Anti-stress Constituents of *Evolvulus alsinoides*: An Ayurvedic Crude Drug

Prasoon Gupta,[a] Akanksha,[a] Kiran Babu Siripurapu,[b] Ausaf Ahmad,[b] Gautam Palit,[b] Ashish Arora,[c] and Rakesh Maurya*,[a]

[a] *Division of Medicinal and Process Chemistry, Central Drug Research Institute;* [b] *Division of Pharmacology, Central Drug Research Institute; and* [c] *Division of Molecular and Structural Biology, Central Drug Research Institute; Lucknow–226 001, India.* Received January 15, 2007; accepted February 10, 2007

Bioactivity-guided purification of *n*-BuOH soluble fraction from the ethanol extract of *Evolvulus alsinoides* resulted in the isolation of two new compounds, 2,3,4-trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-phenyl]-2-propenoate (1) and 1,3-di-*O*-caffeoyl quinic acid methyl ester (2) along with six known compounds, caffeic acid (3), 6-methoxy-7-*O*-β-glucopyranoside coumarin (4), 2-*C*-methyl erythritol (5), kaempferol-7-*O*-β-glucopyranoside (6), kaempferol-3-*O*-β-glucopyranoside (7) and quecetine-3-*O*-β-glucopyranoside (8). The structure of new compounds 1 and 2 were elucidated by spectroscopic analysis, while known compounds were confirmed by direct comparison of their NMR data with those reported in literature. This is the first report of the presence of phenolic constituents in *Evolvulus alsinoides*. The isolated compounds 1—5 and 8 were screened for anti-stress activity in acute stress induced biochemical changes in adult male Sprague–Dawley rats. Stress exposure has resulted in significant increase of plasma glucose, adrenal gland weight, plasma creatine kinase (CK), and corticosterone levels. Compound 1 displayed most promising antistress effect by normalizing hyperglycemia, plasma corticosterone, CK and adrenal hypertrophy, while compounds 2 and 3 were also effective in normalizing most of these stress parameters, however compounds 4, 5 and 8 were ineffective in normalizing these parameters.

Key words *Evolvulus alsinoides*; Shankhpuspi; Convolvulaceae; phenyl propanoid; flavonol glycoside; quinic acid; erythritol

Physiological adaptation during stressful conditions is defined as a biochemical change in an organism that results from exposure to certain environmental conditions or stressors and generates a more effective response to their survival. Long-term stress can induce a range of disorders like hypertension, coronary heart disease,[1] gastric ulcers,[2] immunosuppression,[3] metabolic disorders like diabetes,[4] reproductive dysfunction,[5] mental depression, memory loss and host of other diseases.[6] Due to the nonspecific nature of the stress pathogenesis, a drug having central and peripheral activity is needed to combat stressful conditions. Since ancient times therapeutic approach to combat stress has involved utilization of substances from natural origin. Pharmacological investigations have shown that the basic effect of *Panax ginseng*, *Elutherococcus senticosus* and *Rhodiola rosea*, is their ability to increase non-specific resistance of the organism to various untoward influences.[7] Initial studies on plants originating from folk medicine along with an exponential increase in knowledge regarding the interactions among components of the stress system have encouraged various investigators to evaluate the potential of plant derived anti-stress agents for usage in modern day medicine. Further enrichment of the study on plant derived adaptogens was enabled by the substantial work carried out on plants such as *Ocimum sanctum*,[8] *Emblica officinalis*,[9] *Bacopa monniera*,[10] *Ginkgo biloba*[11] and *Withania somnifera*.[12] In our continuing efforts to identify biologically active secondary metabolites, we have initiated our study on *Evolvulus alsinoides* to identify anti-stress agents.

Evolvulus alsinoides Linn. (Family: Convolvulaceae) commonly known as Shankhpuspi in India. It is an important medicinal plant that grows in the open and grassy places almost throughout the India and subtropical countries of the world.[13,14] Plant extracts have been used in traditional medicine for treatment of bronchitis, asthma[14,15] and brain disorders like insanity, epilepsy, nervous disability, and scrofula.[14—17] *Evolvulus alsinoides* is well known for its memory enhancing property in traditional Indian system of medicine and extensively commercialized as nervin tonic in Asian countries. *Evolvulus alsinoides* extracts have exhibited antioxidant,[18] anti-ulcer,[19] and immunomodulatory activities.[20] Early phytochemical studies of this species resulted in the isolation and identification of chemical constituents such as triacontane, pentatriacontane, β-sitosterol and two alkaloids betaine and shankpushpin.[21—23] Four unidentified alkaloids A, B, C and evolvine have also been described in literature.[21,24] In the view of its importance in traditional medicinal system, no substantial phytochemical and pharmacological works have been reported. In addition, recently we have disclosed adaptogenic and anti-amnesic properties of ethanolic extract of *Evolvulus alsinoides*.[25] Further phytochemical investigation led to the isolation of two new compounds evolvoids A (**1**) and B (**2**) along with six known compounds (**3**—**8**). The structures of isolated compounds were elucidated on the basis of extensive spectroscopic analysis. The anti-stress activity of compounds **1**—**5** and **8** were evaluated in acute stress induced biochemical changes in adult male Sprague–Dawley rats. In the present study, we have described isolation, structure elucidation and anti-stress activity of these compounds.

Results and Discussion

Following the extraction of whole plant of *Evolvulus alsinoides* with ethanol and successive partitioning of the dried extract in $H_2O/CHCl_3$, H_2O/*n*-BuOH, significant anti-stress activity of the *n*-BuOH fraction was observed. However, $CHCl_3$ soluble fraction showed insignificant anti-stress activity while aqueous fraction was found to be inactive. The ac-

* To whom correspondence should be addressed. e-mail: mauryarakesh@rediffmail.com

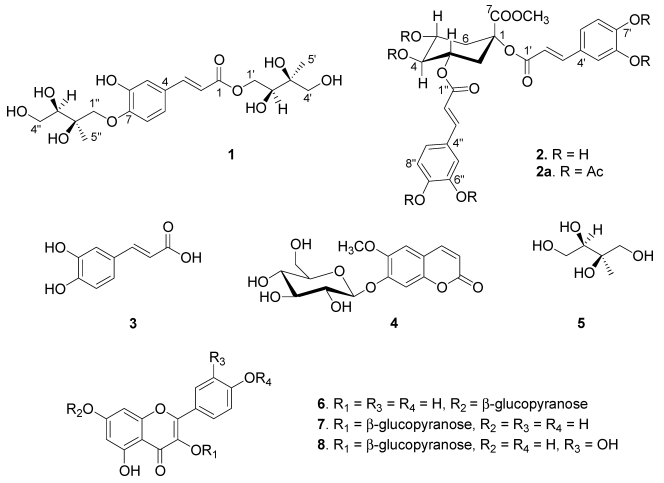

Fig. 1. Structural Formula of Isolated Compounds (**1**—**8**)

tive fraction was then subjected to sequence of normal- and reverse-phase column chromatography, to yield two new compounds **1** and **2** along with six known compounds. The structure of new compounds were established using chemical and spectroscopic (FAB-MS, ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC) studies.

Compound **1** was obtained as yellow solid, possessing elemental composition $C_{19}H_{28}O_{10}$ as concluded from ESI-MS pos. spectrum (*m/z* at 417 $[M+H]^+$) and HR-ESI-MS 416.1709 (Calcd 416.1683). The IR spectrum of **1**, displayed absorption bands for α-β unsaturated C=O (1670 cm^{-1}), aromatic ring (1483, 1341 cm^{-1}) and polyhydroxyl group at (3340 cm^{-1}). The UV absorption bands at (324, 239, 213 nm) were characteristic of caffeoyl unit.[26] The ^{1}H- and ^{13}C-NMR spectra (Table 1) displayed chemical shift pattern of caffeoyl moiety, showed olefinic protons at δ_H 7.61 (d, J=15.8 Hz, H-3; δ_C 147.0) and 6.31 (d, J=15.8 Hz, H-2; δ_C 115.1). The presence of three proton signals at δ_H 7.06 (br s, H-5), 6.97 (d, J=8.2 Hz, H-9) and 6.79 (d, J=8.2 Hz, H-8) in the ^{1}H-NMR spectrum, and resonance of typical carbons at δ_C 127.1 (C-4), 115.1 (C-5), 149.6 (C-6), 147.1 (C-7), 116.4 (C-8) and 122.9 (C-9) including α-β unsaturated carbonyl carbon at 169.5 (C-1) indicated presence of 6,7-disubtituted caffeoyl moiety in the molecule. In addition, the existence of two tetrose sugars (erythritol) were inferred from the ^{1}H- and ^{13}C-NMR spectrum,[27] which showed presence of four oxygenated methylenes, two methines, two methyls and two oxygenated quaternary carbons. Acid/base hydrolysis of **1** afforded erythritol sequentially, which was confirmed by co-TLC with authentic sample **5** isolated from same plant. The ^{1}H- and ^{13}C-NMR signals of both erythritol units were assigned using ^{1}H, ^{13}C, HSQC, HMBC and ^{1}H–^{1}H COSY NMR spectral data. The first erythritol unit was resonated as ABX and AB system at δ_H 4.53 (dd, J=11.2, 6.0 Hz, H-1′a), 4.20 (dd, J=11.2, 2.4 Hz, H-1′b), 3.87 (dd, J=6.0, 2.4 Hz, H-2′) and δ_H 3.49 (d, J=11.0 Hz, H-4′a), 3.63 (d, J=11.0 Hz, H-4′b) and corresponding carbon signals were appeared at δ_C 67.0 (C-1′), 73.6 (C-2′), 68.4 (C-4′) including quaternary carbon at δ_C 77.9 (C-3′). The methyl signal was assigned at δ_H 1.20 (s, H-5′; δ_C 19.2). ^{1}H- and ^{13}C-NMR spectrum exhibited second erythritol unit at δ_H 4.25 (d, J=11.4 Hz, H-1″a), 4.14 (d, J=11.4 Hz, H-1″b); δ_C 70.0 (C-1″) as AB spin system and δ_H 3.65 (br d, J=6.8 Hz, H-3″); δ_C 75.6 (C-3″), 3.91 (dd, J=11.2, 6.8 Hz, H-4″a), 3.61 (br d, J=11.2 Hz, H-4″b); δ_C 63.6 as ABX spin system, whereas methyl signal was assigned at δ_H 1.17 (s, H-5″); δ_C 19.3 (C-5″). The quaternary carbon resonated at δ_C 74.6 (C-2″). HMBC spectrum was utilized to identify the position of attachment (Fig. 2); it gave useful correlation between $H_{a,b}$-1′/C-1, 2′, 3′; H-2′/C-1′, 4′, 5′; $H_{a,b}$-4′/C-5′, 2′ and $H_{a,b}$-1″/C-7, 2″, 3″, 5″; $H_{a,b}$-4″/C-3″, 2″; H-5″/C-1″, 2″, 3″ indicated that first eythritol unit was attached to acid carbonyl (C-1) with ester linkage and second unit was attached to aromatic hydroxyl group (C-7) with ether linkage. The D-configuration of erythritol was determined by optical rotation $[\alpha]_D^{29}$ +24.5° (c=0.02, H_2O) and supported with the data available in literature.[27] Thus, compound **1** was characterized as a 2,3,4-trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methyl-butoxy)-phenyl]-2-propenoate, a new compound named evolvoid A.

Table 1. NMR Spectroscopic Data for Compound **1**

Position	**1** (CD_3OD)[a]			
	δ_H (mult., J in Hz)	δ_C	COSY	HMBC
1	—	169.5		—
2	6.31 (d, 15.8)	115.1	3	C-1, 4
3	7.61 (d, 15.8)	147.0	2	C-1, 5, 9
4	—	127.1	—	—
5	7.06 (br s)	115.1	—	C-3, 7, 9
6	—	149.6	—	—
7	—	147.1	—	—
8	6.79 (d, 8.2)	116.4	9	C-4, 6, 9
9	6.97 (d, 8.2)	122.9	8	C-3, 5, 7, 8
1′a	4.53 (dd, 11.2, 6.0)	67.0	1′b, 2′	C-1, 3′
1′b	4.20 (dd, 11.2, 2.4)	—	1′a, 2′	C-1, 3′
2′	3.87 (dd, 6.0, 2.4)	73.6	1′a, 1′b	C-4′, 5′
3′	—	77.9	—	—
4′a	3.49 (d, 11.0)	68.4	4′b	C-2′, 3′, 5′
4′b	3.63 (d, 11.0)	—	4′a	C-2′, 5′
5′	1.20 (s)	19.2	—	C-2′, 4′
1″a	4.25 (d, 11.4)	70.0	1″b	C-7, 3″, 5″
1″b	4.14 (d, 11.4)	—	1″a	C-7, 3″, 5″
2″	—	74.6	—	—
3″	3.65 (br d, 6.8)	75.6	4″a	C-1″, 4″, 5″
4″a	3.91 (dd, 11.2, 6.8)	63.6	3″, 4″b	C-2″, 3″
4″b	3.61 (br d, 11.2)	—	4″a	C-2″
5″	1.17 (s)	19.3	—	C-2″, 4″

a) ^{1}H-NMR: 200 MHz, ^{13}C-NMR: 300 MHz, 2D NMR: 600 MHz (TMS as internal standard), chemical shifts, multiplicity and coupling constants (*J*, Hz) were assigned by means of ^{1}H, ^{13}C and 2D NMR data.

Compound **2** was obtained as amorphous powder with few complex impurities. Our repeated effort to obtain pure compound by column chromatography was unsuccessful. IR spectrum exhibited absorption bands for aromatic ring (1604, 1514 cm^{-1}), α-β unsaturated carbonyl (1672 cm^{-1}) and ester (1737 cm^{-1}) function in the molecule. The ^{1}H-NMR of **2** (impure sample) showed presence of caffeoyl unit, ester methyl and few aliphatic signal although acetyl signal were absent in the spectrum. Acetylation followed by chromatography led to isolation of pure compound **2a** as polyacetate. However in nature this exists as non-acetylated compound. Compound **2a** exhibited molecular ion peak *m/z* at 783 $[M+H]^+$ in ESI-MS spectrum, corresponding to molecular formula $C_{38}H_{38}O_{18}$ suggested by HR-ESI-MS 782.2073 (Calcd 782.2058).

Analysis of ^{1}H- and ^{13}C-NMR data guided by COSY spectrum (Table 2) displayed presence of two overlapped caffeoyl

units and an oxygenated cyclohexyl ring. On the basis of HMBC, HSQC and COSY spectral data each signals of cyclohexyl ring were assigned, three mutual coupled acetylated methine signals at δ_H 5.28 (ddd, J=3.3, 7.1, 9.2 Hz, H-3; δ_C 68.4), 5.67 (dd, J=7.2, 9.2 Hz, H-4; δ_C 70.0), 5.60 (ddd, J=4.5, 5.4, 7.2 Hz, H-5; δ_C 71.2), two methylenes at δ_H 2.26, 2.63 (m, H-2a,b; δ_C 37.7) and δ_H 2.67, 2.78 (m, H-6a,b; δ_C 37.5), separately coupling with H-3 and H-5 protons. An oxygenated quaternary carbon resonated at δ_C 80.4 (C-1). Additional signal in NMR spectrum at δ_C 172.3 (C=O), gave HMBC correlations with H-2, 6 and methyl group at δ_H 3.73 (s, δ_C 53.4). Other important HMBC correlations, H-2/C-3, 4, 6; H-3/C-1, 5; H-4/C-2, 6 and H-5/C-1, 3, 4 confirmed cyclohexyl ring as quinic acid methyl ester (Fig. 2). The large coupling values of H-3, 4, 5 protons clearly indicated their axial orientation in the ring. The J values of substituted cyclohexane has shown to be in the range of *ca.* 10 Hz for axial–axial, *ca.* 5 Hz for axial–equatorial and *ca.* 2—3 Hz for equatorial–equatorial.[28] The J values were slightly lower than would be expected for a perfect chair conformation, therefore, quinic acid moiety of **2a** may exist in a skewed/boat form. The stereochemistry at C-1 could not be decided by the above method, therefore, relative stereochemistry deduced by the NOESY experiments (Fig. 2). Since axial proton H-3 and H-5 gave diagnostic NOE cross peaks with CO_2CH_3, indicated their axial orientation in the ring. Such correlation is possible only if the orientation of C-1 OH group is equatorial. Other diagnostic cross peaks (Fig. 2) confirmed the relative stereochemistry of cyclohexyl ring in **2a**. Presence of two caffeoyl units confirmed by two set of olefinic protons at δ_H 7.69 (d, J=15.9 Hz, H-3′); 6.51 (d, J=15.9 Hz, H-2′) and δ_H 7.74 (d, J=15.9 Hz, H-3″); 6.57 (d, J=15.9 Hz, H-2″). The J values 15.9 Hz confirmed both are the *trans* isomers. Other ABX aromatic protons of caffeoyl units were overlapped and assigned respectively (Table 2). One caffeoyl unit must be present at C-3 with ester linkage, as evident from HMBC cross peak between H-3/C-1″ (167.2) but no cross peak observed for second caffeoyl unit indicated that it must be attached to C-1 (OH). This was also supported by HMBC correlation of H-4 and H-5 with acetyl carbonyl δ_C 172.3 and 171.3 (due to acetylation, Fig. 2). Thus, above data confirmed the structure of evolvoid B hexaacetate represented by structure **2a** and the corresponding parent structure by **2**, which has not been previously reported.

Table 2. NMR Spectroscopic Data for Compound **2a**

Position	**2a** (CD_3OD)[a]			
	δ_H (mult., J in Hz)	δ_C	COSY	HMBC
1	—	80.4		
2a	2.63 (m)	37.7	2b, 3	C-3, 4, 6, 7
2b	2.26 (m)	—	2a, 3	—
3	5.28 (ddd, 3.3, 7.1, 9.2)	68.4	2a, 2b, 4	C-1, 5, 1″
4	5.67 (dd, 7.2, 9.2)	70.0	3, 5	C-2, 6
5	5.60 (ddd, 4.5, 5.4, 7.2)	71.2	4, 6a, 6b	C-1, 3, 4
6a	2.67 (m)	37.5	5, 6b	C-1, 2, 4, 7
6b	2.78 (m)	—	5, 6a	—
7	—	172.3	—	—
CO_2CH_3	3.73 (s)	53.4		C-7
1′	—	167.1[d]	—	—
2′	6.51 (d, 15.9)	119.5[e]	3′	1′, 4′
3′	7.69 (d, 15.9)	144.2	2′	1′, 5′, 9′
4′	—	134.4	—	—
5′	7.58 (br s)[b]	124.4	—	3′, 7′, 9′
6′	—	147.3	—	—
7′	—	147.7	—	—
8′	7.27 (d, 8.7)[c]	125.3	9′	4′, 6′
9′	7.58 (d, 8.7)[b]	127.8	8′	3′, 5′, 7′
1″	—	167.2[d]	—	
2″	6.57 (d, 15.9)	119.6[e]	3″	1″, 4″
3″	7.74 (d, 15.9)	145.5	2″	1″, 5″, 9″
4″	—	134.4	—	—
5″	7.58 (br s)[b]	124.4	—	3″, 7″, 9″
6″	—	147.3	—	—
7″	—	147.7	—	—
8″	7.29 (d, 8.7)[c]	125.3	9″	4″, 6″, 9″
9″	7.58 (d, 8.7)[b]	127.8	8″	3″, 5″, 7″
Ar-$OCOCH_3$	2.30 (s), 2.31 (s)	171.6, 172.3	—	
Al-$OCOCH_3$	2.00 (s), 2.07 (s)	169.7, 169.9	—	

a) ^{1}H-, ^{13}C- and 2D NMR: 300 MHz, chemical shifts, multiplicity and coupling constants (J, Hz) were assigned by means of ^{1}H-, ^{13}C- and 2D NMR data. *b*) Overlapped signals, *c*—*e*) interchangeable within the row.

Isolated compounds **1**—**5** and **8** were screened for anti-stress activity in acute stress (AS) model at the dose of 40 mg/kg body weight, as shown in Table 3. A significant increase (p<0.001) in the adrenal gland weight after AS was observed when compared to non-stressed (NS) group. Compound **1** 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 con-

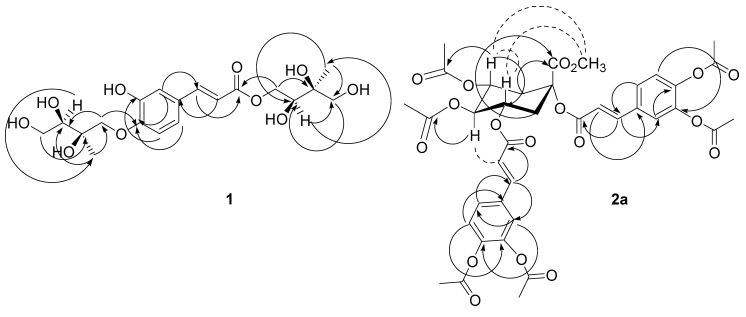

Fig. 2. Key HMBC (→) and NOESY (---) Correlation of Compounds **1** and **2a**

Table 3. Effect of Pure Compounds **1**—**5**, **8** 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	6.17±0.32	81.5±3.37	297.5±17.55	244.5±15.43
AS+Vehicle	9.93±0.84**	134.7±4.68**	1115±29.43**	477.2±22.2**
COMP-**1** (40)	7.23±0.41*	78.17±2.88*	485.3±85.12*	213.3±21.16*
COMP-**2** (40)	8.33±0.35	96.33±5.74*	600±51.81*	320±24.21*
COMP-**3** (40)	9.60±0.41	97.33±7.14*	962.7±61.23	301±22.51*
COMP-**4** (40)	11.15±0.78	123.7±6.83	1128±50.38	468±30.69
COMP-**5** (40)	11.41±0.48	108.7±6.82	1090±62.13	476.2±31.69
COMP-**8** (40)	11.51±0.48	121.2±10.21	1036±88.86	456.5±20.98
PQ (100)	7.33±0.44*	86.5±3.51*	493.3±49.96*	238.5±13.59*

Mmean±S.E.M 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 and * $p<0.01$ when compared with acute stress control group.

trol. Pretreatment with compounds **1**, **2** ($p<0.01$) and PQ ($p<0.01$) were effective in reducing the AS-induced increase in CK levels and compounds **1**, **2** and **3** 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**, **2** and **3** ($p<0.01$) and PQ ($p<0.01$) significantly reduced the increase in corticosterone levels.

In conclusion, compound **1** has shown significant ($p<0.01$) anti-stress activity by normalizing hyperglycemia, corticosterone level, creatine kinase and adrenal hypertrophy. Compound **2** also shown anti-stress activity but having no effect on adrenal hypertrophy similar is the case with compound **3**, which was found to be effective in some of the stress parameters but showing no effect on adrenal hypertrophy and increased CK levels. The compounds **4**, **5** and **8** were found to be ineffective in normalizing these parameters (Table 3). The biological activity profiles of compounds **1**, **2** and **3** are worthy for further investigation to develop anti-stress drug.

Experimental

General Procedures Melting points (uncorr.) were recorded on a Complab melting point apparatus. IR spectra were recorded on a Perkin-Elmer RX-1 spectrophotometer using either KBr pallets or in neat. UV spectra were obtained on a Perkin Elmer λ-15 UV spectrophotometer, optical rotations were measured on a Perkin-Elmer Model 241 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on an Avance DPX-200 and Bruker DRX 300 MHz spectrometer and chemical shift are expressed as δ (ppm) values. The abbreviations of ^{1}H-NMR signals pattern are as follows: s, singlet; d, doublet; dd, double doublet; ddd, doublet of doublet of doublet; t, triplet; m, multiplate. Proton detected heteronuclear correlations were measured using HSQC and HMBC. 2D spectrums were recorded on Varian Inova-600 MHz NMR and Bruker DRX 300 MHz spectrometer. FAB-MS were carried out on Jeol SX 102/DA-6000 mass spectrometer using *m*-nitro benzyl alcohol as matrix. ESI-MS spectra were obtained on LCQ Advantage Max Thermo Finnigan. Elemental analyses were obtained in a Carlo-Erba-1106 CHN elemental analyzer. HR-ESI-MS analysis was carried out on Jeol-MS 600H instrument. Preparative HPLC were performed on Shimadzu CLC-Octa decyl silane RP-18 (ODS) column with (20 mm ID×25 cm length); 8 ml/min flow rate, PDA UV λ 254 and 220 nm as detector. Column chromatography was performed using silica gel (60—120 and 230—400 mesh); TLC: pre-coated silica gel plates 60 F_{254} or RP-18 F_{254} plates with 0.5 or 1 mm film thickness (Merck). Spots were visualized by UV light or by spraying with H_2SO_4–MeOH, anisaldehyde–H_2SO_4 reagents.

Plant Materials 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 CDRI and preserved with voucher specimen number 2659 in the herbarium.

Extraction and Isolation Powdered *Evolvulus alsinoides* whole plant (17 kg) was extracted with ethanol (each 30 l for 24 h×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 rotavapor at 40 °C, 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 rotavapor at 50 °C, yielded 260.0 g 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 (200 g) was subjected to column chromatography (CC) over silica gel (230—400 mesh, 1.8 kg) and eluted with a gradient of chloroform : methanol (95 : 05) to methanol : water (95 : 05) sequentially. Seventy three fractions (800 ml each) were sampled and their composition monitored by TLC, with those showing similar TLC profiles grouped into nine fractions (F-1 to F-9). Further purification of F-1 (5.2 g) over silica gel (60—120 mesh, 130 g), using gradient of EtOAc/MeOH (100 to 80%) afforded **3** (800 mg). Column chromatography of F-3 (20.0 g) over silica gel (60—120 mesh, 450 g) with gradient of $CHCl_3$/MeOH (95 : 5) to (85 : 15), afforded thirteen pooled fractions (F-10 to F-22) on the basis of TLC profiles from total sixty two fractions of 500 ml each. Compound **4** (55 mg) was obtained as amorphous powdered from F-18 at room temperature. Successive purification of mother of F-18 (800 mg) over silica gel (230—400 mesh, 25 g), eluted with isocratic $CHCl_3$/MeOH (90 : 10) resulted in the isolation of compound **5** (463 mg). Rechromatography of F-19 (3.0 g) over silica gel (60—120 mesh, 85 g) using ethyl acetate saturated with water (isocratic) as mobile phase, afforded F-23 to F-29. Fraction F-23 (122 mg) containing **2** with few complex impurities were acetylated using (pyridine/Ac_2O) followed by CC over silica gel (60—120 mesh, 11 g) eluted with isocratic hexane : EtOAc (85 : 15) afforded **2** in their acetate form **2a** (75 mg). Fraction F-26 containing compound **1** (75 mg) in minor quantity was purified by preparative HPLC (RP-18), eluted with gradient of H_2O/MeOH (80—50%). Preparative HPLC (RP-18) of F-27 (120 mg) using gradient H_2O/MeOH (75—40%) yielded **6** (27 mg). Fraction F-20 (4.0 g) was rechromatographed over silica gel (60—120 mesh, 70 g), eluted with EtOAc/Acetone (80—70%) yielded F-30 to F-33 respectively. Further purification of F-31 (200 mg) by preparative HPLC (RP-18) with gradient H_2O/MeOH resulted in purification of compound **7** (30 mg). Compound **8** (70 mg) was eluted from F-21 (285 mg) using same condition as for F-31.

Acid Hydrolysis of 1 The compound **1** (25 mg, 0.06 mmol) was treated with 2 N HCl (5 ml) and refluxed for 30 min. The reaction mixture was worked up in the usual manner. The sugar fraction of compound **1** over CC on an activated silica column yielded 2-*C*-methyl-D-erythritol identified by co-TLC with authentic sample and optical rotation $[\alpha]_D^{29}$ +24.5° (c=0.02, H_2O).

Basic Hydrolysis 1 The compound **1** (2 mg, 0.004 mmol) was dissolved in methanol (3 ml) and NaOMe (5 mg, 0.092 mmol) was added, reaction mixture was stirred at rt. for 3 h. The reaction was quenched with acidic resin amberlite IRC-50 (Rohm and Hass, H^+ form), the resin was removed by simple filtration and filtrate was dried under reduced pressure. The sugar erythritol were identified by co-TLC with authentic sample.

Acetylation of Compound 2 Crude fraction containing **2** (122 mg) was dissolved in dry pyridine (5 ml) and acetic anhydride (5 ml) was added. Reaction mixture was left overnight at room temperature. The reaction mixture was dried under reduced pressure. The crude residue on column chromatography over silica gel (60—120 mesh, 11 g) eluted with isocratic hexane : EtOAc (85 : 15), yielded amorphous powder of **2a** (75 mg).

2,3,4-Trihydroxy-3-methylbutyl 3-[3-Hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)phenyl]-2-propenoate (**1**): Yellow solid. ^{1}H-NMR (CD_3OD, 200 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) see Table 1. IR (KBr) cm^{-1}: 3340, 2888, 1670, 1483, 1341, 1293. UV λ_{max} (MeOH) nm: 324, 239, 213. ESI-MS (pos.): *m/z* 417 $[M+H]^+$. $[\alpha]_D^{29}$ +15.0° (c=0.04, MeOH). Positive HR-ESI-MS: *m/z* $[M]^+$ 416.1709 (Calcd 416.1683 for $C_{19}H_{28}O_{10}$).

1,3-Di-*O*-caffeoyl Quinic Acid Methyl Ester (**2a**): Amorphous powder. ^{1}H-NMR (CD_3OD, 300 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) see Table 2. IR ($CDCl_3$) cm^{-1}: 3351, 2871, 1737, 1672, 1604, 1514. UV λ_{max} (MeOH) nm: 327, 273, 211. ESI-MS (pos.): *m/z* 783 $[M+H]^+$. $[\alpha]_D^{29}$ −50.8° (c= 0.04, $CDCl_3$). Positive HR-ESI-MS: *m/z* $[M]^+$ 782.2073 (Calcd 782.2058 for $C_{38}H_{38}O_{18}$).

Anti-stress Activity. Animals Adult male Sprague–Dawley rats (180—200 g) were obtained from National Animal Laboratory Centre,

CDRI, Lucknow. Animals 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).

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 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, U.S.A. (Cat. No. G7253).

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)]

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, creatine kinase (CK).

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).

Estimation of Corticosterone An HPLC/UV system (Waters, U.S.A.) 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).

Statistical Analysis Mean and S.E.M. 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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