing hyperglycemia, corticosterone level, creatine kinase and adrenal hypertrophy. Compounds **3** and **6** also have shown anti-stress activity but compound **6** had no effect in normalizing hyperglycemia. The

compounds **4** and **5** were found to be ineffective in normalizing these parameters (Table 2). Whilst several thousand compounds belonging to the flavonol structure class have been isolated, triglycoside at C-4′ of the flavonol moiety are rare with less than 10 reported in the literature. The biological activity profiles of compounds **1**, **2** and **3** supplied another chemical scaffold for potential anti-stress drug discovery.

4. Experimental

4.1. General experimental procedures

All NMR spectra were recorded on a Bruker DRX 300 and 400 MHz spectrometer. All chemical shifts (δ) were referenced internally to the residual solvent peak (DMSO-*d*6: ^{1}H, δ 2.5; ^{13}C, δ 39.5 ppm; CD_3OD: 1H, δ 3.34; ^{13}C, δ 49.5 ppm). Short- and long-range ^{1}H–13C correlations were determined with gradient-enhanced inverse-detected HSQC and HMBC experiments respectively. Optical rotations were measured on a Perkin-Elmer Model 241 digital polarimeter (*c* g/100 mL) at 589 nm. UV spectra were obtained on a Perkin–Elmer Lambda l–15 UV–VIS spectrophotometer. IR spectra were recorded on a Perkin–Elmer Nicolet IR-100 spectrophotometer. The high-resolution mass spectra performed HRESIMS analysis was carried out on Jeol-MS 600H instrument. HPLC purifications were performed on Beckman System Gold HPLC system with a 168 UV detector. GC–MS analysis was done using a Shimadzu GC-14A unit coupled with a GCMS-QP 2000 instrument. Column chromatography was performed using silica gel (60—120 and 230—400 mesh); TLC: pre-coated silica gel plates 60 F254 or RP-18 F254 plates with 0.5 or 1 mm film thickness (Merck). Spots were visualized by UV light or by spraying with 1:1 H_2SO_4/MeOH.

4.2. Plant material

The plant material was collected from district 24-Parganas, West Bengal (state of India) in the month of September 2002 and identified as *Evolvulus alsinoides* (LINN.) by Botany Division of Central Drug Research Institute (CDRI) and preserved with voucher specimen number 2659 in the herbarium.

4.3. Extraction and purification procedures

Powdered *Evolvulus alsinoides* whole plant (17 kg) was extracted with ethanol (each 30 L for 24 × 5) at room temperature. The resulting extracts were combined and concentrated under reduced pressure using rotavapor at 40 °C, to give a dark green extract (1.19 kg), which was suspended in distilled water (800 mL) and partitioned with $CHCl_3$ (1000 mL × 7). The $CHCl_3$ soluble extract was concentrated under vacuum using a rotavapor at 40 °C and yielded 380.3 g residue. Water-soluble fraction was further extracted with *n*-BuOH saturated with water (1000 ml × 5). The *n*-BuOH and water-soluble fractions were concentrated under reduced pressure using a rotavapor at 50 °C, and yielded 260.0 and 530.2 g of crude residue respectively. All the extracts were stored in refrigerator till further purification. A portion of *n*-BuOH soluble fraction (60 g) was loaded on polymeric HP-20 resin using cyclic loading method.[18] The HP-20 column was then eluted with 400 mL fractions of (1) 20% Me_2CO/H_2O (2) 40% Me_2CO/H_2O (3) 60% Me_2CO/H_2O (4) 80% Me_2CO/H_2O and finally with Me_2CO. The 20% Me_2CO/H_2O fraction (18.3 g) was then subjected to column chromatography (CC) over silica gel (230–400 mesh, 200.0 g) and eluted with a gradient of chloroform: methanol (95:05) to methanol: water (95:05) sequentially. Seventy three fractions (50 mL each) were sampled and their compositions were monitored by TLC, with those showing similar TLC profiles grouped into nine fractions (F-1 to F-9). Further purification of F-8 (4.3 g) over silica gel column (230–400 mesh, 70 g), using gradients of EtOAc/MeOH (85:15) to (05:95), afforded total eight pooled fractions (F-10 to F-17) on the basis of NMR profiles from total thirty-two fractions of 30 mL each. Fraction 16 (0.521 g) was subjected to preparative C18 reversed-phase HPLC (Gemini 5 μm; 21.2 × 250 mm; 6 mL/min; 40–100% CH_3CN/H_2O over 60 min) to give evolvoside C (**1**, 57.0 mg), evolvoside D (**2**, 63.2 mg) and evolvoside E (**3**, 9.0 mg). The known compounds **4** (81 mg), **5** (61.2 mg), **6** (52.1 mg), **7** (24.3 mg), **8** (12.5 mg) and **9** (16.1 mg) were purified from fractions 11–14 sequentially using semi-preparative C18 reversed-phase HPLC (Gemini 5 μm; 10.0 × 250 mm; 4 mL/min; 50–100% CH_3CN/H_2O over 60 min) methods. Compounds **4-9** were identified by comparison of their ^{1}H and ^{13}C NMR spectra with the literature data.

4.4. Sugar analysis

Acid hydrolysis of **1–9** (0.5–2.0 mg) was carried out by standard procedures (0.5 mL 2 M HCl, 100 °C, 1.5 h). After cooling, particulates were spun down using microcentrifugation and the supernatants were removed. Each reaction mixture was extracted with EtOAc. The aqueous fractions (sugars) were concentrated to dryness for the identification sugar residue on TLC plates comparing with standard sugar samples. The absolute configurations of monosaccharides released by acid hydrolysis of compounds **5, 7–8** were determined by observed optical rotation and found comparable to published report.[21a] The absolute configurations of the constituent monosaccharides, L-rahmnose and D-glucose released by acid hydrolysis of compounds **1–3, 6** and **9** were determined from GC–MS analysis of trimethylsilylated thiazolidine derivatives, which were prepared using the standard method.[21b] Conditions for GC were: capillary column, DB5-MS (30 m × 0.25 mm × 0.25 m), oven temp. programme, 180–300 °C at 6 °C/min; injection temp, 350 °C; carrier gas, He at 1 ml/min. The acid hydrolysates of **1–3, 6** and **9** each gave L-rhamnose and D-glucose, at t_R = 10.2 and 12.3, respectively (identical to authentic samples purchased from Sigma–Aldrich Co.). Compound **4** did not show the hydrolysis product.

4.5. Acetylation of compounds (1–9)

Compounds **1–9** (0.2–0.5 mg) were dissolved separately in dry pyridine (0.5 mL) and acetic anhydride (1 mL) was added. Reaction mixture was left overnight at room temperature. The reaction mixture was dried under reduced pressure and under a stream of N_2 to yield amorphous solids (**1a–9a**). The ESI-MS data of compounds (**1a–9a**) were taken to confirm the number of free hydroxyl groups (Supplementary data).

4.6. Evolvoside C (1)

Hygroscopic yellow solid; $[\alpha]_D^{25}$ −32.0 (*c* 0.02, methanol); IR (KBr) ν_{max} 3432, 2933, 1667, 1590, 1461, 1399 cm^{-1}; UV (MeOH) λ_{max} 364 (ε 2114), 261 (ε 1204) nm; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) see Table 1. HRESIMS *m/z* 779.2023 $[M+Na]^+$ (Calcd for $C_{33}H_{40}O_{20}Na$, 779.2011).

4.7. Evolvoside D (2)

Hygroscopic Yellow solid; $[\alpha]_D^{25}$ −51.2 (*c* 0.04, methanol); IR (KBr) ν_{max} 3451, 2941, 1671, 1581, 1450, 1382 cm^{-1}; UV (MeOH) λ_{max} 362 (ε 3198), 267 (ε 2041) nm; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) see Table 1. HRESIMS *m/z* 793.2206 $[M+Na]^+$ (calcd for $C_{34}H_{42}O_{20}Na$, 793.2197).

4.8. Evolvoside E (3)

Hygroscopic Yellow solid; $[\alpha]_D^{25}$ −41.2 (*c* 0.03, Methanol); IR (KBr) ν_{max} 3440, 2945, 1678, 1586, 1458, 1391 cm^{-1}; UV (MeOH) λ_{max} 365 (ε 5891), 271 (ε 2345) nm; ^1H (400 MHz) and ^{13}C NMR (100 MHz) see Table 1. HRESIMS *m/z* 807.2324 $[M+Na]^+$ (calcd for $C_{35}H_{44}O_{20}Na$, 807.2311).

4.9. Anti-stress activity

4.9.1. Animals

Adult male Sprague–Dawley rats (180—200 g) were kept in raised mesh bottom cages to prevent coprophagy in environmentally controlled rooms (25 ± 2 °C, 12 h light and dark cycle), animals had free access to standard pellet chow and drinking water except during experiments. Experiments were conducted between 09:00 and 14:00 h. Experimental protocols were approved by our institutional ethical committee following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), which complies with international norms of INSA (Indian National Science Academy).

4.9.2. Administration of drug

Suspension of pure compounds in 0.1% sodium carboxy methyl cellulose were prepared and administered by oral gavage using a ball ended feeding needle at a dose of 40 mg/kg, once daily for 3 d in case of acute stress (AS). Drug was prepared fresh daily before administration. A freshly prepared aqueous suspension of crude powder of ginseng root *Panax quinquifolium* was used as a standard at a dose of 100 mg/kg body weight and purchased from Sigma, USA.

4.9.3. Stress protocol

The rats were divided into control non-stress group, AS group, and drug-treated groups. Each group consists of 6 rats. A parallel group of rats were fed with vehicle for the same number of treatment days but were not immobilized and they were used as non-stress control group to obtain baseline data for various parameters. On the second day after feeding drug or vehicle, animals were fasted overnight with free access to water. On the third day, 45 min after feeding the drug or vehicle, rats were stressed except the non stress group. AS was produced by immobilizing animals for 150 min once only and sacrificed immediately by cervical dislocation. Briefly, immobilization stress was produced by restraining each naive animal inside an acrylic hemi cylindrical plastic tube (4.5 cm diameter, 12 cm long) for 150 min.[34]

4.9.4. Biochemical estimations

The blood was collected in EDTA coated tubes, through cardiac puncture after the stress regime and centrifuged at 2000 rpm × 20 min at 4 °C and plasma was separated. The plasma was used to estimate corticosterone, glucose and creatine kinase (CK).

4.9.5. Estimation of glucose and CK

Auto analyzer (Synchron Cx-5, Beckman) was used to estimate glucose and CK with their respective kits (Beckman Coulter International, Nyon, Switzerland).

4.9.6. Estimation of corticosterone

An HPLC/UV system (Waters, USA) was used for quantification of plasma corticosterone by the method of Woodword and Emery with modifications.[35] Dexamethasone was used an internal standard. The mobile phase consisted of methanol/water (70:30) at a flow rate of 1.2 ml/min and corticosterone was detected at 250 nm using UV detector. The chromatogram was recorded and analyzed with Breeze software (3.20 version).

4.9.7. Statistical analysis

Mean and SEM. were calculated. The data was analyzed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's multiple comparison test. Data of ulcer was analyzed by non-parametric ANOVA followed by Dunn's multiple comparison tests. p <0.05 was considered to be statistically significant.

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Supplementary data

Supplementary data (HMBC data for evolvosides D and E (**2** and **3**) together with structure and MS data of compounds **1a–9a**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.12.040.

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