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Anti-Stress Effects of *Urtica dioica* L. Leaf Extract against Experimentally Induced Oxidative Stress in Wistar Rats

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ABSTRACT: Background: *Urtica dioica* L. (stinging nettle, Urticaceae) is a widely used sub-Himalayan medicinal herb rich in flavonoids, phenolic acids, and other free-radical-scavenging phytoconstituents. Although antioxidant, anti-inflammatory and neuroprotective properties of the plant have been reported repeatedly, no study has yet examined the anti-stress activity of an extract prepared from nettle grown in Uttarakhand.

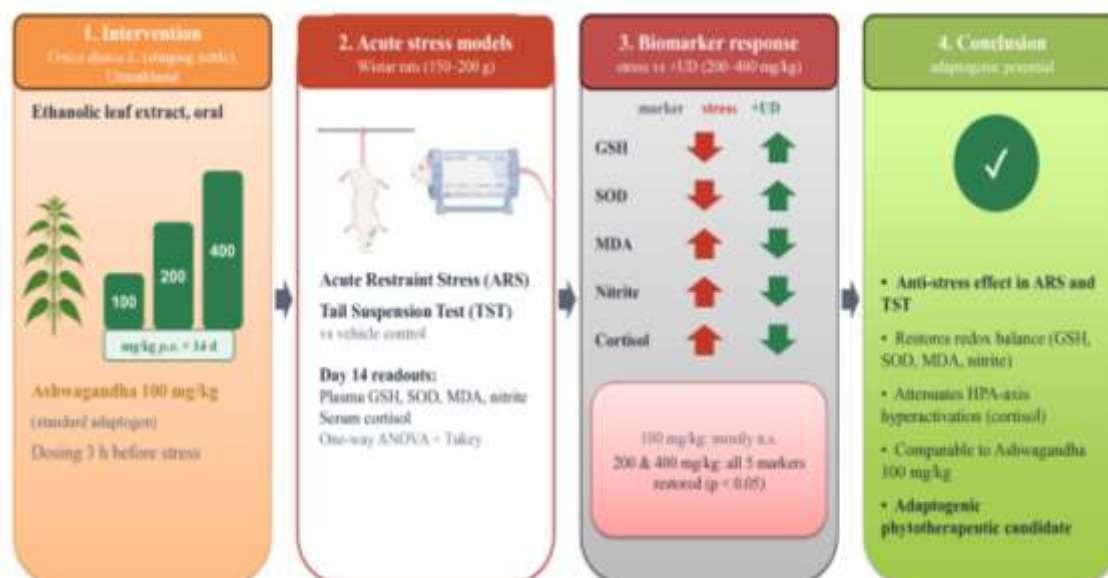
Objective: The present work assessed the anti-stress potential of an ethanolic leaf extract of *Urtica dioica* (UD) using two acute rodent stress models: Acute Restraint Stress (ARS) and the Tail Suspension Test (TST).

Methods: Wistar rats (150–200 g) received oral UD extract at 100, 200 or 400 mg/kg, or Ashwagandha (100 mg/kg, standard adaptogen), three hours before induction of ARS or TST over 14 days. On day 14th Plasma glutathione (GSH), malondialdehyde (MDA), nitrite, superoxide dismutase (SOD), and serum cortisol were measured and Group comparisons used one-way ANOVA with Tukey's post hoc test.

Results: Both ARS and TST significantly depleted GSH and SOD and elevated MDA, nitrite, and cortisol relative to vehicle controls ($p < 0.05$). Oral UD reversed these changes dose-dependently in both models; the 100 mg/kg dose was generally non-significant relative to the negative control, while 200 and 400 mg/kg produced significant, dose-dependent restoration of all five biomarkers. Ashwagandha (100 mg/kg) produced comparable or numerically greater normalisation than UD 400 mg/kg across most parameters.

Conclusion: The ethanolic leaf extract of Uttarakhand-grown *Urtica dioica* exhibits dose-dependent anti-stress and antioxidant activity in acute stress paradigms, restoring redox balance and attenuating HPA-axis hyperactivation, supporting its further development as an adaptogenic phytotherapeutic candidate.

Graphical Abstract



INTRODUCTION

Oxidative stress, arising from an imbalance between reactive oxygen species (ROS) generation and endogenous antioxidant defences, damages cellular macromolecules through lipid peroxidation, protein oxidation, and DNA breakage, and is strongly implicated in aging, diabetes, cardiovascular disease, neurodegeneration, and stress-related disorders. The brain is particularly vulnerable to oxidative injury owing to its high oxygen consumption and lipid-rich composition[1]. Psychological and physiological stressors compound this burden by activating the hypothalamic–pituitary–adrenal (HPA) axis, elevating circulating glucocorticoids, and increasing metabolic ROS production, thereby directly linking chronic stress to oxidative tissue damage, neuroinflammation, and impaired hippocampal function³. This has intensified interest in safe, plant-derived antioxidant and adaptogenic agents capable of restoring redox and neuroendocrine homeostasis under stress[2].

Urtica dioica L. (stinging nettle, family Urticaceae) is a perennial medicinal herb widespread across the temperate sub-Himalayan belt, including Uttarakhand, and has long been used in traditional and Ayurvedic medicine for arthritis, diabetes, inflammation, and general debility. Its leaves are rich in bioactive phytoconstituents- flavonoids (rutin, quercetin, kaempferol), phenolic acids (chlorogenic acid), catechins, carotenoids, sterols, tannins, and compounds such as scopoletin, carvacrol, and serotonin, with reported total polyphenol and flavonoid contents of ~100–117 mg GAE/g and ~199–277 mg QE/g dry matter, respectively[3]. These constituents confer potent free-radical scavenging, metal-chelating, and lipid peroxidation-inhibiting activity through hydrogen/electron-donation mechanisms [4].

Ethanolic and hydroalcoholic *Urtica dioica* extracts show marked in vitro antioxidant activity (DPPH, ABTS, FRAP, nitric oxide assays) and consistent in vivo efficacy across oxidative-damage models, improving antioxidant enzyme status in hypertensive rats, attenuating pulmonary and hepatic oxidative markers in an asthma model, and elevating glutathione, glutathione peroxidase, and superoxide dismutase while lowering malondialdehyde and neuroinflammation in streptozotocin-induced diabetic rat hippocampus[5]. Nettle extract has also been shown to raise hippocampal brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels, supporting neuronal plasticity and cognitive protection in aging and neurodegeneration models. Its flavonoid and phenolic constituents further suppress pro-inflammatory mediators such as TNF- α and IL-1 β , and modulate dopaminergic and serotonergic signaling, contributing to stress-adaptive and mood-regulatory effects[6].

Collectively, these findings suggest that the antioxidant, anti-inflammatory, and neuroprotective actions of *Urtica dioica* are mechanistically interlinked with its anti-stress potential, since HPA-axis activation and oxidative damage share a common redox-based pathway. However, most existing studies have used *Urtica dioica* sourced from Europe, Iran, or North Africa, while extracts from Uttarakhand-grown nettle a region noted for its high-altitude phytochemical diversity, remain largely unexplored

for anti-stress efficacy[7]. The present study therefore evaluates the anti-stress potential of an ethanolic extract of *Urtica dioica* leaves, collected from Uttarakhand, against experimentally induced oxidative stress in Wistar rats using two acute stress paradigms Acute Restraint Stress (ARS) and the Tail Suspension Test (TST)[8].

MATERIALS AND METHODS

Chemicals used

Ethanol 70% was purchased at from Merck (Darmstadt, Germany), Ashwagandha was purchased from Sigma-Aldrich (Steinheim, Germany) & other analytical-grade chemicals were used.

Plant material

Urtica dioica was collected in June month from local farmers of Village Chandla, District Tehri, Garhwal Himalayas, Uttarakhand, India. The collected plant material was authenticated by Dr. Sunita Garg, Former Chief Scientist and Head, and Mr. R. S. Jayasomu, Chief Scientist and Head, RHMD, CSIR-NIScPR, authentication number: NIScPR/RHMD/Consult/2023/4555-56-4.

Preparation of ethanolic extract

50g of *Urtica dioica* Leaves were shade-dried, and the solvent used was 500 mL of ethanol; then, using a Soxhlet apparatus, the yield was obtained to be 16.16% at 50°C

Total flavonoid content:-

Flavonoids were quantified using the aluminium chloride colorimetric technique. 1 mg/ml of ethanolic extract sample of *Urtica dioica* Leaves was dissolved in methanol, 1 ml of $AlCl_3$ (2%) in methanol was added, and after incubation for 10 min, the absorbance was measured at 430 nm[9]. The analyses were replicated ($n = 3$), and the contents were given as mean values, plus or minus the standard deviation. Flavonoids were measured as quercetin equivalents and expressed as milligrams of each compound per 100 g of dry weight of ethanolic extract[10].

Total phenolic content

The Folin–Ciocalteu phenol assay was employed for the determination of total phenolic content. The ethanolic extract sample of *Urtica dioica* Leaves was prepared at a concentration of 5 mg/ml in 30% DMSO. A sample (20 μ l) of the plant extract and a standard solution of gallic acid were mixed with Folin–Ciocalteu phenol reagent (100 μ l) and sodium carbonate (80 μ l)[11]. After a 30-min incubation, the absorbance was measured at 765 nm using a microplate reader (Synergy LX, Bio-Tek, Instrument, Inc., U.S.A.). The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of plant extract[12].

Experimental animals

In the present investigation, adult Wistar rats (150–200 g), aged 3–4 months, were procured from the central animal house facility of ISF College of Pharmacy (Moga, India). The rats were housed in polyacrylic cages under a controlled atmosphere and maintained under standard laboratory conditions (25 ± 2 °C room temperature and $60 \pm 10\%$ relative humidity) with a 12-hour dark/light reverse cycle (lights on at 7:00 a.m.)[13]. Animals were fed a commercial pellet diet with water ad libitum. All behavioural indicators were assessed during the animals' active period, between 9:00 a.m. and 6:00 p.m. The study protocol was duly approved by the Institutional Animal Ethics Committee (ISFCP/IAEC/CPCSEA/2018/386) and was performed in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA)[14].

Experimental design (in vivo activity)

Animals were acclimatized to laboratory conditions for 5 days before testing. Three doses of the *Urtica dioica* (UD) ethanolic extract (100, 200, and 400 mg/kg, p.o.) were selected for evaluation. Animals were divided into six groups ($n = 6$ per group) for each stress model: Group 1 (Vehicle) received normal saline; Group 2 (Negative Control) was exposed to the respective stress paradigm (ARS or TST) without extract treatment; Groups 3–5 (*Urtica dioica* I, II, III) received *Urtica dioica* extract at 100, 200, and 400 mg/kg p.o., respectively, together with stress exposure; and Group 6 (Positive Control) received Ashwagandha (100 mg/kg, p.o.) together with stress exposure[15]. *Urtica dioica* extract and Ashwagandha were administered orally 3 hours

before stress induction, and animals were fasted for 4 hours before treatment. At the end of the study, animals were lightly anaesthetized with ether, and blood was collected from the retro-orbital vein for biochemical estimation.

Study design

Table 1. In vivo experimental plan for ARS and TST (14-day protocols, n = 6 animals/group).

Group (N)	Treatment for ARS (14 days)	Treatment for TST (14 days)
Vehicle (Normal Control)	Normal saline, <i>p.o.</i>	Normal saline, <i>p.o.</i>
Negative Control	ARS alone	TST alone
UD I (100 mg/kg)	UD 100 mg/kg <i>p.o.</i> + ARS	UD 100 mg/kg <i>p.o.</i> + TST
UD II (200 mg/kg)	UD 200 mg/kg <i>p.o.</i> + ARS	UD 200 mg/kg <i>p.o.</i> + TST
UD III (400 mg/kg)	UD 400 mg/kg <i>p.o.</i> + ARS	UD 400 mg/kg <i>p.o.</i> + TST
Positive Control	Ashwagandha 100 mg/kg <i>p.o.</i> + ARS	Ashwagandha 100 mg/kg <i>p.o.</i> + TST

In vivo screening models for stress

Acute Restraint Stress (ARS)

Acute restraint stress was induced by placing animals individually in well-ventilated restrainers for 2 hours. *Urtica dioica* extracts and Ashwagandha were administered orally 3 hours before stress exposure. After completion of the experimental period, blood samples were collected for estimation of GSH, MDA, nitrite, SOD, and serum cortisol levels[16].

Tail Suspension Test (TST)

Rats were suspended individually by the tail using adhesive tape fixed approximately 1–2 cm from the tail tip and hung 50 cm above the floor for 6 minutes. The duration of immobility was recorded. *Urtica dioica* extracts and Ashwagandha were administered orally 3 hours before stress exposure. After completion of the experimental period, blood samples were collected for estimation of GSH, MDA, nitrite, SOD, and serum cortisol levels [17].

In vivo biochemical estimation

For blood collection, animals were anaesthetized with ether, and blood was collected in tubes. Samples were centrifuged at 12,000 rpm for 15 minutes, and the separated plasma was used for biochemical assessments[18].

Estimation of reduced glutathione (GSH)

The Ellman method was used to measure reduced GSH in blood plasma. The supernatant (1 mL) was precipitated with 4% sulfosalicylic acid (1 mL) and allowed to digest for 1 hour at 4 °C. The sample was centrifuged for 15 minutes at 1200 rpm; 1 mL of supernatant, 2.7 mL of phosphate buffer (0.1 mmol/L, pH 8), and 0.2 mL of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were added. The yellow colour developed was measured at 412 nm using a UV spectrophotometer, and GSH content was expressed as $\mu\text{mol/g protein}$ [19].

Measurement of lipid peroxidation (MDA)

Lipid peroxidation was assessed via the thiobarbituric acid reactive substances (TBARS) method¹⁴. Malondialdehyde (MDA), a terminal product of polyunsaturated fatty acid breakdown, reacts with thiobarbituric acid (TBA) to generate a pink MDA-TBA complex, measured spectrophotometrically at its absorption maximum of 530–535 nm. Values were expressed as nmol/mg protein[20].

Estimation of nitrite

Nitric oxide production was estimated indirectly via the Griess reaction. Acidified nitrite generates a nitrosating agent that combines with sulfanilic acid to form a diazonium ion, which is subsequently coupled with N-(1-naphthyl) ethylenediamine to form an azo-chromophore measured at 540–570 nm. Values were expressed as $\mu\text{g/mL protein}$ [21].

Estimation of superoxide dismutase (SOD)

SOD activity was measured following the pyrogallol autoxidation-inhibition method. The assay mixture comprised 0.05 M phosphate buffer (pH 7.0), 0.019 M H₂O₂, and 0.05 mL PMS in a total volume of 3 mL; absorbance change was recorded at 240 nm, and activity was expressed as U/mg protein[22] .

Estimation of blood cortisol level

Blood was collected from the retro-orbital vein under aseptic precautions and allowed to clot for 30 minutes at room temperature. Serum was separated by centrifugation at 12,000 rpm for 5 minutes and stored at –20 °C until analysis. Serum cortisol was estimated using a Krishgen Biosystems ELISA kit (standard diluent, biotinylated CORT antibody, TMB substrate, and streptavidin–HRP conjugate), with final absorbance read at 450 nm. Values were expressed as ng/mL.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism software (Version 10.0). Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation with $p < 0.05$ was considered statistically significant.

RESULTS

Total flavonoid and phenolic content

Table 2. Quantitative Estimation of Total Flavonoid and Phenolic Content in Ethanolic Extract of *Urtica dioica*

Phytochemical	Quantity
Total flavonoid content (as quercetin equivalent)	266.47 mg/100 g dry extract ($R^2 = 0.994$)
Total phenolic content (as gallic acid equivalent)	103.74 mg/100 g dry extract ($R^2 = 0.996$)

Acute Restraint Stress (ARS)

(a) Estimation of reduced glutathione (GSH)

On day 14, animals subjected to acute restraint stress showed a marked reduction in glutathione levels compared to the vehicle control group. Further oral administration of *Urtica dioica* extract at a dose of 100mg/kg do not produce any significant changes but *Urtica dioica* 200 and 400 mg/kg/ *p.o.*, significantly and dose dependently restored the level of glutathione as compared to negative control . However, oral administration of Ashwagandha significantly improved glutathione level as compared to the high dose of *Urtica dioica* 400 in ARS animals, as shown in Figure 1.

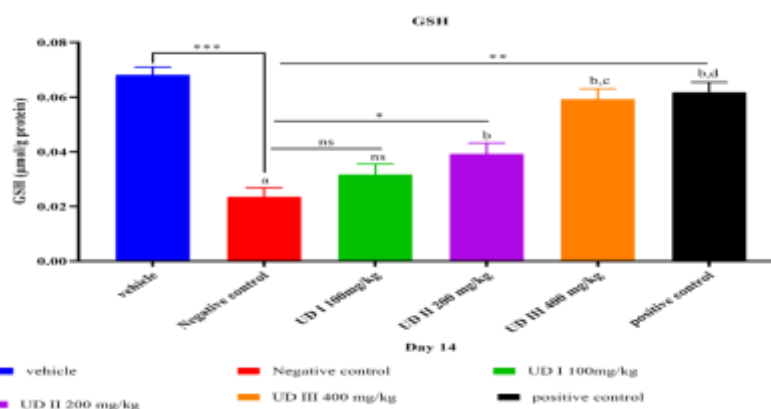


Figure 1. Effect of extract *Urtica dioica* (UD) on glutathione level in blood plasma in acutely restrained test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200mg/kg), ^d $p < 0.05$ vs UD (400mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(b) Measurement of lipid peroxidation (MDA)

On day 14, animals subjected to acute restraint stress showed a marked increase in MDA levels compared to the vehicle control group. Further oral administration of *Urtica dioica* extract at a dose of 100mg/kg do not produce any significant changes, but *Urtica dioica* 200 and 400 mg/kg/ oral significantly and dose-dependently restored the level of MDA as compared to the negative control. However, oral administration of Ashwagandha significantly lowered MDA levels as compared to the high dose of UD 400 in ARS animals, as shown in Figure 2.

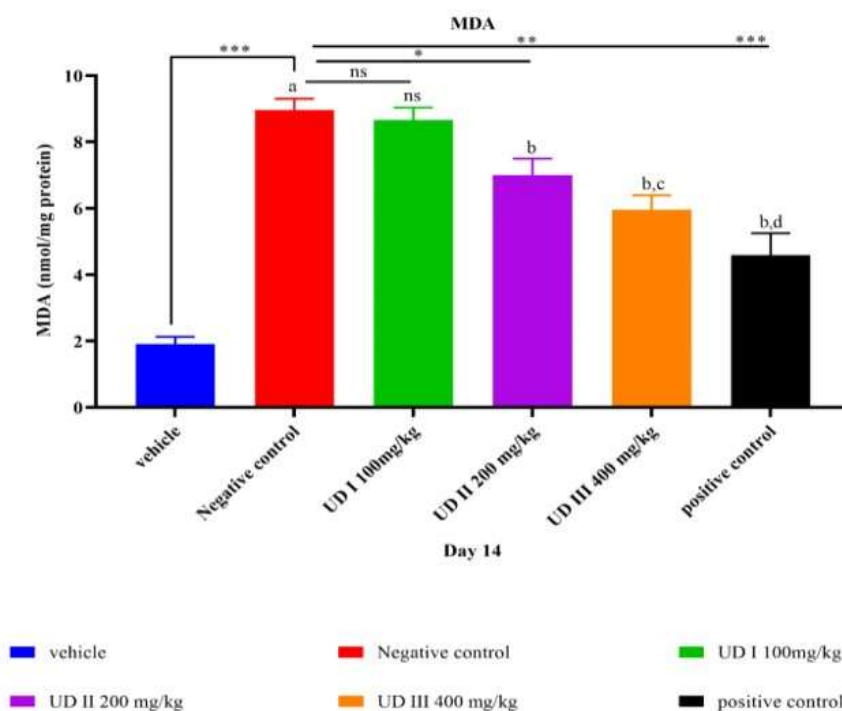


Figure 2. Effect of extract UD on MDA levels in blood plasma in acutely restrained test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200mg/kg), ^d $p < 0.05$ vs UD (400mg/kg). Further, UD (100mg/kg) found non-significant compared to Negative control.

(c) Estimation of nitrite

On day 14, animals subjected to acute restraint stress showed a marked increase in nitrite levels compared to the vehicle control group. Further oral administration of UD extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ *p.o.* significantly and dose-dependently restored the level of Nitrite as compared to the negative control. However, oral administration of Ashwagandha significantly improved nitrite levels as compared to the high dose of UD 400 in ARS animals, as shown in Figure 3.

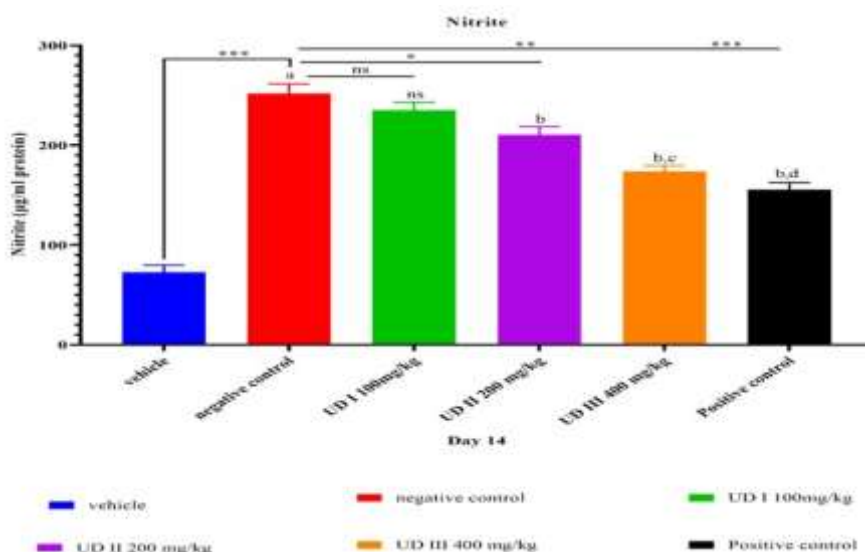


Figure 3. Effect of extract UD on nitrite levels in blood plasma in acutely restrained test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200 mg/kg), ^d $p < 0.05$ vs UD (400 mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(d) Estimation of reduced superoxide dismutase (SOD)

On day 14, animals subjected to acute restraint stress showed a marked decrease in SOD levels compared to the vehicle control group. Further oral administration of *Urtica dioica* extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ *p.o.* significantly and dose-dependently restored the level of SOD as compared to the negative control. However, oral administration of Ashwagandha significantly improved SOD level as compared to the high dose of UD 400 in ARS animals, as shown in Figure 4.

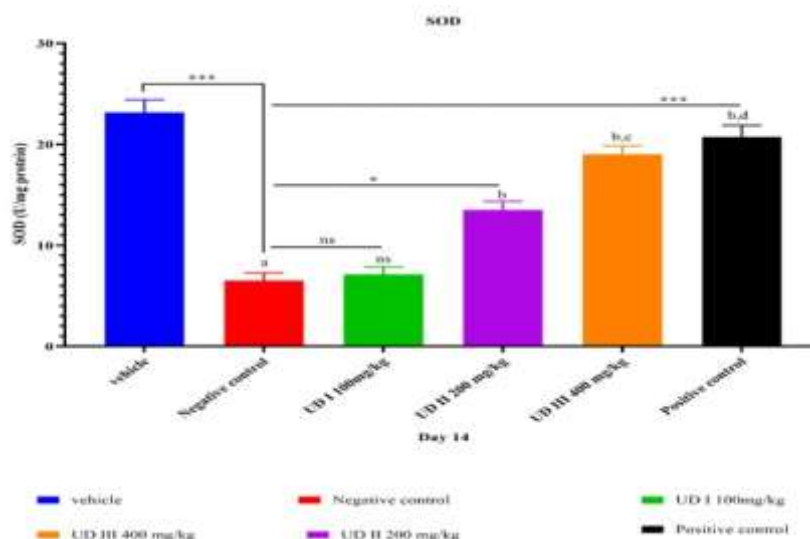


Figure 4. Effect of extract UD on SOD level in blood plasma in acutely restrained test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200 mg/kg), ^d $p < 0.05$ vs UD (400mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(e) Estimation of Blood cortisol level

On day 14, animals subjected to acute restraint stress showed a marked increase in Cortisol levels compared to the vehicle control group. Further oral administration of UD extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ oral significantly and dose-dependently restored the level of cortisol as compared to the negative control. However, the effect after oral administration of Ashwagandha and a high dose of UD 400 in ARS animals was found to be non-significant, as shown in Figure 5.

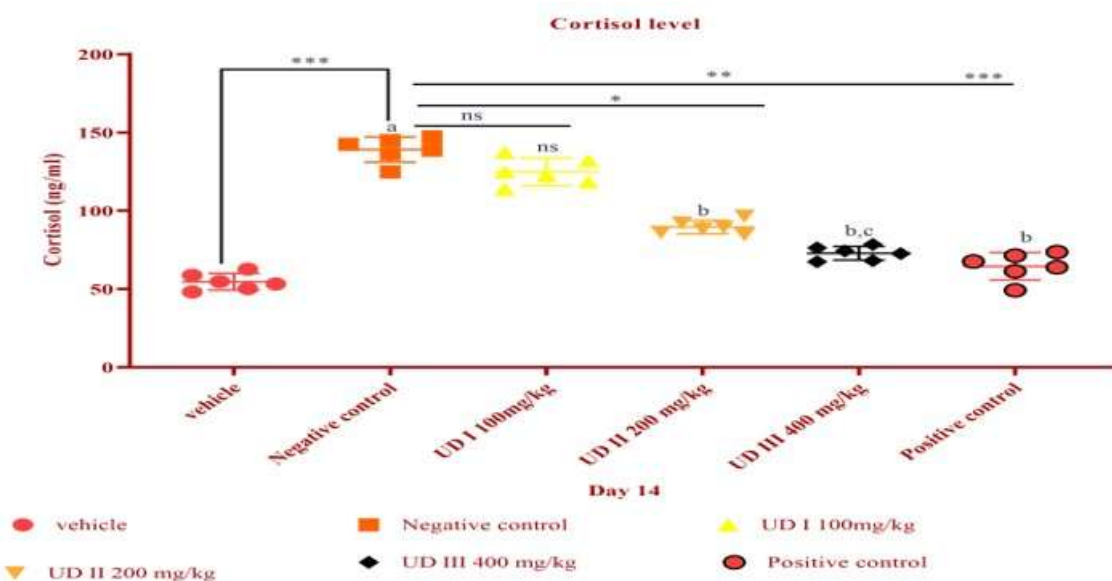


Figure 5. Effect of extract UD on cortisol level in blood plasma in acutely restrained test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200mg/kg).

Further, UD (100mg/kg) found non-significant compared to the negative control.

Tail Suspension Test (TST)

(a) Estimation of reduced glutathione (GSH)

On day 14, animals subjected to the tail suspension test showed a marked decrease in glutathione levels compared to the vehicle control group. Further oral administration of UD extract at dose of 100mg/kg do not produce any significant changes but UD 200 and 400 mg/kg/ oral, significantly and dose dependently restored the level of glutathione as compared to negative control. However, oral administration of Ashwagandha significantly improved glutathione levels as compared to the high dose of UD 400 in TST animals, as shown in Figure 6.

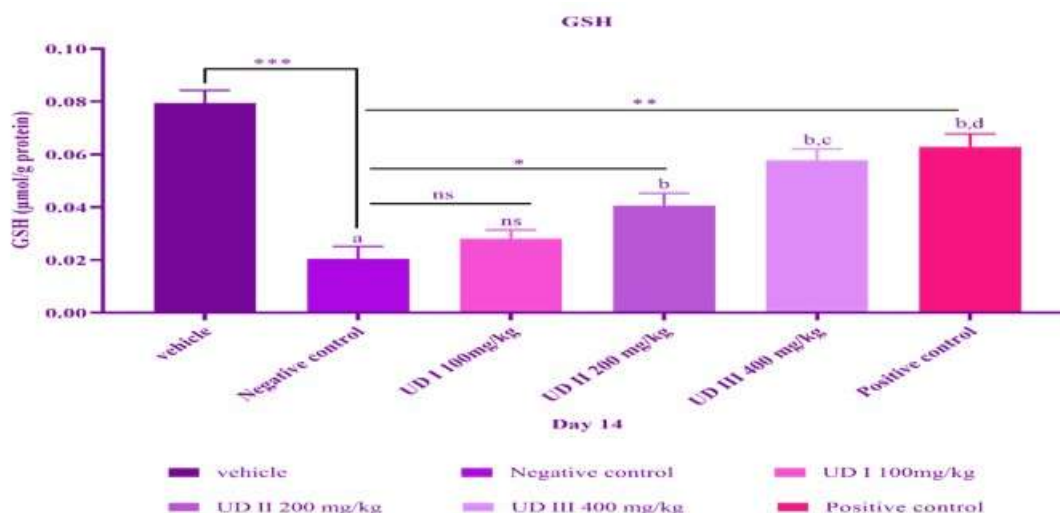


Figure 6. Effect of UD extract on GSH levels in blood plasma in tail suspension test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation with $p < 0.05$ was considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200 mg/kg), ^d $p < 0.05$ vs UD (400mg/kg).

Further, UD (100mg/kg) was not significant compared to the negative control.

(b) Measurement of lipid peroxidation (MDA)

On day 14, animals subjected to the tail suspension test showed a marked increase in MDA levels compared to the vehicle control group. Further oral administration of UD extract at 100mg/kg did not produce significant changes, but UD 200 and 400mg/kg p.o. significantly and dose-dependently restored MDA levels compared with the negative control. However, oral administration of Ashwagandha significantly improved MDA levels as compared to the high dose of UD 400 in TST animals, as shown in Figure 7.

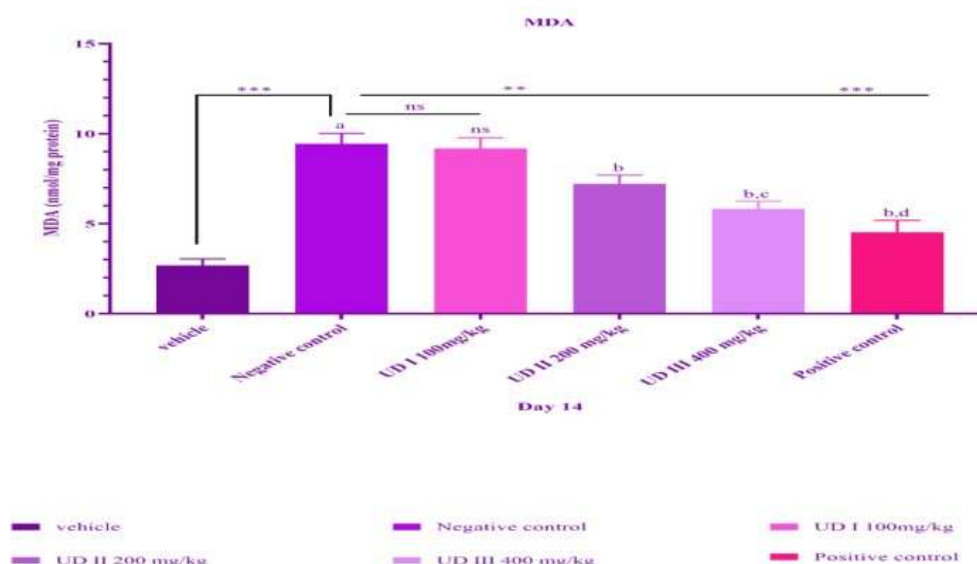


Figure 7. Effect of extract UD on MDA levels in blood plasma in tail suspension test rats. Data were statistically analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, and $p < 0.05$ was considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200 mg/kg), ^d $p < 0.05$ vs UD (400 mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(c) Estimation of nitrite

On day 14, animals subjected to the tail suspension test showed a marked increase in Nitrite levels compared to the vehicle control group. Further oral administration of UD extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ *p.o.* significantly and dose-dependently restored the level of Nitrite as compared to the negative control. However, oral administration of Ashwagandha significantly improved nitrite levels as compared to the high dose of UD 400 in TST animals, as shown in Figure 8.

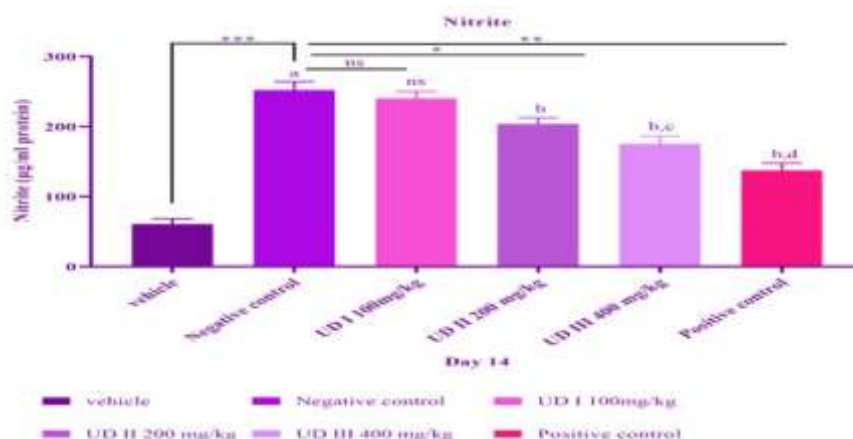


Figure 8. Effect of extract UD on nitrite levels in blood plasma in tail suspension test rats. Data were statistically analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200mg/kg), ^d $p < 0.05$ vs UD (400mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(d) Estimation of reduced superoxide dismutase (SOD)

On day 14, animals subjected to the tail suspension test showed a marked decrease in SOD levels compared to the vehicle control group. Further oral administration of UD extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ *p.o.* significantly and dose-dependently restored the level of SOD as compared to the negative control. However, oral administration of Ashwagandha significantly improved SOD level as compared to the high dose of UD 400 in TST animals, as shown in Figure 9.

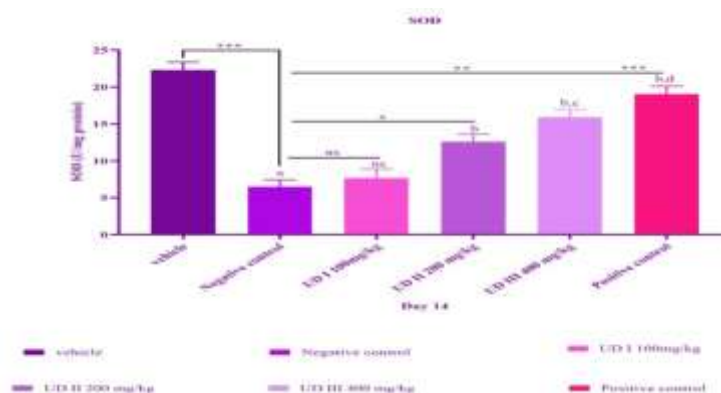


Figure 9. Effect of extract UD on SOD level in blood plasma in tail suspension test rats. Data were statistically analyzed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200mg/kg), ^d $p < 0.05$ vs UD (400mg/kg).

Further, UD (100mg/kg) was not significantly different from the negative control.

(e) Estimation of Blood Cortisol level

On day 14, animals subjected to the tail suspension test showed a marked increase in cortisol levels compared to the vehicle control group. Further oral administration of UD extract at a dose of 100mg/kg do not produce any significant changes, but UD 200 and 400 mg/kg/ *p.o.* significantly and dose-dependently decreased the level of cortisol as compared to the negative control. However, the effect after oral administration of Ashwagandha and a high dose of UD 400 in TST animals was found to be non-significant, as shown in Figure 10.

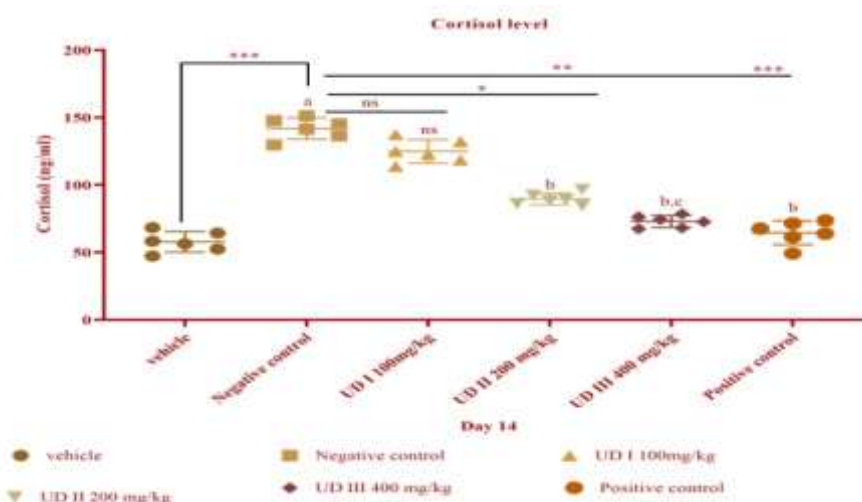


Figure 10. Effect of extract UD on cortisol level in blood plasma in tail suspension test rats. Data were statistically confirmed by one-way ANOVA followed by Tukey's post hoc test. Values are expressed as mean $\pm$ standard deviation, with $p < 0.05$ considered statistically significant: ^a $p < 0.001$ vs vehicle, ^b $p < 0.05$ vs Negative control, ^c $p < 0.01$ vs UD (200 mg/kg).

Further, UD (100mg/kg) found non-significant compared to the negative control.

DISCUSSION

Acute Restraint Stress (ARS)

Exposure to acute restraint stress produced the classical biochemical signature of an acute stress response: significant depletion of GSH and SOD, and significant elevation of MDA, nitrite, and cortisol, relative to vehicle-treated controls, consistent with rapid HPA-axis activation and a parallel burst of lipid peroxidation and nitrosative stress in restraint paradigms.

Oral UD at 200 and 400 mg/kg significantly reversed all five biomarkers in a dose-dependent manner, whereas 100 mg/kg failed to produce significant change relative to the negative control for most parameters, indicating a clear minimum-dose threshold for measurable antioxidant and anti-stress activity. At the highest dose tested (400 mg/kg), *Urtica dioica* approached — but in several parameters did not fully match- the effect of the standard adaptogen Ashwagandha, suggesting that *Urtica dioica*'s protective ceiling under acute restraint stress may sit somewhat below that of a clinically validated reference adaptogen at the doses tested here.

Tail Suspension Test (TST)

The tail suspension paradigm, which layers behavioural despair onto physical restraint, produced a comparable pattern of oxidative and neuroendocrine disruption to ARS significant depletion of GSH and SOD and elevation of MDA, nitrite, and cortisol relative to vehicle controls.

As in the ARS model, *Urtica dioica* reversed these changes dose-dependently, with 400 mg/kg again producing the strongest recovery among the three doses tested. For serum cortisol specifically, *Urtica dioica* 400 mg/kg and Ashwagandha were statistically comparable, mirroring the pattern seen for cortisol in the ARS model and suggesting that UD's HPA-axis-modulating activity generalises across at least two distinct acute-stress modalities (physical restraint and behavioural despair).

Overall discussion

Across both acute stress paradigms tested, a consistent biochemical pattern emerged: stress exposure depleted endogenous antioxidant defences (GSH, SOD) while elevating lipid peroxidation (MDA), reactive nitrogen species (nitrite), and cortisol, and oral *Urtica dioica* extract reversed this pattern in a dose-dependent manner in both models. The 400 mg/kg dose was consistently the most effective of the three doses tested, approaching the standard adaptogen Ashwagandha for most biomarkers and matching it for cortisol suppression in both ARS and TST.

These findings are consistent with the hypothesis that *Urtica dioica*'s anti-stress activity is mediated by its flavonoid- and phenolic-rich phytochemical profile acting on free-radical scavenging and antioxidant enzyme restoration, in parallel with an attenuating effect on HPA-axis output. Because only acute (14-day) stress models were evaluated in this study, whether this protective profile is sustained, diminished, or altered under chronic stress exposure remains an open question for future work.

Limitations of the study

This study has several limitations. First, only two acute stress models (ARS, TST) were evaluated; no chronic stress paradigm was included, so the durability of *Urtica dioica*'s protective effect beyond 14 days of exposure is unknown. Second, biochemical markers were assessed only at a single terminal endpoint (day 14), so the time-course of *Urtica dioica*'s protective action could not be characterized. Third, HPA-axis activity was inferred solely from downstream serum cortisol; upstream markers such as ACTH or corticotropin-releasing hormone, and central (hypothalamic/hippocampal) tissue markers, were not measured. Fourth, no behavioural or cognitive assays beyond immobility duration in the TST were performed, limiting direct evidence for any neuroprotective or anxiolytic claims. Fifth, the extract's phytochemical standardization is incomplete in the current dataset (see Materials and Methods notes above); without total flavonoid/phenolic quantification and, ideally, HPLC or LC-MS fingerprinting of the specific UD batch used, attribution of activity to "flavonoids and phenolics" remains inferential. Sixth, plant material provenance (collection site, season, authentication) is not yet documented in this draft and must be added prior to submission. Finally, as with all rodent studies, direct extrapolation of these findings to humans requires confirmation through standardized-extract pharmacokinetic and clinical studies.

CONCLUSION

This study demonstrates that the ethanolic leaf extract of Uttarakhand-grown *Urtica dioica* exerts a genuine, dose-dependent anti-stress and antioxidant effect in two acute rodent stress paradigms (Acute Restraint Stress and the Tail Suspension Test), restoring GSH and SOD while suppressing MDA, nitrite, and cortisol relative to untreated stressed controls. At 400 mg/kg, UD approached the effect of the standardized adaptogen Ashwagandha across most biomarkers and matched it for cortisol suppression in both models.

These effects are most plausibly mediated by the extract's flavonoid- and phenolic-rich phytochemical profile acting on free-radical scavenging, lipid peroxidation, and HPA-axis regulation, positioning *Urtica dioica* as a promising nutraceutical and adaptogenic phytotherapeutic candidate for mitigating acute stress-associated oxidative damage. Future work should prioritize phytochemical fingerprinting of the specific extract batch, evaluation under chronic stress paradigms, inclusion of upstream HPA markers and behavioural/cognitive endpoints, and dose-optimization studies, ultimately supporting translational and clinical evaluation in humans.

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Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

Ethical approval was obtained from the Institutional Animal Ethics Committee under protocol number ISFCP/IAEC/CPCSEA/2018/386, Department of Pharmacology, Central Animal House, ISF College of Pharmacy, Moga, Punjab.

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