Constituents of *Ocimum sanctum* with Antistress Activity[§]

Prasoon Gupta,[†] Dinesh Kumar Yadav,[†] Kiran Babu Siripurapu,[‡] Guatam Palit,[‡] and Rakesh Maurya*[,†]

Division of Medicinal and Process Chemistry and Division of Pharmacology, Central Drug Research Institute, Lucknow-226 001, India

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Three new compounds, ocimumosides A (**1**) and B (**2**) and ocimarin (**3**), were isolated from an extract of the leaves of holy basil (*Ocimum sanctum*), together with eight known substances, apigenin, apigenin-7-*O*-*β*-D-glucopyranoside, apigenin-7-*O*-*β*-D-glucuronic acid (**4**), apigenin-7-*O*-*β*-D-glucuronic acid 6″-methyl ester, luteolin-7-*O*-*β*-D-glucuronic acid 6″-methyl ester, luteolin-7-*O*-*β*-D-glucopyranoside, luteolin-5-*O*-*β*-D-glucopyranoside, and 4-allyl-1-*O*-*β*-D-glucopyronosyl-2-hydroxybenzene (**5**), and two known cerebrosides. The structures of the new compounds were determined on the basis of extensive 1D and 2D NMR spectroscopic analysis. The new compounds (**1**–**3**) and the known compounds **4** and **5** were screened at a dose of 40 mg/kg body weight for acute stress-induced biochemical changes in male Sprague–Dawley rats. Compound **1** displayed promising antistress effects by normalizing hyperglycemia, plasma corticosterone, plasma creatine kinase, and adrenal hypertrophy. Compounds **2** and **5** were also effective in normalizing most of these stress parameters. In contrast, compounds **3** and **4** were ineffective in normalizing any of these effects.

Stress response is a nonspecific demand to maintain the steady state required for successful adaptation. This consists of a repertoire of physical or mental reactions that attempt to counteract the effects of the stressors in order to re-establish homeostasis. Prolonged adaptation response with consistent physical and mental hostile conditions leads to various psychosomatic disorders such as ulceration, blood pressure, stroke, diabetes, anxiety, depression, and other psychosomatic disorders.[1,2] With changing lifestyles, stressful conditions are faced by most of the world population. Stress stimulates the hypothalamus pituitary adrenal (HPA) axis, causing activation of various central and peripheral mediators.[3] The role of important monoamines like noradrenaline (NA), dopamine (DA), and 5-hydroxytryptamine (5-HT) is well established in modulating various behavioral and biochemical responses during stressful conditions.[4] In view of the nonspecific nature of the stress response, a drug having central and peripheral activities would be in great demand. Thus, the identification of new effective and safe antistress agents is desperately needed. Plants and plant-derived metabolites have a long history of clinical relevance in a variety of diseases, and their use in traditional medicine systems all over the world is being re-examined.[5] In the course of a program aimed at the discovery of antistress agents from medicinal plants, we have initiated an investigation of *Ocimum sanctum* leaves.

Ocimum sanctum L. (Lamiaceae), a well-known herbal medicine, is widely distributed throughout the world.[6] This plant has been worshipped since Vedic times and is considered sacred for its medicinal properties in the Indian subcontinent and is well recognized in Ayurvedic medicine in India.[6] Its leaves have long been used to treat a variety of ailments, including ozena, skin diseases, and gastric and hepatic disorders and are used as a diaphoretic, an antiperiodic, and an expectorant.[6] *O. sanctum* is an important botanical supplement used in combination with other plants for the treatment of various stress-induced disorders in India and other Asian countries. Numerous studies have shown the immunomodulatory and antistress potential of *O. sanctum* leaf extracts.[7,8] Its mechanism of action has also been studied.[7,8]

Previous phytochemical investigations on *O. sanctum* have described the isolation of two principal groups of compounds, terpenoids and phenolic derivatives.[9–12] To date, over 25 terpenoid and fatty acid derivatives have been isolated from this plant, and they show anticancer,[13] anti-HIV,[14] antimicrobial,[15] and anti-inflammatory effects.[15] Phenolic substances also have been isolated from the leaves of *O. sanctum*, including hydroxycinnamic acid derivatives, benzoic acid derivatives, flavonoids and their glycosides, and eugenol and eugenol glycosides. Some of these compounds have been reported to exhibit antioxidant,[16] antimicrobial,[16] anti-inflammatory,[17] antistress,[8] anthelmintic, and radio-protective activities.[10]

As a result of the increasing use of *O. sanctum* for stress-induced disorders and as an immunomodulator, it was considered important to better understand the phytochemical constituents of this plant responsible for its antistress activity. In a preliminary pharmacological study, antistress activity was found for the ethanol extract of *O. sanctum* leaves in acute stress (AS) and chronic unpredictable stress (CUS) models. On bioassay-guided fractionation of an ethanol extract, activity was localized in a *n*-butanol-soluble fraction. Further work has led to the isolation and identification of three new compounds designated as ocimumoside A (**1**), ocimumoside B (**2**), and ocimarin (**3**), along with 10 known compounds from this *n*-butanol-soluble fraction. Compounds **1**–**3** and two of the known compounds, **4** and **5**, were evaluated for their ability to inhibit acute stress-induced biochemical changes such as hyperglycemia, plasma corticosterone, creatine kinase, and adrenal hypertrophy in rats. In addition, this is the first report of the presence of glycoglycerolipids (**1**, **2**), a coumarin (**3**), and cerebrosides 1-*O*-(*β*-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-8(*Z*)-octadecene-1,3,4-triol,[18] and 1-*O*-(*β*-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxytetracosanoyl amino]-8 (*Z*)-octadecene-1,3,4-triol[18] from *O. sanctum*.

Results and Discussion

The dried and powdered leaves of *O. sanctum* were extracted with ethanol and concentrated at 45 °C. The resulting dried ethanol extract was suspended in water and sequentially partitioned with chloroform and *n*-butanol. The *n*-butanol-soluble fraction was separated by a combination of chromatographic procedures, which afforded three new compounds (**1**–**3**), along with 10 known substances. The structures of the new compounds were established using chemical and spectroscopic (FABMS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC) studies, whereas the known compounds were identified by comparison of their spectroscopic data (UV, MS, and NMR) with published reports.

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* Corresponding author. Tel: +91-(522)-2612411-18, ext. 4235. Fax: +91-(522)-2623405/2623938/2629504. E-mail: mauryarakesh@rediffmail.com.

[†] Division of Medicinal and Process Chemistry.

[‡] Division of Pharmacology.

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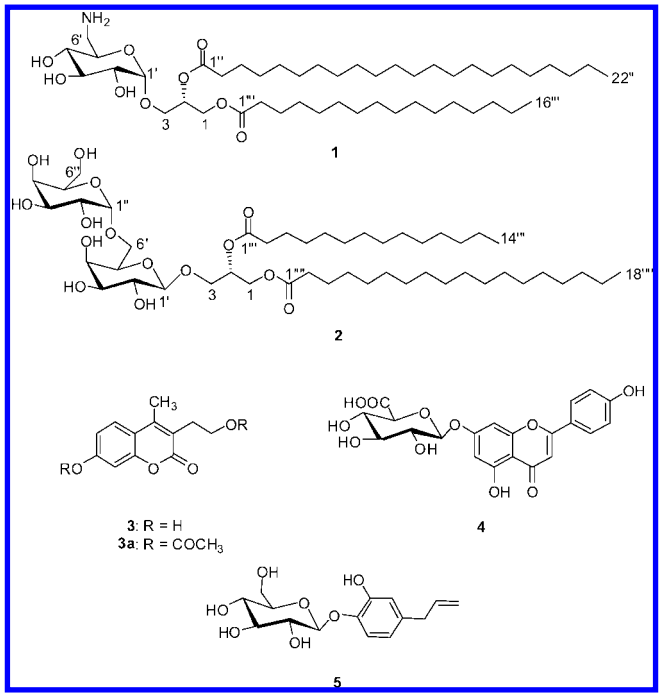

The ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** suggested certain common structural features attributable in each case to a sugar moiety, a glycerol system, and long aliphatic chains. Both compounds gave a positive Feigel test for glycosides. Compounds **1** and **2** revealed strong IR absorption bands for a hydroxyl group and an ester carbonyl, respectively, at ν_{max} 3416 and 1732 cm^{-1} (**1**) and 3417 and 1737 cm^{-1} (**2**).

Compound **1** was isolated as a white, amorphous powder and gave quasimolecular ion peaks at *m/z* 814 [M + H]$^+$ and 836 [M + Na]$^+$ in the positive FABMS, corresponding to a molecular formula $C_{47}H_{91}NO_9$, and this was supported from its NMR data and by elemental analysis. Assignment of all the ^{13}C and ^{1}H NMR signals for three spin systems is as shown in Table 1. The first spin system was assigned to a glycerol moiety (δ_H 4.14 and 3.90 (δ_C 62.8); δ_H 4.90 (δ_C 69.8); δ_H 3.90 and 3.54 (δ_C 64.8)); the second group of signals was attributable to two long-chain saturated fatty acids, where the terminal methyl signals appeared at δ_H 0.80 (6H, t, J = 6.9 Hz, δ_C 14.0), and the third spin system indicated the presence of a glycosyl moiety. Acid hydrolysis of **1** did not lead to the identification of any of the normal pyranose sugars. In the ^{1}H NMR spectrum, most of the glycosyl proton signals were overlapped with a solvent signal. Therefore, using the anomeric proton at δ_H 4.33 (d, J = 3.6 Hz) as a starting point, a sequence of four oxymethines at δ_H 3.31–3.27 (m) (δ_C 71.7, 73.0, 74.2, and 68.6) and one deshielded methylene at δ_H 2.65 and 2.83 (δ_C 54.4) was identified. The relatively upfield ^{13}C NMR resonance of C-6′ (δ_C 54.4) was indicative of the presence of an amino group at this position.[19] The α-glucopyranose nature of this sugar was determined by the small coupling constant, $J_{1'2'}$ = 3.6 Hz. Analysis of these data confirmed the assignment of the sugar unit as 6-deoxy-6-amino-α-glucopyranose. The HMBC experiment furnished useful data for solving the structure of **1** (Table 1). The sugar residue could be linked to the C-3 of the glycerol, as indicated by the HMBC correlation between anomeric proton H-1′ and C-3, while other HMBC cross-peaks indicated the attachment of acyl groups at the C-1 and C-2 position (H-2/C-1‴, H$_2$-2‴/C-1‴, H$_2$-1/C-1″, H$_2$-2″/C-1″) of the glycerol moiety.

According to its molecular formula, the two acyl moieties of **1** must represent a total of 38 carbon atoms. To infer the exact nature of the fatty acids, **1** was hydrolyzed in methanol/NaOMe (2 h). After the usual workup, the nonpolar organic extract was analyzed by GC-MS and FABMS, and two ion peaks [M + H]$^+$ at *m/z* 271 and 355 were observed, corresponding to hexadecanoic acid methyl ester and docosanoic acid methyl ester, repectively. Upon regioselective enzymic hydrolysis of **1** with lipase enzyme type III in dioxane/H$_2$O (1:1) at 37 °C for 4 h,[20] only hexadecanoic acid was obtained, as analyzed by ESIMS and compared with an authentic sample. Thus, it was concluded that a hexadecanoyl residue was attached to the C-1 position of the glycerol moiety in **1**.

Table 1. NMR Spectroscopic Data for Compound **1**[a]

position	δ_H (mult., J in Hz)	δ_C	HMBC	COSY
1a	4.14 (dd, 12.4, 4.4)	62.8	2, 3, 1‴	1b
1b	3.90 (dd, 11.3, 4.2)		2, 3, 1‴	1a, 2
2	4.90 (m)	69.8	1, 3, 1″	1b, 3b
3a	3.90 (dd, 10.5, 6.5)	64.8	1′, 1, 2	3b
3b	3.54 (dd, 10.5, 7.3)		1′, 1, 2	2, 3a
1′	4.33 (d, 3.6)	98.4	3, 5′, 3′	2′
2′	3.31–3.27 (m)[b]	71.7	4′	1′, 3′
3′	3.31–3.27 (m)[b]	73.0	1′, 5′	2′, 4′
4′	3.31–3.27 (m)[b]	74.2	6′, 2′	3′, 5′
5′	3.31–3.27 (m)[b]	68.6	1′, 3′	6′a,b; 4′
6′a	2.65 (dd, 11.2, 4.2)	54.4	4′, 5′	5′, 6′b
6′b	2.83 (dd, 10.8, 4.5)		4′, 5′	5′, 6′a
1″		172.6		
2″	2.44 (t, 6.4)	33.7	1″	3″
3″	2.16 (m)[c]	31.4	1″	2″
1‴		172.4		
2‴	2.23 (t, 7.2)	33.5	1‴,	3‴
3‴	2.16 (m)[c]	29.2	1‴	2‴
–CH$_2$	1.42–1.16 (m)	29.2–22.2		
CH$_3$	0.80 (6H, t, 6.9)	14.0		

[a] Recorded in DMSO-d_6 at 300 MHz (TMS as internal standard); chemical shifts, multiplicity, and coupling constants (J, Hz) were assigned by means of ^{1}H, ^{13}C NMR and 2D NMR data. [b] Overlapped with H$_2$O signals. [c] Overlapped signals that may be interchanged.

The aqueous phase was subjected to acid hydrolysis (2 N HCl, 30 min), and after workup and purification over an activated carbon column, methyl 6-deoxy-6-amino-α-D-glucopyranoside was obtained. The D-configuration of this sugar was confirmed by comparing its optical rotation ([α]$^{28}_D$ +139 in H$_2$O) with that reported in the literature for an authentic sample ([α]$^{25}_D$ +147 in H$_2$O).[21]

The absolute configuration of **1** was based on its ^{1}H NMR data and optical rotation ([α]$^{29}_D$ +50.2 in MeOH) data. In the CD spectrum, a negative Cotton effect was observed at 225 nm, which was shown to be identical in all respects to a diacylglycosylglycerolipid with known absolute stereochemistry.[22] Consequently, the structure of **1** was characterized as (2*S*)-1-*O*-hexadecanoyl-2-*O*-docosanoyl)-3-*O*-[6-deoxy-6-amino-α-D-glucopyranoside]glycerol, a new compound that has been named ocimumoside A. Compound **1** bears a rare 6-deoxy-6-aminoglucose moiety.[19,22]

Compound **2** exhibited quasimolecular ion peaks at *m/z* 915 [M + Na]$^+$ and *m/z* 910 [M + NH$_4$]$^+$ in the ESIMS, corresponding to the elemental formula $C_{47}H_{88}O_{15}$. It showed an almost identical NMR pattern to **1** except for the presence of additional sugar carbons and the absence of a signal at δ_C 54.4 in the ^{13}C NMR spectrum. Two anomeric carbon signals appeared at δ_C 103.8 (C-1′) and 99.6 (C-1″), whereas anomeric protons resonated at δ_H 4.50 (d, J = 6.0 Hz) and 4.65 (d, J = 3.0 Hz) in the ^{1}H NMR spectrum (Table 2). The downfield-shifted carbon signal of C-6′ (at δ_C 66.8) compared to C-6″ (at δ_C 60.7) indicated a glycosidic linkage between C-1″ and C-6′. This was supported by a HMBC correlation between H-1″/C-6′, and the coupling constant of H-1″/H-2″ (J = 3.0 Hz, axial–equatorial) inferred an α-glycosidic linkage. Other sugar protons and carbons (Table 2) were identical to digalactoside,[23] a substituent confirmed by acid hydrolysis of **2** followed by co-TLC with an authentic sample. Another spin system was assigned to a glycerol moiety by ^{1}H and ^{13}C, ^{1}H–^{1}H COSY, and HSQC NMR spectroscopy (δ_H 4.33 and 4.11, δ_C 62.5; δ_H 5.08, δ_C 70.4; δ_H 3.77 and 3.56, δ_C 69.7). The remaining NMR signals were

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

position	δ_H (mult., J in Hz)	δ_C	HMBC	COSY
1a	4.33 (dd, 12.0, 6.0)	62.5	2, 3, 1‴″	1b
1b	4.11 (dd, 12.0, 3.0)		2, 3, 1‴″	1a, 2
2	5.08 (m)	70.4	1, 3, 1‴	1b, 3b
3a	3.77 (dd, 12.3, 6.0)	69.7	1′, 1, 2	3b
3b	3.56 (dd, 9.3, 3.1)		1′, 1, 2	2, 3a
1′	4.50 (d, 6.0)	103.8	3, 5′, 3′	2′
2′	3.51–3.44 (m)[b]	73.0	4′	1′, 3′
3′	3.51–3.44 (m)[b]	70.0	1′, 5′	2′, 4′
4′	3.54 (bs)	68.1	6′, 2′	3′, 5′
5′	3.59 (t, 5.7)	73.2	1′, 3′	6′a,b; 4′
6′a	3.51–3.44 (m)[b]	66.8	1″, 4′	5′
6′b	3.51–3.44 (m)[b]		1″, 4′	5′
1″	4.65 (d, 3.0)	99.6	5″, 6′a, 3″	2″
2″	3.80 (m)	69.7	4″	1″, 3″
3″	3.63 (dd, 9.2, 3.5)	68.5	1″, 5″	2″, 4″
4″	3.69 (dd, 3.2, 1.6)	69.0	6″, 2″	3″, 5″
5″	3.59 (t, 5.7)	71.4	1″, 3″	6″a,b; 4″
6″a	2.71 (dd, 11.1, 5.2)	60.7	4″	6″b, 5″
6″b	3.44 (m)[b]		4″	6″a, 5″
1‴		172.7		
1‴″		172.4		
2‴	2.29 (t, 6.2)	33.7	1‴	3‴
2‴″	2.29 (t, 6.2)	33.5	1‴″	3‴″
3‴	1.46 (m)[c]	27.5	1‴	
3‴″	1.46 (m)[c]	27.5	1‴″	
$-CH_2$	1.35–1.15 (m)	30.8–22.2		
CH_3	0.81 (6H, t, 6.1)	14.0		

[a] Recorded in DMSO-d_6 at 300 MHz (TMS as internal standard); chemical shifts, multiplicity, and coupling constants (J, Hz) were assigned by means of ^1H, ^{13}C NMR and 2D NMR data. [b] Overlapped with H_2O signals. [c] Overlapped signals that may be interchanged.

assigned for two long-chain fatty acid units, which accounted for 32 carbon atoms according to the molecular formula and ^{13}C NMR spectrum. To determine the length of these fatty acids, compound **2** was treated with methanol/NaOMe and afforded glyceryldigalactose (**2a**) and two fatty acid residues. The fatty acid residues were determined by GC-MS as methyl tetradecanoate and methyl octadecanoate. The HMBC spectrum indicated that two acylating fatty chains were attached to C-1 and C-2 of the glycerol moiety, whereas C-3 was involved in a β-glycosidic linkage with a sugar residue (J = 6.0 Hz, H-1′). Compound **2a**, $[\alpha]^{29}_D$ +83 in H_2O, was shown to be identical in all respect to (2*R*)-1-*O*-[α-D-galactopyranosyl-(1″→6′)-*O*-β-D-galactopyranosyl] glycerol,[24] confirming the configurations of both the sugar and glycerol moieties in the molecule. The sequence of acyl chains between C-1 and C-2 was determined by regioselective enzymatic hydrolysis of **2** (as above),[20] with only octadecanoic acid being obtained, as determined by ESIMS. Consequently, **2** was characterized as (2*S*)-1-*O*-octadecanoyl-2-*O*-tetradecanoyl)-3-*O*-[α-D-galactopyranosyl-(1″→6′)-*O*-β-D-galactopyranosyl] glycerol and is a new compound that has been given the trivial name ocimumoside B.

Compound **3** was obtained as a brown, amorphous powder, and its molecular formula, $C_{12}H_{12}O_4$, was established by positive FABMS at *m/z* 221 $[M + H]^+$ and from its NMR data. The UV absorption bands, UV (MeOH) λ_{max} (log ε) 320 (2.61), 204 (2.72) nm, were characteristic of a coumarin derivative.[25] The IR spectrum exhibited absorption bands at ν_{max} 3533, 1720, 1616, 1570, and 1358 cm^{-1} and clearly indicated the presence of an α,β-unsaturated carbonyl ester, a hydroxyl group, and an aromatic ring in the molecule. On acetylation, it formed a diacetate, **3a** (FABMS at *m/z* 305 $[M + H]^+$), confirming the presence of two free hydroxyl groups. The ^1H NMR spectrum (Table 3) showed signals at δ_H 7.54 (1H, d, J = 8.8 Hz), 6.78 (1H, dd, J = 8.6, 2.2 Hz), and 6.65 (1H, d, J = 2.0 Hz) and indicated the presence of an ABX system. In addition, two sharp triplets appeared at δ_H 3.48 (2H, t, J = 7.0 Hz) and 2.67 (2H, t, J = 6.8 Hz) and a singlet at δ_H 2.33 (3H, s), due to the presence of hydroxyl ethyl and methyl groups, respectively. Its ^{13}C NMR spectrum (Table 3) showed 12 carbons

Table 3. NMR Spectroscopic Data for Compound **3**[a]

position	δ_H (mult., J in Hz)	δ_C	HMBC
2		161.5	
3		118.8	
4		148.6	
5	7.54 (d, J = 8.8)	126.7	4, 7, 10
6	6.78 (dd, J = 2.2, 8.6)	113.1	7, 8, 10
7		160.4	
8	6.65 (d, J = 2.0)	102.1	6, 7, 10
9		153.6	
10		112.9	
CH_3	2.33 (s)	15.2	3, 4, 10
$\underline{CH_2}$-CH_2-OH	2.67 (t, J = 6.8)	31.1	2, 3, 4
CH_2-$\underline{CH_2}$-OH	3.48 (t, J = 7.0)	59.7	3

[a] Recorded in DMSO-d_6 at 200 MHz (TMS as internal standard). Assignments are based on HSQC and HMBC experiments.

in the molecule, and their multiplicity was assigned by a DEPT experiment, revealing the presence of six quaternary carbons at δ 161.5 (CO), 160.4, 153.6, 148.6, 118.8, and 112.9, three aromatic methines at δ 126.7, 113.1, and 102.1, two methylenes at δ 59.7 and 31.1, and one methyl group at δ 15.2, respectively. The HMBC experiment provided useful correlations between CH_3/C-3, C-4, C-10 and CH_2/C-2, C-3 and confirmed the position of the methyl and hydroxy ethyl groups in **3**. From the above data, the structure of **3** was elucidated as 7-hydroxy-3-(2-hydroxyethyl)-4-methyl-2*H*-1-benzopyran-2-one, a new naturally occurring compound named ocimarin.

The structures of several known compounds isolated were identified as apigenin,[26] apigenin-7-*O*-β-D-glucuronic acid (**4**),[27] apigenin-7-*O*-β-D-glucuronic acid 6″-methyl ester,[28] luteolin-7-*O*-β-D-glucuronic acid 6″-methyl ester,[29] 4-allyl-1-*O*-β-D-glucopyronosyl-2-hydroxybenzene (**5**),[12] apigenin-7-*O*-β-D-glucopyranoside,[26] luteolin-7-*O*-β-D-glucopyranoside,[30] and luteolin-5-*O*-β-D-glucopyranoside.[31] In addition, two known cerebrosides, 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-8(*Z*)-octadecene-1,3,4-triol and 1-*O*-(β-D-glucopyranosyl)-(2 *S*,3 *S*,4 *R*,8 *Z*)-2-[(2′*R*)-2′-hydroxytetracosanoylamino]-8(*Z*)-octadecene-1,3,4-triol, were isolated and characterized using detailed spectroscopic (^1H–^1H COSY, HSQC, HMBC, and NOESY) and chemical degradation studies.[18]

A variety of stress situations have been employed in animals to evaluate antistress activity. Stress-induced effects mainly depend on duration and type of stressors.[32] 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, neuroendocrine, and immunological pathology.[33] 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.[34]

It is know that AS exposure not only stimulates and ensures the supply of glucose but also increases creatine kinase (CK) activity.[35] 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.[36] Prominent changes during stress are adrenocorticoid hypersecretion, increased plasma corticosterone, and enlarged pituitary and adrenal size.[37,38] In our work, various type of stress also increased the adrenal gland weight and plasma corticosterone.[3]

In the present investigation, the EtOH extract and its *n*-butanol-soluble fraction of *O. sanctum*, at a dose of 200 mg/kg body weight, were significantly ($p < 0.05$) effective in normalizing acute stress (AS)- and chronic unpredictable stress (CUS)-induced hyperglycemia, plasma corticosterone, plasma creatine kinase, and adrenal gland weight in the rat model used. A chloroform-soluble fraction of *O. sanctum* was ineffective in normalizing most of these biochemical changes and only effective in reducing the acute stress-induced hyperglycemia,

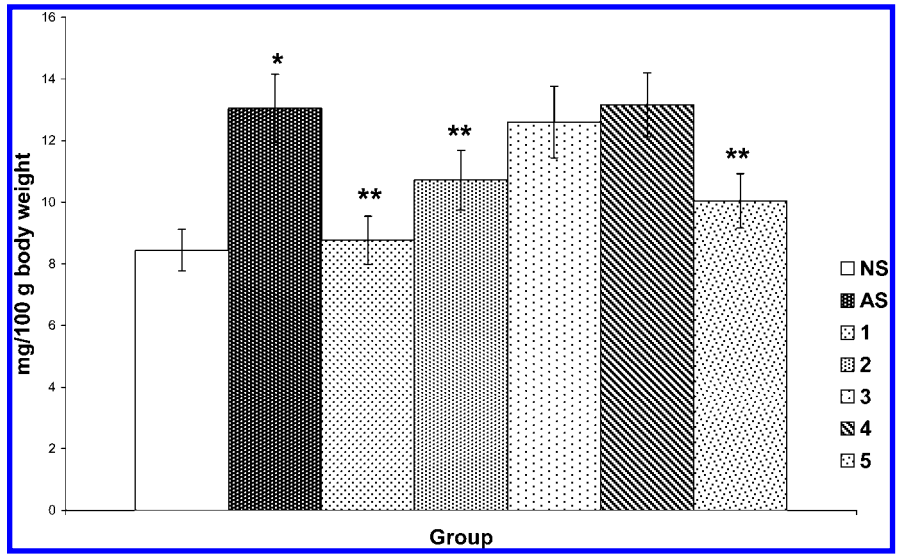

Figure 1. Effect of compounds **1**–**5** at 40 mg/kg body weight on stress-induced changes in the rat adrenal gland. The stress group was compared with the nonstress control group, and the drug-treated groups were compared with an acute stress group. Results are represented as means ± SEM with $n = 6$ in each group (*$p < 0.01$ when compared to the nonstress (NS) group and **$p < 0.05$ when compared to the acute stress (AS) group).

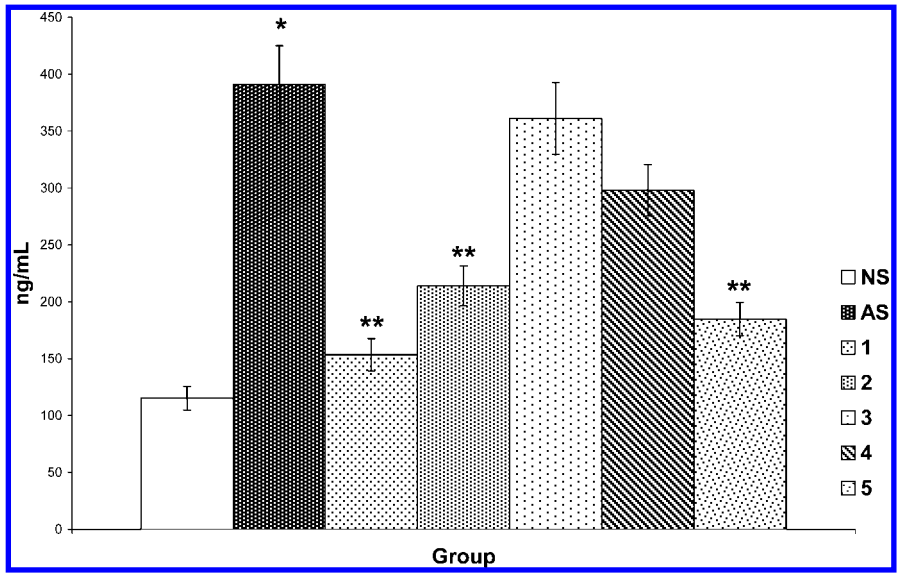

Figure 2. Effect of compounds **1**–**5** at 40 mg/kg body weight on stress-induced changes in rat plasma corticosterone levels. The stress group was compared with a nonstress control group, and the drug-treated groups were compared with the acute stress group. Results are represented as means ± SEM with $n = 6$ in each group (*$p < 0.01$ when compared to the nonstress (NS) group and **$p < 0.05$ when compared to the acute stress (AS) group).

adrenal gland weights, and the CUS-induced plasma creatine kinase levels. Moreover, the aqueous fraction of the plant was found ineffective in normalizing any of the stress-induced changes. The antistress effects of the EtOH extract and the *n*-butanol-soluble fraction were comparable to those of a standard drug, *Panax quinquifolium* (PQ), at a dose of 100 mg/kg body weight (Figures S1–S4, Supporting Information).

Several isolated compounds (**1**–**5**) from *O. sanctum* were tested for antistress activity in the acute stress model at a dose of 40 mg/kg body weight. A significant increase ($p < 0.01$) in the adrenal gland weight after AS was observed when compared to a nonstress (NS) group. Compounds **1** and **5** were significantly ($p < 0.05$) effective in reducing the stress-induced adrenal hypertrophy. Similarly, AS ($p < 0.01$) exposure resulted in increased plasma corticosterone when compared to the NS control. Pretreatment with compounds **1**, **2**, and **5** ($p < 0.05$) reduced significantly the increase in corticosterone levels. The plasma CK and glucose levels were also increased by AS significantly ($p < 0.01$) when compared to the NS control. Prior treatments of compounds **1**, **2**, and **5** ($p < 0.01$) were effective in reducing the AS-induced increase in CK levels. Compounds **1** and **5** were observed to reduce increased glucose levels.

In conclusion, compound **1** has shown significant ($p < 0.05$) antistress activity by normalizing hyperglycemia, corticosterone levels, creatine kinase, and adrenal hypertrophy. Compound **2** also showed antistress activity but had no effect on plasma glucose levels. Compound **5** was found to be effective in most of the stress parameters evaluated but less effective on plasma glucose levels. Compounds **3** and **4** were ineffective in normalizing these parameters (Figures 1–4). The other isolated compounds from *O. sanctum* could not be tested due to the insufficient amounts obtained. The biological activity profiles of compounds **1**, **2**, and **5** described herein are worthy of further investigation.

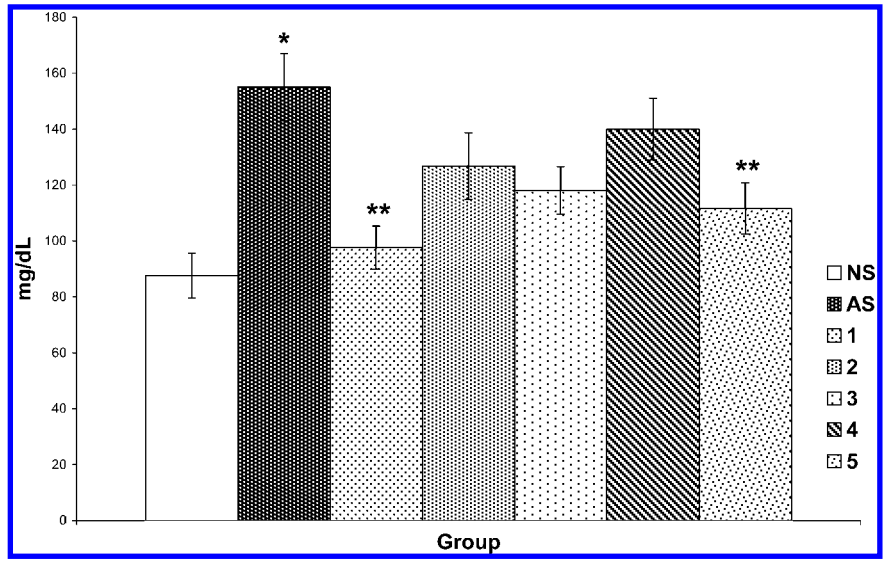

Figure 3. Effect of compounds **1**–**5** at 40 mg/kg body weight on stress-induced changes in rat plasma glucose levels. The stress group was compared with the nonstress control group, and the drug-treated groups were compared with the acute stress group. Results are represented as means ± SEM with $n = 6$ in each group (*$p < 0.01$ when compared to the nonstress (NS) group and *$p < 0.05$ when compared to the acute stress (AS) group).

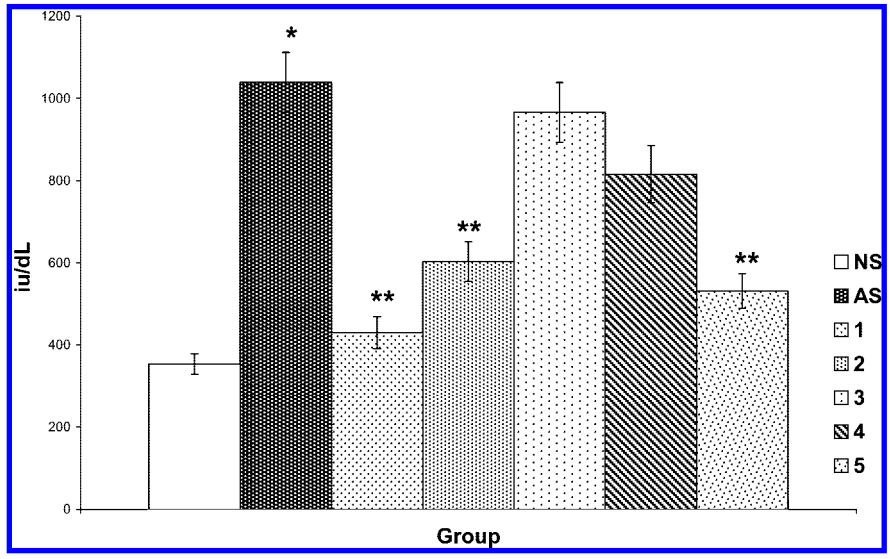

Figure 4. Effect of compounds **1**–**5** at 40 mg/kg body weight on stress-induced changes in rat plasma creatine kinase levels. The stress group was compared with a nonstress control group, and the drug-treated groups were compared with an acute stress group. Results are represented as means ± SEM with $n = 6$ in each group (*$p < 0.01$ when compared to the nonstress (NS) group and **$p < 0.05$ when compared to the acute stress (AS) group).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 241 digital polarimeter. UV spectra were obtained on a Perkin-Elmer λ-15 UV spectrophotometer. IR spectra were recorded on a Perkin-Elmer RX-1 spectrophotometer using either KBr pellets or when neat. The CD spectrum was obtained on JASCO J-810 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on an Avance DPX-200 or a Bruker DRX 300 MHz NMR spectrometer; proton-detected heteronuclear correlations were measured using the HSQC and HMBC techniques. All 2D-NMR spectra were recorded on a Varian Inova 600 MHz NMR spectrometer. FABMS were carried out on a JEOL SX 102/DA-6000 mass spectrometer and ESIMS on a Advantage Max LCQ Thermo-Finnigan mass spectrometer. HRESIMS analysis was carried out on a JEOL-MS 600H instrument. Elemental analyses were obtained in a Carlo-Erba-1106 CHN elemental analyzer. GC analyses were performed on a Perkin-Elmer XL auto-system using a SE-30 capillary column (10 ft), with 30 mL of N_2 as carrier gas at 60–250 °C. 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 was carried out on precoated 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 or anisaldehyde–H_2SO_4 reagents.

Plant Material. The plant material was collected from Ajamgarh, Uttar Pradesh, India, in November 2002 and was confirmed as *Ocimum sanctum* by staff members of the Botany Division of CDRI, where a voucher specimen (No. 38) is preserved in the herbarium.

Extraction and Isolation. Powdered leaves of *Ocimum sanctum* (10 kg) were placed in a glass percolator with ethanol (25 L) and allowed to stand at room temperature overnight. The percolate was collected. This process of extraction was repeated four times. The combined extract was filtered, concentrated at 45 °C under a vacuum, and afforded an ethanol extract (C003, 820 g). This ethanol extract (800 g) was suspended in distilled water (400 mL) and then extracted successively with chloroform (600 mL × 7) and *n*-butanol saturated with water (500 mL × 7). The chloroform-, *n*-butanol-, and water-soluble extracts were concentrated at 45 °C and afforded chloroform

(F004, 99 g), *n*-butanol (F005, 445.7 g), and aqueous (F006, 270.4 g) residues, respectively.

A portion of the *n*-butanol-soluble fraction (300 g) was subjected to column chromatography over silica gel (230–400 mesh, 2.1 kg) and eluted with a gradient solvent system composed of chloroform–methanol (95:05) to methanol–water (95:05). Ninety-three fractions (600 mL each) were obtained, and their composition was monitored by TLC, with those showing similar TLC profiles grouped into six major fractions (F-1 to F-6). Fraction F-1 afforded compound **3** (190 mg) by crystallization from methanol. Further purification of fraction F-2 (30.4 g) over silica gel (230–400 mesh, 300 g), using a gradient solvent system of $CHCl_3$–MeOH (95 to 50%), afforded 65 fractions (500 mL each), and these were grouped into seven further fractions (F-7 to F-13) on the basis of their TLC profiles. Purification of fraction F-7 (1.5 g) over silica gel (60–120 mesh, 40 g), using $CHCl_3$–MeOH (95:5), afforded apigenin (40 mg). Compound **4** (445 mg) was obtained as an amorphous solid from fraction F-4 at room temperature. Column chromatography of fraction F-9 (6.0 g), over silica gel (230–400 mesh, 160 g), using ethyl acetate saturated with water (isocratic) as mobile phase, afforded 42 fractions (200 mL each), which were pooled into five further fractions on the basis of their TLC profiles (F-14 to F-18). Fraction F-18, containing a mixture of two compounds (300 mg), was purified over Sephadex LH-20, eluted with water–methanol (1:1) and resulting in the collection of 55 subfractions, 10 mL in volume. Subfractions 24–32 yielded 1-*O*-(*β*-D-glucopyranosyl)-(2 *S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxydocosanoylamino]-8(*Z*)-octadecene-1,3,4-triol (35 mg), and subfractions 33–38 yielded 1-*O*-(*β*-D-glucopyranosyl)-(2*S*,3*S*,4*R*,8*Z*)-2-[(2′*R*)-2′-hydroxytetracosanoylamino]-8(*Z*)-octadecene-1,3,4-triol (18 mg). Column chromatography of F-10 (4.2 g) over silica gel (60–120 mesh, 120 g), using ethyl acetate saturated with water as mobile phase, afforded 31 fractions of 100 mL each, with those showing similar TLC profiles grouped into five additional fractions (F-19 to F-23). Further purification of fraction F-20 (700 mg) over silica gel (230–400 mesh, 20 g) using ethyl acetate saturated with water (isocratic) as mobile phase afforded apigenin-7-*O*-*β*-D-glucuronic acid 6″-methyl ester (40 mg). Purification of fraction F-11 (3.2 g) over silica gel (230–400 mesh, 90 g) using ethyl acetate saturated with water as mobile phase afforded a total of 35 fractions, 50 mL in volume. These were grouped into six major fractions (F-24 to F-29) on the basis of their TLC profiles. Fraction F-25 (150 mg) was purified by medium-pressure liquid chromatography on reversed-phase silica gel (RP-18), eluted with a gradient of MeOH–H_2O (1:1) to methanol, to generate 40 subfractions, 15 mL in volume. Subfractions 25–31 gave a yellow, amorphous solid of luteolin-7-*O*-*β*-D-glucuronic acid 6″-methyl ester (35 mg). Fraction F-12 (3.7 g) was rechromatographed over silica gel (230–400 mesh, 100 g), eluted with gradient of ethyl acetate and methanol 95–80%, and yielded fractions F-30 to F-34. Fraction 34 was kept in a refrigerator overnight and yielded a further quantity of **1** (62 mg). Column chromatography of F-22 (1.1 g) using EtOAc–MeOH (95:5) as eluent over silica gel (100–200 mesh, 25 g) afforded **5** (500 mg). Purification of F-28 (300 mg) on reversed-phase silica gel (RP-18), eluted with H_2O–MeOH (6:4), afforded 60 fractions, 15 mL in volume. Subfractions 38–45 yielded compound **2** (48 mg). Purification of fraction F-30 (1.2 g) over silica gel (100–200 mesh, 24 g), using a gradient solvent system of $CHCl_3$–MeOH (95:05) to $CHCl_3$–MeOH (70:30), yielded apigenin-7-*O*-*β*-D-glucopyranoside (30 mg, fractions 10–15), luteolin-7-*O*-*β*-D-glucopyranoside (35 mg, fractions 21–29), and luteolin-5-*O*-*β*-D-glucopyranoside (27 mg, fractions 35–40), respectively.

Ocimumoside A (1): white, amorphous powder; $[\alpha]^{29}_D$ +50.2 (*c* 0.04, MeOH); UV (*c* 0.04, MeOH) λ_{max} (log ε) 225 (3.00) nm; IR (KBr) ν_{max} 3416, 3406, 2910, 1732, 1673 cm^{-1}; CD (*c* 0.04, MeOH) ($\Delta\varepsilon$ –3.5) nm; ^1H (DMSO-d_6, 300 MHz) and ^{13}C (DMSO-d_6, 75 MHz) NMR spectra, see Table 1; FABMS (positive-ion) *m/z* 814 $[M + H]^+$, 836 $[M + Na]^+$; *anal*. C 66.21%, H 11.31%, N 1.73%, calcd for $C_{47}H_{91}O_9N$, C 66.33%, H 11.26%, N 1.72%.

Ocimumoside B (2): white, hygroscopic solid; $[\alpha]^{29}_D$ + 70.0 (*c* 0.04, MeOH); IR (KBr) ν_{max} 3417, 2910, 1737, 1042 cm^{-1}; ^1H (DMSO-d_6, 300 MHz) and ^{13}C (DMSO-d_6, 75 MHz) NMR spectra, see Table 2; ESIMS (positive ion) *m/z* 915 $[M + Na]^+$, 910 $[M + NH_4]^+$; HRESIMS *m/z* $[M]^+$ 892.6143 (calcd for $C_{47}H_{88}O_{15}$, 892.6123).

Ocimarin (3): brown, amorphous powder; UV (MeOH) λ_{max} (log ε) 320 (2.61), 204 (2.72) nm; IR (KBr) ν_{max} 3533, 2927, 1720, 1616, 1570, 1027 cm^{-1}; ^1H (DMSO-d_6, 200 MHz) and ^{13}C (DMSO-d_6, 50 MHz) NMR spectra, see Table 3; FABMS (positive-ion) *m/z* 221 $[M + H]^+$, 243 $[M + Na]^+$; *anal*. C 65.41%, H 5.41%, calcd for $C_{12}H_{12}O_4$, C 65.45%, H, 5.49%.

Acid and Basic Hydrolysis of 1 and 2. Compounds **1** (15 mg, 0.018 mmol) and **2** (10 mg, 0.011 mmol) were separately dissolved in methanol (3 mL), and NaOMe (5 mg, 0.092 mmol) was added; the reaction mixtures were stirred at rt for 3 h. The reaction mixtures were quenched with the acidic ion exchange resin Amberlite IRC-50 (Rohm and Hass, H^+ form), with the resin removed by filtration, and the filtrate was dried under reduced pressure and partitioned between chloroform and water. The organic layers from compounds **1** and **2** were analyzed by GC-MS. The aqueous layer of compound **1** was treated with 2 N HCl (5 mL) and refluxed for 30 min. The reaction mixture was worked up in the usual manner and the sugar fraction isolated on an activated carbon column to give methyl 6-deoxy-6-amino-α-D-glucopyranoside (2.1 mg), identified by comparison with an authentic sample (TLC) and by its optical rotation ($[\alpha]^{28}_D$ +139 in H_2O). The aqueous layer of **2** afforded compound **2a**, which had optical rotation $[\alpha]^{29}_D$ +83 in H_2O and was identical in all respect to (2*R*)-1-*O*-[α-D-galactopyranosyl-(1″→6′)-*O*-*β*-D-galactopyranosyl] glycerol.[24]

Enzymatic Hydrolysis of 1 and 2. Compounds **1** and **2** (2 mg) were each dissolved in 4 mL of dioxane–H_2O (1:1) and treated with lipase enzyme type III (2 mg, 46 unit, from a *Pseudomonas* species, lot 093K0698, Sigma-Aldrich) at 37 °C, with shaking for 4 h. The reaction mixtures of compounds **1** and **2** were quenched with 5% AcOH (1 mL), and the product was dried under reduced pressure. The crude residues of compounds **1** and **2** were dissolved in water and extracted with EtOAc, concentrated under reduced pressure, and analyzed by ESIMS.

Acetylation of Compound 3. Compound **3** (10 mg) was dissolved in dry pyridine, (2.0 mL) and acetic anhydride (2 mL) was added, with the reaction mixture left overnight at room temperature. The reaction mixture was dried under reduced pressure. The crude residue was dissolved in methanol and cooled, and an amorphous powder of **3a** (13 mg) was obtained: ^1H NMR ($CDCl_3$, 200 MHz) δ_H 7.63 (1H, d, J = 8.0 Hz, H-5), 7.09 (1H, d, J = 1.2 Hz, H-8), 7.07 (1H, dd, J = 8.0, 1.2 Hz, H-6), 4.30 (2H, t, J = 6.6 Hz, CH_2OH), 3.06 (2H, t, J = 6.6 Hz, CH_2–CH_2OH), 2.33 (3H, s, CH_3), 2.46 and 2.02, (3H each, acetoxyl methyl); FABMS *m/z* 305 $[M + H]^+$.

Antistress Activity Determination. Animals. Adult male Sprague–Dawley rats (180–200 g) were obtained from the National Animal Laboratory Centre, CDRI, Lucknow, India. Animals were kept in raised-mesh-bottom cages to prevent coprophagy in environmentally controlled rooms (25 ± 2 °C, 12 h light and dark cycle), and animals had free access to standard pellet chow and drinking water except during experiments. Experiments were conducted between 9 a.m. and 2 p.m. Experimental protocols were approved by an institutional ethical committee following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), which complies with the international norms of INSA (Indian National Science Academy).

Test Compound Administration. Suspensions of test materials from *O. sanctum* in 0.1% sodium carboxymethylcellulose were prepared and administered orally at a dose of 200 mg/kg (for the ethanol extract and fractions) once daily for three days and 40 mg/kg (for isolated compounds) once only, in case of acute stress (AS), and for seven days in case of chronic unpredictable stress (CUS), 45 min prior to a stress session. Control animals received an equivalent volume of vehicle for the same period. After the stress regimen, animals were sacrificed by decapitation on the seventh day immediately after the last stress session. A freshly prepared aqueous suspension of the crude powder of ginseng root, *P. quinquifolium*, was used as a standard at a dose of 100 mg/kg body weight and was purchased from Sigma, St. Louis, MO (cat. no. G7253).

Stress Protocol. The rats were divided into nonstress (NS), AS, and CUS groups as well as drug-treated groups for both AS and CUS groups. Each group consists of six rats. In the AS model, 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, rats were stressed. A parallel group of vehicle-treated rats without exposure to any kind of stress and maintained under normal conditions, served as control nonstress group. In the CUS groups the drugs were fed daily 45 min prior to the stress regime for seven days, with the rats fasted overnight on the sixth day after completion of the experimental regimens of drug feeding and stress exposure.

AS was produced by immobilizing animals for 150 min once only and sacrificed immediately. CUS was produced with variable stressors with modifications as described by Katz and co-workers.[39] The stressors included immobilization, forced swimming, soiled cage bedding, foot shock, day–night reversal, and fasting. Animals were subjected to two stressors of variable intensity every day in an unpredictable manner to avoid habituation for seven consecutive days.[3] Briefly, immobilization stress was produced by restraining each naive animal inside an acrylic hemicylindrical plastic tube (4.5 cm diameter, 12 cm long) for 150 min. In the swimming stress test, animals were allowed to swim in a glass jar (35.5 cm long, 20.2 cm diameter) containing water at 25 °C for 20 min. In soiled cages, the bedding was wet with water to produce overnight inconvenience. In the foot shock stress test, animals were subjected to foot shock (2 mA) in an agressometer (Techno Electronics, Lucknow, India) for 20 min on a grid floor with a shock interval of 2 s. Day and night reversal was produced by keeping the animals in the dark during the day and in high-intensity light during the night.

Gastric Ulceration. Immediately after the stress session, rats were killed by decapitation, the stomachs were split open along the greater curvature, and the number of discrete ulcers was noted using a magnascope under 5× magnification. Ulcers were scored according to the method of Gupta et al., and the mean ulcer severity score was calculated.[40]

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).

Estimation of Corticosterone. An HPLC/UV system (Waters, Milford, MA) was used for quantification of plasma corticosterone by the method of Woodward and Emery, with modifications.[41] Dexamethasone was used as 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 a UV detector. The chromatogram was recorded and analyzed with Breeze software (3.20 version).

Estimation of Glucose and Creatine Kinase. An autoanalyzer (Synchron Cx-5, Beckman) was used to estimate glucose and creatine kinase with their respective kits (Beckman Coulter International, Nyon, Switzerland).

Statistical Analysis. Data are represented as mean and SEM and analyzed using one-way analysis of variance (ANOVA) followed by Newman–Keul's multiple comparison test. Data for the ulcers were analyzed by nonparametric ANOVA followed by Dunn's multiple comparison tests, with $p < 0.05$ considered to be statistically significant.

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Supporting Information Available: Figures S1–S4, providing antistress activity profile of ethanol extract C003 and its fractions F004-F006 on AS- and CUS-induced changes in adrenal gland weight, plasma glucose, corticosterone levels, and creatine kinase. This information is available free of charge via the internet at http://pubs.acs.org.

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