PHYTOTHERAPY RESEARCH
Phytother. Res. **30**: 805–814 (2016)
Published online 22 February 2016 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5584

Anti-stress Activity of *Ocimum sanctum*: Possible Effects on Hypothalamic–Pituitary–Adrenal Axis

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The present study investigated anti-stress potential of *Ocimum sanctum* in chronic variable stress (CVS) paradigm. Further, the possible mechanism of anti-stress was explored *in vitro* using cell and cell-free assays. Rats were administered *O. sanctum* followed by CVS regimen for a period of 16 days. On days 4, 8, 12, and 16, body weight and immobility time in forced swim test were measured. In addition, the possible inhibitory effect of *O. sanctum* and ursolic acid on cortisol release and CRHR1 receptor activity were studied in cell-based assays, while inhibitory effects on 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and catechol-*O*-methyltransferase (COMT) were studied in cell-free assays. CVS group demonstrated less body weight gain and higher immobility time than *O. sanctum* administered groups, while oral administration of *O. sanctum* significantly increased body weight gain and decreased the immobility time. Further, *O. sanctum* and its constituents inhibited cortisol release and exhibited a significant CRHR1 receptor antagonist activity. Also, they had specific inhibitory activity towards 11β-HSD1 and COMT activity. Thus, *O. sanctum* was found to be effective in the management of stress effects, and anti-stress activity could be due to inhibition of cortisol release, blocking CRHR1 receptor, and inhibiting 11β-HSD1 and COMT activities. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: anti-stress; catechol-*O*-methyltransferase; cortisol; corticotropin-releasing factor receptor 1; forced swim test; *Ocimum sanctum*.

INTRODUCTION

Stress becomes an integral and inevitable component of present life situations affecting the general health of the individuals. It is defined as psychological, physiological, and behavioral response by individuals when they perceive a lack of equilibrium between the demands placed upon them and their ability to meet those demands. In addition, the societal, financial costs, and the healthcare costs involved due to the stress-related disorders are extensive (Bakker *et al.*, 2006), and hence, it is clear that intervention for the management of stress is of paramount importance. Despite the high prevalence of disabling adjustment disorder and severe challenge to healthcare systems, an approved synthetic intervention for general stress is lacking till date. Alternatively, herbal medicines have gained global attention in recent years for human health ailments and could be potential sources for alleviating stress (Darbinyan *et al.*, 2000; Kalman *et al.*, 2008; Milesi *et al.*, 2009).

One of the predominant traditional remedy for anti-stress activity is *Ocimum sanctum* (synonym – *Ocimum tenuiflorum*). *O. sanctum*, also known as Holy Basil or Tulsi, belongs to the family Lamiaceae. It is widely distributed in India and is considered as a sacred plant. The word Tulsi in Sanskrit connotes 'the incomparable ones' with enormous potential for treating and preventing diseases (Singh *et al.*, 2010). Pharmacological properties of *O. sanctum* have been attributed to various phytochemical constituents such as eugenol, ursolic acid, rosmarinic acid, apigenin, myretenal, luteolin, β-sitosterol, and carnosic acid (Pattanayak *et al.*, 2010; Baliga *et al.*, 2013). Available literature indicates that pre-treatment with eugenol for 7 days decreased the 4-h restraint stress-induced increase in stomach ulcer index and plasma corticosterone, indicating a preferential effect on the hypothalamic–pituitary–adrenal (HPA) axis (Garabadu *et al.*, 2011). Yet another study by Yi *et al.* (2013) examined the effect of oleanolic acid in mice exposed to the repeated forced swimming test. The duration and latency of immobility affected by oleanolic acid (10, 20, and 40 mg/kg) were evaluated in the forced swimming test repeated at intervals on days 1, 7, and 14. The findings demonstrated that sub-chronic and chronic oleanolic acid treatment reduced the immobility time and increased the latency to immobility (Yi *et al.*, 2013).

Herbs under adaptogen category are better suited for combating stress, and Tulsi is considered to be an adaptogen, balancing different processes in the body and helpful for adapting to stress (Hershoff and Rotelli, 2001; Chandrasekhar *et al.*, 2012). Several preclinical and clinical studies have reported the anti-stress effects of *O. sanctum* (Ravindran *et al.*, 2005; Bathala *et al.*, 2012; Saxena *et al.*, 2012). Although preclinical evidence on the anti-stress activity of *O. sanctum* is available (Ravindran *et al.*, 2005; Tabassum *et al.*, 2010; Bathala *et al.*, 2012), studies that evaluated *O. sanctum* in chronic variable stress (CVS) paradigm are not reported. In addition, stress causes dysregulation of HPA axis and catecholamines (Ramanathan

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Received 13 April 2015
Revised 14 January 2016
Accepted 17 January 2016

et al., 2011; Rasheed and Alghasham, 2012). HPA axis activation in stress initiates increased corticotropin-releasing factor (CRF) release by hypothalamus, and consequently, CRF binds to the CRHR1, a predominant receptor in anterior pituitary that subsequently induces the synthesis and release of cortisol (McEwen, 2005; Sharma *et al.*, 2013). On the other hand, enzyme known as 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays an important role in the conversion of inactive cortisone to active cortisol (Sun *et al.*, 2011). Further, stress has profound effect on the catecholamines. Although *O. sanctum* is reported to modulate catecholamines (Tabassum *et al.* 2010), the effect of *O. sanctum* on a catechol-*O*-methyltransferase (COMT) inhibition that spares catecholamines needs to be elucidated. Hence, the effect of O. *sanctum* on CRHR1 activity, cortisol release, 11β-HSD1, and COMT provides insights on the action mechanism.

Keeping in view the aforementioned, the present study was conducted to investigate the anti-stress effects of *O. sanctum* in CVS paradigm that closely mimics the stress conditions in human. Also, the effects of *O. sanctum* and its constituents on CRHR1 activity and cortisol release were evaluated in cell-based assays; the effects on 11β-HSD1 and COMT activity were elucidated in cell-free assays to understand the possible action mechanism.

MATERIALS AND METHODS

Plant material. *Ocimum sanctum* Linn. was procured from regions of Tamil Nadu, India. It was identified and authenticated at National Institute of Science Communication And Information Resources, New Delhi. A voucher specimen (no. 106) was deposited in our herbarium. Extract of whole plant of *O. sanctum* Linn. was developed and named as OciBest by M/s Natural Remedies Pvt. Ltd., Bangalore, India. Coarse ground whole plants of *O. sanctum* (300 kg) were charged into a stainless-steel jacketed extractor fitted with a reflux condenser. Methanol (1200 L) was added to the extractor, and the contents were refluxed for 3 h by providing steam in the jacket. The liquid extract was drained from the extractor into a separate vessel, and fresh methanol (1000 L) was added to the extractor containing the marc. The extraction procedure as in the preceding text was carried out two times, and the liquid extracts from each extraction step were separately subjected to distillation under vacuum (at <55 °C) until a thick paste with a total solid content of 40–50% (w/w) was obtained. Thick paste obtained from the three extraction steps was mixed and dried under vacuum (<65 °C) to obtain lumps of the extract. The extract lumps were then milled and sieved (#40) to obtain a uniform-powdered extract of *O. sanctum* (around 27 kg). Methanol was stripped off from the marc by passing the steam and heating at 80 °C. After removal of methanol, demineralized water (1200 L) was added in the extractor containing marc, and the contents were refluxed for 3 h by providing steam in the jacket. The extraction of marc with water was carried out totally three times. The liquid aqueous extracts were drained from the extractor, combined, and passed into a concentrator and were subjected to distillation under vacuum (at <75 °C) until the total solid content in the liquid reached about 15–20% (w/v). The concentrated liquid was then spray dried to obtain the successive water extract of *O. sanctum* (around 45 kg). The alcohol and water extracts were then analyzed for the content of active constituents and blended to obtain OciBest with the required levels of active constituents. The analysis methods were briefed in the report published by Chandrasekaran *et al.* (2013). The extract was ensured to comply with phytochemical specifications, namely, (1) ociglycoside-I (>0.1% w/w), (2) rosmarinic acid (>0.2% w/w), and triterpene acids such as (3) oleanolic acid and (4) ursolic acid (>2.5% w/w) (Saxena *et al.*, 2012).

Analysis method. Standards (1–4) and *O. sanctum* (OciBest) extract were prepared in methanol [high-performance liquid chromatography (HPLC) grade; Qualigens, Mumbai, India]. The analytical method was validated for specificity, linearity, precision, accuracy, and range of quantification. Standards and *O. sanctum* extract solutions were injected to the HPLC system (Model LC 2010 A; Shimadzu, Kyoto Japan) consisting of quaternary pump with ultraviolet detector, auto-injector, and column oven with class LC software. The stationary phase used for standards 1 and 2 was (Phenomenex Luna column, Luna column procured from Phenomenex India private Limited, Hyderabad, India) (C18, 5 µm, 250 × 4.6 mm) and Phenomenex Luna column (C18, 2.5 µm, 100 × 3 mm) for standards 3 and 4. The mobile phase used for the detection of standards 1 and 2 was a gradient mixture of acetonitrile (solvent B) and 0.001 N monopotassium phosphate in HPLC-grade water (solvent A). Solvents A and B were mixed in such a manner that the concentration of solvent B was increased from 10% to 30% as linear gradient in the first 18 min. From 18 to 25 min, the concentration of solvent B was increased from 30% to 85% as a linear gradient at a flow rate of 1.5 mL/min. The detection wavelength was set at 278 nm. The mobile phase used for standards 3 and 4 was a degassed mixture of 67 volumes of acetonitrile and 33 volumes of water containing 0.25% ammonium acetate at a flow rate of 0.3 mL/min. The detection wavelength was set at 205 nm. HPLC chromatograms of *O. sanctum* (OciBest) were recorded (Fig. 1(a) and (b)), and the quantification of standards 1–4 was achieved by external standard method.

Animals and experiment design. Animals were provided free access to rodent dry feed pellets (M/s VRK Nutritional Solutions, Pune, India) and UV purified water *ad libitum*. Rats were accommodated in polypropylene cages in a group of three under standard laboratory conditions of 12-h light and 12-h dark cycle at 24 ± 2 °C with 40–70% relative humidity.

Male and female albino Wistar rats ($n = 30$) aged 8–10 weeks and weighing 190–210 g were randomly assigned to five groups consisting of six animals in each group. Each group consisted of three male and three female rats. Three rats of the same group were housed in one cage. It was ensured that male and female rats were housed separately and not co-habited. The animals of a given group were always placed in the same cage and not mixed with other group rats. Male and female albino Wistar rats were randomly assigned to five groups consisting of six animals in each group. Group I (control) was administered vehicle (demineralized water at 10 mL/kg b.w.),

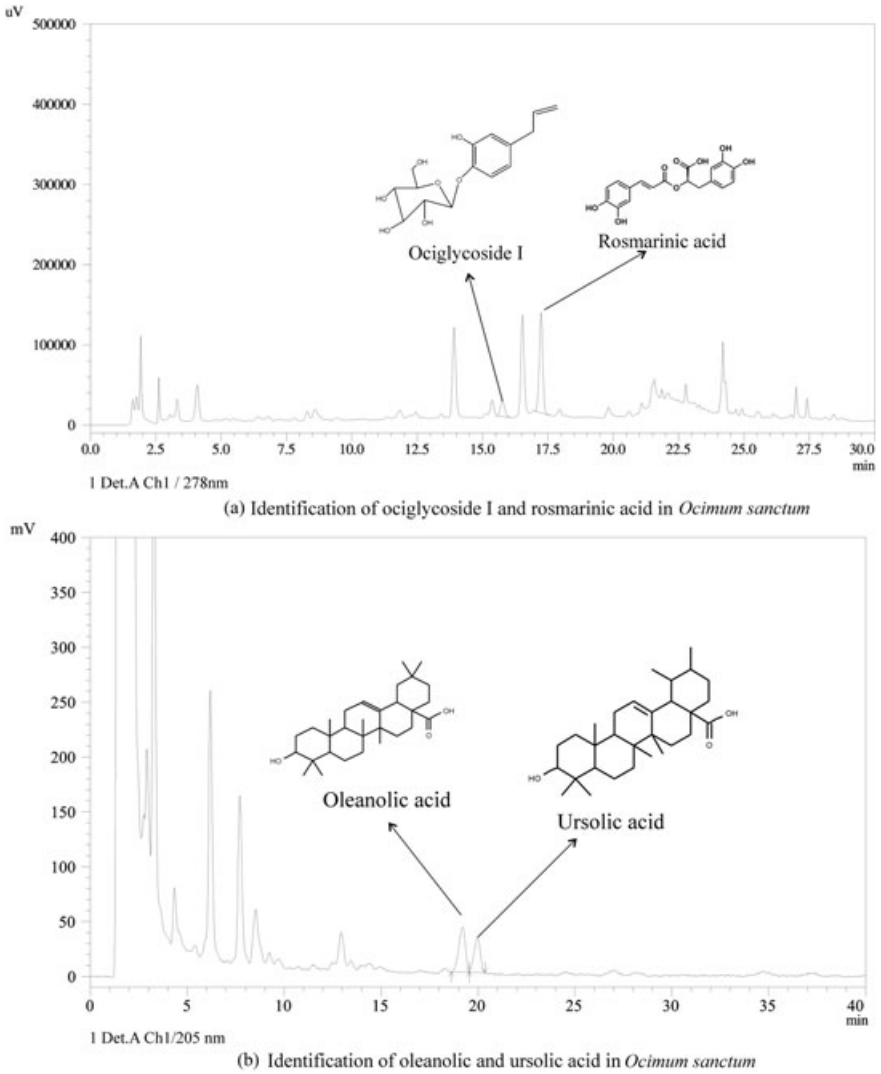

Figure 1. HPLC of *Ocimum sanctum*. (a) Identification of ociglycoside I and rosmarinic acid in *O. sanctum*. (b) Identification of oleanolic acid and ursolic acid in *O. sanctum*.

while groups II to V rats were subjected to CVS regimen for 16 days (Gouirand and Matuszewich, 2005; Ittiyavirah and Anurenj, 2014) as briefed in Table 1. Group II served as CVS control, whereas groups III to V were treated with *O. sanctum* extract at the dose levels of 50, 100, and 200 mg/kg b.w., respectively. All the treatments were administered daily by oral gavage during 16-day period of variable stress regimen. On days 4, 8, 12, and 16, animal's body weight was recorded and further subjected to forced swim test to record the immobility time manually. On day 16, serum was collected, and rats were anesthetized, and adrenal glands (left and right) were dissected out, blotted, and weighed. The adrenal weight was calculated relative to the body weight [relative adrenal weight = (absolute adrenal weight/body weight) * 100]. The animal experiment was conducted taking into consideration the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines. The experimental protocol was approved by the Institutional Animal Ethics Committee of Natural Remedies Private Limited, Bangalore, constituted as per the Committee for the Purpose of Control and Supervision on Experiments on Animals, India.

Forced swim test. Individual rats were placed in a cylinder filled with water (25 ± 1 °C, 25 cm in depth) and were exposed to 15 min of forced swim (training session) and then were removed and dried before being returned to cages. Rats were placed in the cylinder again for a 5-min period (test session) on days 4, 8, 12, and 16. The immobility time defined as the total time rat remains floating passively in water in a slightly hunched but upright position with its head just above the water surface was recorded (Mezadri *et al.*, 2011; Yi *et al.*, 2013; Ittiyavirah and Anurenj, 2014).

In vitro studies

Cells and culture conditions. Human adreno-carcinoma (NCI-H295R) cell line was procured from American Type Culture Collection (Manassas, Virginia, USA). GeneBLAzer® CRHR1-CRE-bla CHO-K1 cells contain the human CRHR1 stably integrated into the CellSensor® CRE-bla CHO-K1 cell line procured from Invitrogen (Carlsbad, CA, USA). CellSensor® CRE-bla CHO-K1 cells contain a beta-lactamase reporter gene under control of the cyclic adenosine monophosphate response element (CRE) response element. The GeneBLAzer® CRHR1-CRE-bla CHO-K1 cells are functionally validated for Z′ and EC_{50} (half maximal effective concentration) concentrations of CRF. Cells were cultured in growth media and maintained at 37 °C under 5% CO_2 humidified air.

Table 1. Chronic variable stress paradigm

Day	Treatment
1	5-min cold water swim (4–8 °C)
	Overnight fasting
2	4 h of wet bedding
	1-min tail pinch
3	2-h immobilization
	Lights on overnight
4	Forced swim test
5	5-min cold water swim (4–8 °C)
	Overnight fasting
6	4 h of wet bedding
	1-min tail pinch
7	2-h immobilization
	Lights on overnight
8	Forced swim test
9	5-min cold water swim (4–8 °C)
	Overnight fasting
10	4 h of wet bedding
	1-min tail pinch
11	2-h immobilization
	Lights on overnight
12	Forced swim test
13	5-min cold water swim (4–8 °C)
	Overnight fasting
14	4 h of wet bedding
	1-min tail pinch
15	2-h immobilization
	Lights on overnight
16	Forced swim test

Chemicals and reagents. GeneBLAzer® CRHR1 CHO-K1 DA kit, Dulbecco's Modified Eagle's medium, fetal bovine serum, and dialyzed fetal bovine serum were purchased from Invitrogen. Antalarmin hydrochloride, corticotrophin-releasing factor (CRF), insulin, forskolin, thiazolyl blue tetrazolium bromide (MTT), S-(5′-adenosyl)-L-methionine chloride, L-cysteine, 3,5-dinitrocatechol, β-nicotinamide adenine dinucleotide phosphate, tris hydrochloride, cortisone, and carbenoxolone were procured from Sigma (St. Louis, Missouri, USA). ITS + Universal Culture Supplement Premix and Nu-serum supplement from BD Biosciences (Franklin Lakes, New Jersey, USA), liver microsomes from Xenotech (Kansas city, Kansas, USA), dimethyl sulfoxide from RANKEM (Gurgaon, India), and esculetin from Extrasynthase (Genay, France) were obtained. Cortisol-Homogenous Time-Resolved Fluorescense (HTRF) Kit was purchased from Cisbio (Bedford, Massachusetts, USA). Other chemicals such as sodium dihydrogen phosphate, disodium hydrogen orthophosphate dehydrate, magnesium chloride, hexahydrate, and EDTA disodium salt were purchased from HiMedia (Mumbai, India).

Cytotoxicity assay. Cytotoxicity of *O. sanctum* and ursolic acid in human adreno-carcinoma (NCI-H295R) cells and Chinese hamster ovary (CHO-K1) cells were measured by MTT assay. For the assays, 96-well microplates were seeded with 200-μL medium containing 10,000 cells (NCI-H295R or CHO-K1) and incubated for 24 h; subsequently, cells were treated with appropriate serial fold dilutions of *O. sanctum* or ursolic acid. The final concentrations of *O. sanctum* were ranging from 3.125 to 100 μg/mL for NCI-H295R cells and 0.16–100 μg/mL for CHO-K1 cells, while for ursolic acid, the final concentrations were ranging from 0.3125 to 10 μM for NCI-H295R cells and 0.1–10 μM for CHO-K1 cells. After incubation, 10 μL of yellow MTT solution (5 mg/mL) was added to each well and incubated for 1 h. Consequently, the supernatant was removed, and 200 μL of dimethylsulfoxide was used to dissolve the formazan crystal. The cell viability was calculated by reading the absorbance of each well at 570 nm.

CRHR1 receptor assay. A frozen vial of CHO-K1 cells containing CRHR1 receptor was thawed quickly at 37 °C in a water bath, suspended in 10 mL of pre-warmed growth medium, and centrifuged at 200 *g* for 5 min. The cell pellet was resuspended gently in 5 mL growth medium to break any cell aggregates. Cells (10,000 cells per well) were plated immediately on 384-well, black-wall, clear-bottom assay plate containing growth medium and incubated for 20 h at 37 °C and 5% CO_2. Post incubation, the cells were treated with *O. sanctum* or ursolic acid at different non-cytotoxic concentrations of 0.16 to 100 μg/mL and 0.1 to 10 μM, respectively, for 30 min, followed by 4-h incubation with CRF (50 pM). Antalarmin hydrochloride was used as a standard antagonist to CRHR1 receptor. The CRHR1 receptor activation was quantified as β-lactamase expression using BMG FLUOstar Optima (BMG LABTECH, Ortenberg, Germany) by adding 8 μL of the LiveBLAzer–FRET B/G (Invitrogen, Carlsbad, California, USA) substrate mixture. Coumarin fluorescence was detected using 409-nm excitation/460-nm emission spectra, while coumarin–fluorescein complex were detected using 409-nm excitation/530-nm emission spectra (Zlokarnik *et al.*, 1998; Xing *et al.*, 2000; Ransom *et al.*, 2003).

Cortisol release assay. H295R human adrenal tumor cells were grown in 75-cm^2 flasks at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's medium and Ham's Nutrient Mixture F-12 (1:1) containing 15 mM HEPES, 1% ITS + Premix, and 2.5% Nu-serum. The medium was changed thrice a week. Cells used for experiments were subcultured from 80% confluent stock cultures into 48-well culture plates for ≥18 h before the start of the experiment. Forty-eight-hour incubations were conducted on each set of plates with 500 μL of growth media in presence of *O. sanctum* or ursolic acid and 10 μM forskolin (Liakos *et al.*, 2003; OECD, 2011). At the end of the incubation period, the cell supernatant was removed from each well and frozen at −80 °C for cortisol measurement. The concentration of cortisol in the cell supernatants was determined by HTRF technique.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzymatic assay. The conversion of cortisone to active cortisol by 11β-HSD1 activity was measured by HTRF cortisol kit. Briefly, the substances to be tested (*O. sanctum* or ursolic acid) at various concentrations were dissolved in 20 mM Tris HCL and 5 mM EDTA buffer pH 6.0 and are added to the reaction mixture containing liver microsomal preparation (0.1 mg/mL), cortisone 266 nM, and NADPH 333 μM. The assay plates were incubated for 2 h at 37 °C. Following incubation, HTRF conjugate (anti-cortisol cryptate and cortisol-d_2) was added and incubated for another 2 h at room temperature. The cortisol built during the enzymatic reaction will compete with d_2-labeled cortisol

for the binding to the cryptate conjugate, resulting in a loss in HTRF signal (detection step). Signal is expressed as ratio 665 nm:620 nm calculated using PHERAstar (BMG LABTECH, Ortenberg, Germany) plate reader. Carbenoxolone is used as a reference standard.

Catechol-O-methyltransferase (COMT) activity assay. The COMT activity assay was determined using the method reported by Kurkela *et al.* (2004). Briefly, test substances (*O. sanctum* or ursolic acid) at various concentrations and esculetin (45 μM) were dissolved in dimethyl sulfoxide and diluted with assay buffer (100 mM phosphate, 5 mM $MgCl_2$, 20 mM L-cysteine, pH 7.4) to obtain a reaction mixture of total volume 50 μL. The assay plate was incubated for a period of 5 min at 37 °C. The reaction was initiated by the addition of 7.5 μM S-(5′-adenosyl)-L-methionine chloride, at 37 °C, and the reaction was followed for 60 min using a FLUOstar (BMG LABTECH, Ortenberg, Germany) with an excitation filter of 380 nm and an emission filter of 460 nm. The change in fluorescence caused by enzymatic *O*-methylation of esculetin to scopoletin was used as a measure of inhibitory activity of COMT. Dinitrocatechol is used as a reference standard.

Statistical analysis. The results were expressed as mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance, followed by *post hoc* Dunnett's test. *P*-value < 0.05 was considered as statistically significant. IC_{50} was calculated using log-probit analysis.

RESULTS

Effect of *O. sanctum* on CVS

The immobility time during the forced swim test was significantly increased at all the time intervals in rats exposed to CVS as compared with control. On the other hand, administration of *O. sanctum* at all the dose levels has significantly reduced the immobility time as compared with CVS group (Fig. 2). All *O. sanctum*-treated groups with respect to immobility time showed a dynamic pattern over days that were indistinguishable from control group that was not subjected to CVS. The body weight of rats in CVS group reduced significantly during the study period except on day 8 as compared with the control group rats. Further, CVS group rats demonstrated less body weight gain than the control group rat, and the difference between CVS and control group was statistically different (Table 2). On the other hand, *O. sanctum* administration at 200 mg/kg significantly increased the body weight gain and nonsignificantly increased the body weight at all dose levels from day 8 till the end of the study period as compared with the CVS group. A nonsignificant increase in the relative weight of adrenal gland and cortisol levels was observed in CVS group when compared with control rats. Groups treated with *O. sanctum* at 200 mg/kg demonstrated a decrease in the relative weights and cortisol levels that were comparable with control group rats (Tables 2 and 3).

Effect of *O. sanctum* on CRHR1 receptor

Ocimum sanctum and ursolic acid did not affect the cell viability of CHO-K1 cells at the range of tested concentrations. At the non-cytotoxic concentrations ranging from 20 to 100 μg/mL, *O. sanctum* exhibited a significant concentration-dependent CRHR1 receptor antagonist activity. On the other hand, maximum percent antagonism of 63 was observed at a concentration of 100 μg /mL. The major phytoconstituent of *O. sanctum*, ursolic acid, exhibited a significant concentration-dependent CRHR1 receptor antagonist activity at the concentrations of 5 and 10 μM with a maximum antagonism of 49% at

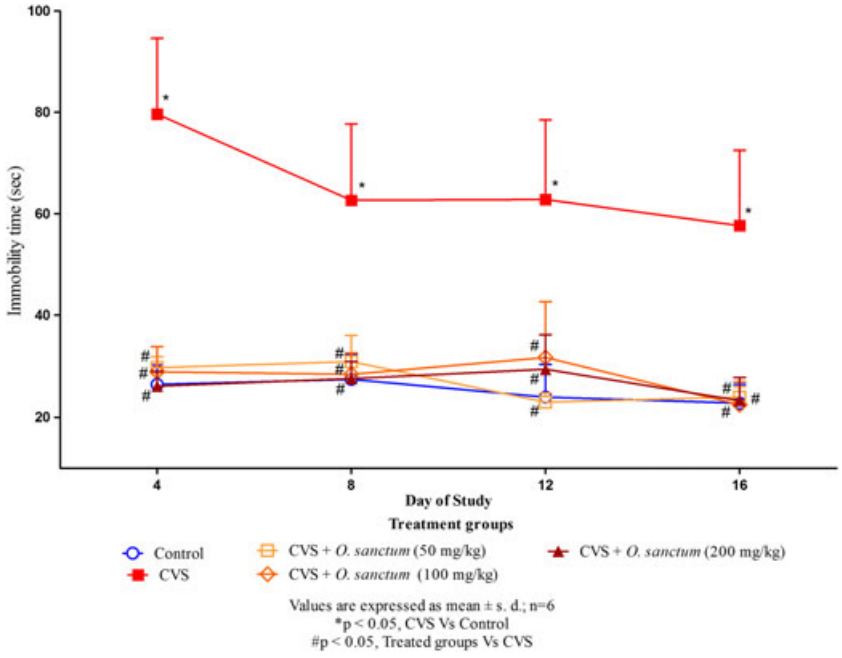

Figure 2. Effect of *Ocimum sanctum* extract on immobility time in rats. Chronic variable stress was induced, and immobility time was determined in forced swim test. All the data are represented as mean ± standard error of the mean. An asterisks indicates a significant ($^{*}p < 0.05$) difference between control group and chronic variable stress (CVS), while hash indicates a significant ($^{\#}p < 0.05$) difference from CVS. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Table 2. Effect of *Ocimum sanctum* extract on body and adrenal weight of rats

Treatment groups	Body weight (g)					Body weight gain (g)	Relative adrenal weight (mg/100 g b.w)
	Day 0	Day 4	Day 8	Day 12	Day 16		
Control	198.67 ± 2.27	215.50 ± 3.67	224.17 ± 5.99	235.33 ± 4.42	244.83 ± 4.42	46.17 ± 5.50	19.74 ± 2.82
CVS	195.00 ± 4.21	197.33 ± 6.50[1]	200.00 ± 8.10	206.67 ± 7.38[1]	216.83 ± 7.98[1]	21.83 ± 6.84[1]	22.79 ± 2.20
CVS + *O. sanctum* (50 mg/kg)	197.33 ± 3.52	204.33 ± 3.07	214.33 ± 1.87	221.33 ± 2.16	229.83 ± 1.96	32.50 ± 4.33	18.27 ± 0.72
CVS + *O. sanctum* (100 mg/kg)	195.33 ± 0.99	197.00 ± 4.75	210.17 ± 5.54	221.17 ± 4.67	233.83 ± 3.98	38.50 ± 4.37	20.85 ± 1.78
CVS + *O. sanctum* (200 mg/kg)	188.50 ± 2.05	199.67 ± 3.19	211.67 ± 2.78	220.67 ± 3.22	231.33 ± 4.18	42.83 ± 4.32[2]	19.66 ± 1.65

Data are presented in mean ± standard error of the mean (n = 6).
CVS, chronic variable stress.
[1]$p \leq 0.05$ CVS versus control;
[2]$p \leq 0.05$ CVS + *Ocimum sanctum* versus CVS.

Table 3. Effect of *Ocimum sanctum* on serum cortisol of rats

Treatment groups	Serum cortisol (ng/mL)
Control	3.56 ± 0.29
CVS	4.41 ± 0.43
Stress + *O. sanctum* (50 mg/kg)	5.36 ± 0.65
Stress + *O. sanctum* (100 mg/kg)	4.05 ± 0.44
Stress + *O. sanctum* (200 mg/kg)	3.95 ± 0.39

Data are presented in mean ± standard error of the mean (n = 6).
CVS, chronic variable stress.

10 μM. The standard drug, antalarmin hydrochloride, at a concentration of 10 μM produced 50% antagonistic effect on CRHR1 receptor (Fig. 3).

Effect of *O. sanctum* on cortisol release

Ocimum sanctum and ursolic acid did not show any toxicity at the tested concentrations of 6.25 to 100 μg/mL and 0.625 to 10 μM, respectively, to NCI-H295R cells (data not shown). *Ocimum sanctum* exhibited a concentration-dependent decrease in the cortisol levels between the concentrations of 6.25 to 100 μg/mL. Highest percent inhibition of 89 was obtained at a concentration of 100 μg/mL. Even at lower concentration of 6.25 μg/mL, *O. sanctum* showed approximately 50% inhibition. Ursolic acid showed a dose-dependent decrease in forskolin-induced cortisol release at the tested concentration of 5–10 μM with a maximum of 31% inhibition at the highest tested concentration of 10 μM (Fig. 4).

Effect of *O. sanctum* on 11β-HSD1 activity

Ocimum sanctum exhibited inhibitory activity on 11β-HSD1 at all the tested concentrations (25–200 μg/mL). Highest percent inhibition of 96.99 was obtained at a concentration of 200 μg/mL. At a concentration of 51.86 μg/mL, *O. sanctum* showed 50% inhibition, while the reference standard, carbenoxolone, at a concentration of

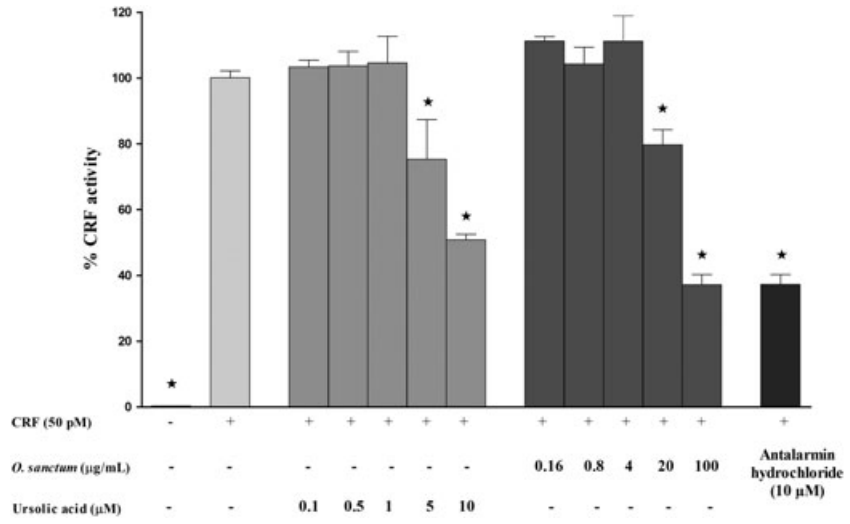

Figure 3. Effect of *Ocimum sanctum* extract and ursolic acid on corticotropin-releasing factor (CRF) stimulated cortisol secretion in H295R human adrenal tumor cells. Human CRF receptor 1 (CRHR1) stably integrated CHO-K1 cells were stimulated with CRF (50 pM) in the presence or absence of *Ocimum sanctum* extract/ursolic acid. The CRHR1 receptor activation was quantified as β-lactamase expression by fluorescence signals. All the data are represented as mean ± standard error of the mean. An asterisk indicates a significant (*$p < 0.05$) difference from CRF-produced fluorescence signals.

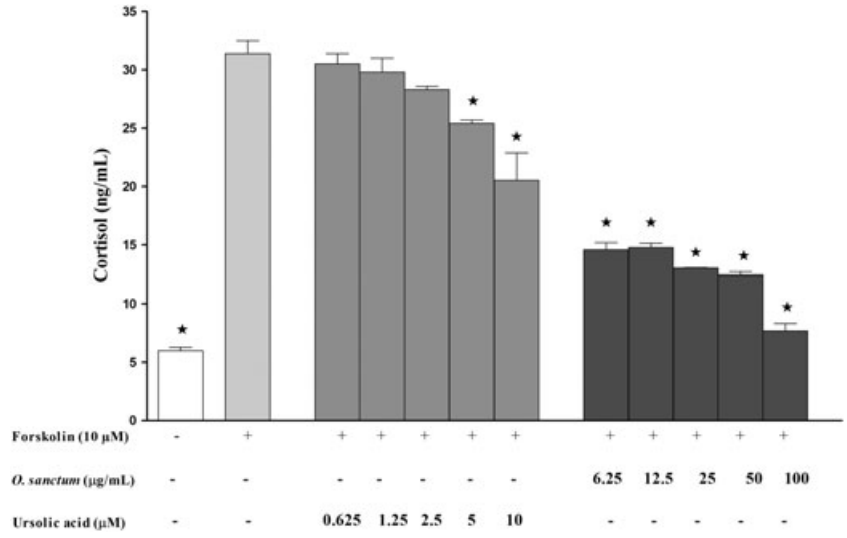

Figure 4. Effect of *Ocimum sanctum* extract and ursolic acid on forskolin-stimulated cortisol secretion in H295R human adrenal tumor cells. H295R human adrenal tumor cells were stimulated with forskolin (10 μM) in the presence or absence of *Ocimum sanctum* extract/ursolic acid. After 48 h, the cortisol content in culture supernatant was analyzed by Homogenous Time-Resolved Fluorescense technique, and cortisol release was expressed as ng/mL. All the data are represented as mean ± standard error of the mean. An asterisk indicates a significant ($*p < 0.05$) difference from forskolin-stimulated cortisol levels.

Table 4. Effect of *Ocimum sanctum* on 11-beta-hydroxysteroid dehydrogenase type 1 activity

S.No	Test substances	Concentration of the test substance	% Inhibition	IC_{50} (half maximal inhibitory concentration)
1.	Carbenoxolone (reference std)	600 nM	61.44	—
2.	*O. sanctum* (μg/mL)	25	22.20	51.86 μg/mL (45.71–58.33)
		50	41.98	
		100	78.73	
		200	96.99	

600 nM demonstrated 61.44% inhibition (Table 4). Ursolic acid was found to be ineffective at the tested concentrations of 5 and 25 μg/mL (data not shown).

Effect of *O. sanctum* on COMT activity

Ocimum sanctum exhibited inhibitory activity on COMT at all the tested concentrations. At a concentration of 11.65 μg/mL, *O. sanctum* showed 50% inhibition, while the reference standard, 3, 5-dinitrocatechol, at a concentration of 24.91 nM demonstrated 50% inhibition. However, ursolic acid did not demonstrate any COMT inhibitory activity at the tested concentrations (Table 5).

DISCUSSION

Ocimum sanctum has been traditionally used for its anti-stress effect (Gupta *et al.*, 2002). Although preclinical and clinical studies on the anti-stress activity of *O. sanctum* are available, the studies used single stressor, and hence, the present study investigated the anti-stress activity of *O. sanctum* in CVS model that closely mimics the condition in humans (Gouirand and Matuszewich, 2005). Further, to delineate the plausible action mechanism, cortisol release and CRHR1 receptor activity in cell-based assays and 11β-HSD1 and COMT activity in cell-free assays were investigated.

Chronic variable stress is a widely implemented and highly effective experimental model for stress induction in which animals undergo a variety of stressors at over (typically) 2 to 5 weeks (Hill *et al.*, 2012), and this experimental model provides a good tool for investigating the effect of intervention on stress (Mizoguchi *et al.*, 2003; Ramanathan *et al.*, 2011; Ittiyavirah and Anurenj, 2014). Hence, the present study employed CVS paradigm to induce stress, and the anti-stress effects of *O. sanctum* in CVS were evaluated using immobility time in forced swim test. On the other hand, body weight and adrenal weight were also considered as measures to evaluate the anti-stress potential of *O. sanctum.* Stressed rats are accompanied by behavior such as increased immobile posture or passive immobility and decreased exploration of novelty. An intervention that reduces the duration of immobility is said to be effective in the management of stressful conditions (Calil and Marcondes, 2006; Ohnishi *et al.*, 2010). Administration of *O. sanctum* caused a significant decrease in the immobility time, indicating its potential as an effective intervention for stress-induced depression and stress management. The findings on the reduction of immobility time are consistent with the findings reported by Tabassum *et al.* (2010) and Bathala *et al.* (2012).

Chronic variable stress is reported to cause a number of well-characterized effects in rats including reduced body weight and change in the serum cortisol levels. In

Table 5. Effect of *Ocimum sanctum* and its phytoactives on catechol-*O*-methyltransferase activity

S.No	Test sample name	Concentration	Inhibition (%)	IC_{50}
1	3,5-Dinitrocatechol (nM)	5	10.84	24.91 nM (21.79–28.32)
		10	20.33	
		20	42	
		40	51.05	
		80	82.83	
		100	100	
2	*Ocimum sanctum* (μg/mL)	5	30.55	11.65 μg/mL (8.88–14.57)
		10	50.75	
		25	64.49	
		50	75.99	
		100	90.56	
3	Ursolic acid (μg/mL)	1	0	
		2.5	0	
		5	0	
		10	5.38	
		20	13.25	

the present study, application of CVS for 16 days resulted significant decrease in body weight, body weight gain, and nonsignificant increase in cortisol levels. Although there was a numerical difference, CVS did not statistically increase the cortisol level. Our results are in agreement with published reports by Kavous *et al.* (2013), Kim *et al.* (2008), Anderson *et al.* (1996), Brodish and Odio (1989), and Patterson-Buckendahl *et al.* (2007). These effects on body weight was reversed by *O. sanctum* administration, indicating the anti-stress activity of *O. sanctum*. Further, the *in vitro* studies demonstrated that *O. sanctum* inhibited cortisol release indirectly via antagonistic effect on CRHR1 receptor and directly by inhibiting 11β-HSD1, suggesting that probably *O. sanctum* would have acted through these mechanisms.

The plausible action mechanism is investigated in CRHR1 receptor assay. Corticotrophin-releasing factor (CRF) is a primary hormone in the fight or flight response targeting a membrane-bound G protein-coupled receptor and remains a key regulator of the HPA axis. In response to chronic stress where the threshold of the organisms exceeds, CRF initiates a cascade of events that culminate in the release of glucocorticoids and thus stress-related disorders. The physiological actions of the CRF are mediated through three distinct receptor subtypes, namely, CRHR1, CRHR2, and CRHR3; however, CRHR1 remains the primary receptor for CRF and thus a key regulator of the HPA axis. Therefore, CRHR1 plays an integral role in the initiation of an array of events leading to stress, making CRHR1 antagonists as a possible therapeutic target for stress management (Gilligan *et al.*, 2000; Keller *et al.*, 2000; Smith and Vale, 2006; Hoare *et al.*, 2008). In this study, the antagonizing effect of *O. sanctum* and ursolic acid on CRF-induced CRHR1 receptor activity in CHO-K1 DA cells was studied. Both *O. sanctum* and its major phytoconstituent, ursolic acid, exhibited a significant concentration-dependent CRHR1 receptor antagonist activity. Thus, the study findings suggest that *O. sanctum* and ursolic acid act as a corticotropin-releasing hormone receptor 1 antagonist attenuating adrenocorticotropic hormone-mediated effects.

The effect of *O. sanctum* on forskolin-induced cortisol release in NCI-H295R cells was investigated. Forskolin induced cortisol release from NCI-H295R cells and increased the cortisol secretion by fivefold compared with solvent control cells. These results are in a good agreement with previously reported effects of forskolin stimulation in primary adrenal as well as in NCI-H295R cells (Bird *et al.*, 1993, 1998). *Ocimum sanctum* or ursolic acid inhibited the cortisol release from forskolin-treated NCI-H295R cells. Treatment of *Ocimum sanctum* and its major active constituent, ursolic acid, exhibited a significant decrease in the cortisol levels in forskolin-induced cortisol release assay. Forskolin-induced high cortisol secretion could be due to the cAMP-responsive elements and/or due to the mRNA-stabilizing role of cAMP, as described previously for the phosphoenolpyruvate carboxykinase mRNA in LLC-PK cells by Dhakras *et al.* (2006). We hypothesize that *O. sanctum* inhibited the secretion of cortisol by destabilizing the cAMP-responsive elements. This cortisol secretion inhibitory effect of *O. sanctum* could be contributed by ursolic acid. Similar findings by Sembulingam *et al.* (1997) stated that *O. sanctum* significantly reduced the cortisol level induced by subjecting rodents to acute and chronic stress. Another study demonstrated the anti-stress activity of Ocimumoside A and B isolated from *O. sanctum* by reducing plasma cortisol level in a preclinical animal model (Ahmad *et al.*, 2012).

11β-Hydroxysteroid dehydrogenase type 1 is an enzyme that converts inactive cortisone to active cortisol, and excess cortisol formed during the stress conditions leads to Cushing's syndrome, obesity, insulin resistance, hypertension, dyslipidaemia, major depressive disorder, Alzheimer's disease, osteoporosis, and glaucoma (Katz *et al.*, 2013). Hence, an HSD1 inhibitor by blocking regeneration of the cortisol has therapeutic potential in ameliorating the stress-related diseases. *Ocimum sanctum* was found to inhibit 11β-HSD1, indicating its potential in aiding to cope with the changes in the environment.

Thus, possibly, the aforementioned mechanisms, that is, CRHR1 antagonist activity, inhibition of cortisol release and inhibition of 11β-HSD1 by O. sanctum could have contributed to the management of stress-induced changes including body weight and depression in rats.

In addition to dysregulation of HPA axis, stress also affects catecholamine system. Compelling evidence exists that stress leads to decrease in dopamine and norepinephrine levels (Cabib and Puglisi-Allegra, 1996; Konstandi *et al.*, 2000; Ramanathan *et al.*, 2011). Hence, the present study investigated COMT inhibitory activity that spares the catecholamines and supports the individual to meet the demands placed and cope up with the stressful stimuli. *Ocimum sanctum* demonstrated COMT inhibitory activity and the COMT inhibitory effect of extract may be attributed to the phytoactives of *O. sanctum* other than ursolic acid.

CONCLUSION

The study results indicate a potential of *O. sanctum* extract (OciBest) in the management of stress-induced effects and probable mechanism could be due to combined effects on the inhibition of cortisol secretion and COMT activity. Directly, *O. sanctum* extract inhibited cortisol secretion and at the same exerted antagonistic effect on CRHR1 receptor indirectly, in turn reducing the cortisol release from adrenal cells. In addition, the study findings also indicate that the inhibitory activity of *O. sanctum* extract could be attributed to phytoactives present.

Conflict of Interest

All authors are employed by Natural Remedies (Bangalore, India).

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