Prev. Nutr. Food Sci. 2024;29(4):414-421
https://doi.org/10.3746/pnf.2024.29.4.414
ISSN 2287-8602

Evaluation of the Anxiolytic and Antidepressant Effects of Standardized Ashwagandha (*Withania somnifera*) Root Extract in Wistar Rats

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ABSTRACT: Ashwagandha (*Withania somnifera*) is a popular herb in Ayurveda, the traditional medicine system in India. It is known to exert stress-mitigating properties and has been extensively studied for its safety and efficacy in various disorders. This *in vivo* study assessed the effects of Ashwagandha root extract (ARE) on stress in rats. The anxiolytic and antidepressant effects of ARE were assessed using the elevated plus maze test, sucrose preference test, and forced swim test. The rats were divided into the following groups: control group (no disease), disease control group (no treatment), standardized ARE group (test; ARE administered in doses of 27, 54, and 108 mg/kg body weight), and fluoxetine group (active control). Biochemical parameters in the serum [monoamine oxidase (MAO)-A, MAO-B, serotonin, cortisol, adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), interleukin (IL)-6, tumor necrosis factor (TNF)-α, and brain-derived neurotrophic factor (BDNF)] and brain tissue (serotonin) were estimated at the end of 36 days to understand the potential mechanism behind the anxiolytic and antidepressant effects of ARE. The behavior test results indicated significant improvement in anxiety and depression-like behavior with ARE treatment in a rat model exposed to a validated protocol of chronic variable stress. The results of biochemical analyses revealed a significant increase in serotonin and BDNF levels and a decrease in CRH, ACTH, and cortisol levels. The inflammatory markers IL-6 and TNF-α were also significantly reduced with ARE treatment. ARE demonstrated notable effects on anxiety and depression markers in rats, indicating its potential as a prophylactic and therapeutic agent.

Keywords: antidepressant, anxiolytic, Ashwagandha, stress, *Withania somnifera*

INTRODUCTION

Anxiety and depression are prevalent stress-related mood disorders that pose a significant challenge to public health worldwide (World Health Organization, 2017). They involve feelings of uneasiness and deep sadness, along with a loss of interest, which can greatly impact the quality of life (Jansson-Fröjmark and Lindblom, 2008; Tripathi, 2021).

Traditional medication for anxiety and depression often results in delayed and varied side effects; thus, alternative treatments with better safety profiles are needed (Bandelow et al., 2017; Hidese et al., 2019). Herbal remedies, including Ashwagandha (*Withania somnifera*), offer a holistic approach with fewer reported side effects, making them attractive for research (Arya et al., 2023).

Ashwagandha (*W. somnifera*) is a widely used herb in Ayurveda and is known for its wide range of therapeutic effects, including antioxidant, anxiolytic, memory enhancement, and anti-inflammatory properties (Mishra et al., 2000). Ashwagandha roots are highly valued because they are rich in withanolides, which are thought to contribute to Ashwagandha's benefits (Kaur et al., 2017). Seepers et al. (2021) described the benefits of *W. somnifera* for stress and stress-related neuropsychiatric disorders, such as anxiety, depression, and insomnia. However, the mechanisms and effects of Ashwagandha on various biomarkers in the blood and brain in these disorders remain to be elucidated.

Research conducted on Ashwagandha has mainly fo-

Received 23 August 2024; Revised 15 October 2024; Accepted 4 November 2024; Published online 31 December 2024
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cused on its therapeutic properties. Several clinical trials have shown that Ashwagandha effectively reduces the symptoms of anxiety and depression and cortisol levels in participants under chronic stress, indicating that Ashwagandha could be a natural treatment option (Speers et al., 2021; Akhgarjand et al., 2022). However, the potential of Ashwagandha for prevention purposes has not been widely studied. Ashwagandha's adaptogenic properties, which help the body cope with stress, suggest its potential for preventive use (Singh et al., 2011).

In the present study, we aimed to examine the preventive and therapeutic effects of Ashwagandha on anxiety and depression in rats by assessing the behavioral and biochemical markers associated with anxiety and stress conditions.

MATERIALS AND METHODS

Animals

Wistar rats of either sex, weighing 150 to 200 g, were obtained from Nutrivet Life Sciences. The animals were acclimated to the environment and handled daily by the same individual for 7 days before the commencement of stress procedures. The rats were placed in a laboratory and housed in polypropylene cages under standard conditions (23°C±2°C, 12-h light/dark cycle, 50%±10% humidity) with free access to food and water. All experimental protocols were approved by the Institutional Animal Ethics Committee of Bharati Vidyapeeth (Deemed to be University) Medical College (BVDUMC/5458/2023/001/005).

Experimental protocol

***Drugs and chemicals*:** Ashwagandha root extract (ARE) was prepared in accordance with the standardized manufacturing process and provided by the sponsor, KSM-66 Ashwagandha. ARE was manufactured from Ashwagandha plant roots using a water extraction process. The herb-to-extract ratio was 15:1. Every batch of this extract was standardized to a withanolide A content of >0.3% by high-performance liquid chromatography. Fluoxetine hydrochloride was sourced from Intas Pharmaceuticals Ltd. Other chemicals and consumables were obtained from Sharad Agencies.

***Study groups*:** Seventy-two rats were randomly divided into nine groups, with four males and four females in each group, and housed in separate cages (Table 1). The daily dosages for ARE were extrapolated from the human dose of 600 mg/d (Ghosh, 2005). Fluoxetine was orally administered at a dose of 10 mg/kg.

***Stress induction*:** Stress was induced using the unpredictable chronic mild stress (UCMS) model, which utilizes various alternating stressors in a semirandom manner to ensure unpredictability. These stressors included a 24-h period of food deprivation, water deprivation, isolation, high-density housing, cage tilt, soiled bedding, immobility, overnight illumination, and exposure to foreign objects (Fig. 1).

***Prophylactic groups (Pr-groups)*:** The elevated plus maze (EPM) test and sucrose preference test (SPT) were conducted at the start to establish baseline data. ARE was administered for the first 15 days, followed by repeated EPM test and SPT on day 15. ARE treatment continued along with unpredictable stress for 3 weeks. On day 29, the EPM test and SPT were conducted again. The animals continued to receive ARE treatment and stress until day 36, at which point they were exposed to the EPM test, forced swim test (FST), and SPT. Blood samples were collected for biochemical analysis under ketamine anesthesia (100 mg/kg intraperitoneally) from the retro-orbital plexus. After sacrifice, the brains of rats were removed for dopamine and serotonin assessment.

***Therapeutic groups (Th-groups)*:** The EPM test and SPT were conducted at baseline, followed by 15 days of exposure to unpredictable stress without treatment. ARE treatment began on day 15 and continued for the next 3

Table 1. Study group design

Group	Dosage
Control	Saline
Disease control	Saline
Prophylactic low dose (Pr-LD)	ARE 27 mg/kg
Prophylactic medium dose (Pr-MD)	ARE 54 mg/kg
Prophylactic high dose (Pr-HD)	ARE 108 mg/kg
Therapeutic low dose (Th-LD)	ARE 27 mg/kg
Therapeutic medium dose (Th-MD)	ARE 54 mg/kg
Therapeutic high dose (Th-HD)	ARE 108 mg/kg
Positive control (fluoxetine)	Fluoxetine 10 mg/kg

ARE, Ashwagandha root extract.

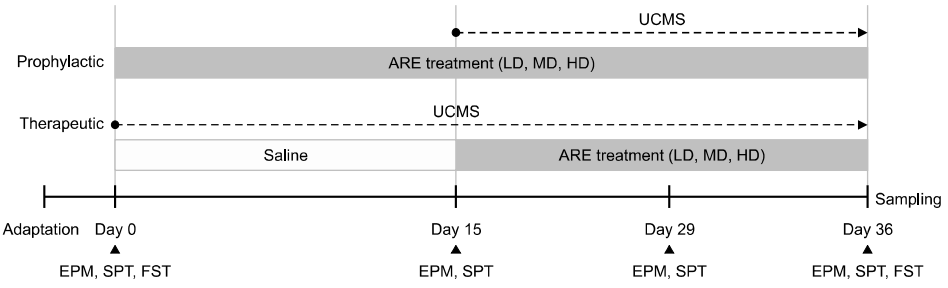

Fig. 1. Design of the stress induction experiment. ARE, Ashwagandha root extract; UCMS, unpredictable chronic mild stress; EPM, elevated plus maze; SPT, sucrose preference test; FST, forced swim test; LD, low dose; MD, medium dose; HD, high dose.

weeks, along with unpredictable stress. The EPM test and SPT were repeated on days 29 and 36, along with unpredictable stress. The FST was performed on day 36, and blood samples were collected from the retro-orbital plexus under ketamine anesthesia (100 mg/kg intraperitoneally) for biochemical analysis. After the animals were sacrificed, their brains were removed for serotonin assessment, and their spleen and adrenal glands were removed for weight analysis.

Behavioral tests

***Elevated plus maze test*:** The EPM test was used to assess anxiety in rats. The rats were placed at the center of the maze facing an open arm, and their behavior, including their first choice of arm, the number of entries into the open or closed arms, and the time spent in each arm, was monitored. An entry was counted when all four paws crossed into an arm. All precautions were taken to maintain constant experimental atmosphere, avoiding external stimuli. A test was conducted 1 h after treatment using a maze tracking system (Stoelting Co.).

***Forced swim test*:** The rats were placed in a vertical Plexiglas cylinder filled with water (25°C; depth 30 cm) for 5 min. The depression indicator immobility time was measured while a rat floated still with its nose above water. The water was changed after each test, and the rats were tested for 1 h posttreatment. Struggling or attempting to swim was considered an indication of potential antidepressant effects (Yankelevitch-Yahav et al., 2015).

***Sucrose preference test*:** To establish baseline preference, the animals were habituated to the sucrose solution before the stress protocol. During testing, the animals were deprived of food and water for 18 h and, then presented with two premeasured bottles containing 1% sucrose solution or water for 24 h. Sucrose preference was measured on days 0, 15, 29, and 36 using the following formula: sucrose preference=[sucrose intake/(sucrose intake+water intake)]×100 (Verharen et al., 2023).

***Biochemical tests*:** Serum monoamine oxidases (MAO-A, MAO-B), serotonin (serum and brain tissue), cortisol, adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), interleukin (IL)-6, tumor necrosis factor (TNF)-α, and brain-derived neurotrophic factor (BDNF) were measured using enzyme-linked immunosorbent assay in an ISO-certified laboratory.

Statistical analysis

One-way analysis of variance was used to evaluate differences between groups. Post hoc analyses were performed using Tukey's, Sidak's, and Dunnett's multiple comparison tests. The Kruskal-Wallis test was used for skewed data. The results are expressed as the mean±SD of the mean, with statistical significance considered at $P<0.05$. Statistical analyses were performed using the Windows-based statistical program Stata version 13.1 (StataCorp.).

RESULTS

Effects of the prophylactic and therapeutic administration of Ashwagandha root extract on sucrose preference

When water and sucrose were given as a choice for drinking at baseline, 100% of the animals preferred to drink sucrose. In the prophylactic ARE groups, sucrose preference remained unchanged from the control after 15 days without stress, indicating no impact on sucrose preference under nonstress conditions. However, when the ARE Th-groups were exposed to chronic unpredictable stress for 15 days without treatment, there was a significant decrease in sucrose preference ($P<0.001$) compared with that in the healthy control ($P<0.001$). On day 29, sucrose preference in the prophylactic ARE groups maintained a similar level to that of the control group, showing that ARE pretreatment preserved sucrose preference even under stress. Moreover, the therapeutic administration significantly reversed the stress-induced decrease of sucrose preference compared with that in the disease control group ($P<0.001$). On day 36, sucrose preference in all study groups was comparable to that in the healthy control, whereas it remained low in the disease control group (Fig. 2).

Effects of the prophylactic and therapeutic administration of Ashwagandha root extract on immobility time in the forced swim test

The disease control group exhibited a significantly increased immobility period ($P<0.001$) compared with the control, showing behavioral despair. By contrast, the prophylactic administration of ARE significantly suppressed the increase in the immobility time under stressful conditions. Notably, the immobility period in the ARE Pr-groups (108 mg/kg) was comparable to that in the healthy control. The therapeutic treatment significantly reduced stress-induced immobility compared with the disease control group. In the Pr- and Th-groups, a dose-dependent effect was observed on the immobility period in the FST, and prophylactic treatment seemed to be more effective than therapeutic use (Fig. 3).

Effects of the prophylactic and therapeutic administration of Ashwagandha root extract on the time spent in the open and closed arms in the elevated plus maze test

At baseline, the time spent by rats in the closed and open arms was almost similar in all groups. The findings on day 15 showed that the prophylactic ARE groups [low dose (LD), medium dose (MD), and high dose (HD)] exhibited significantly higher time spent in the open arms (LD, $P<0.05$; MD, $P<0.05$; and HD, $P<0.01$) than

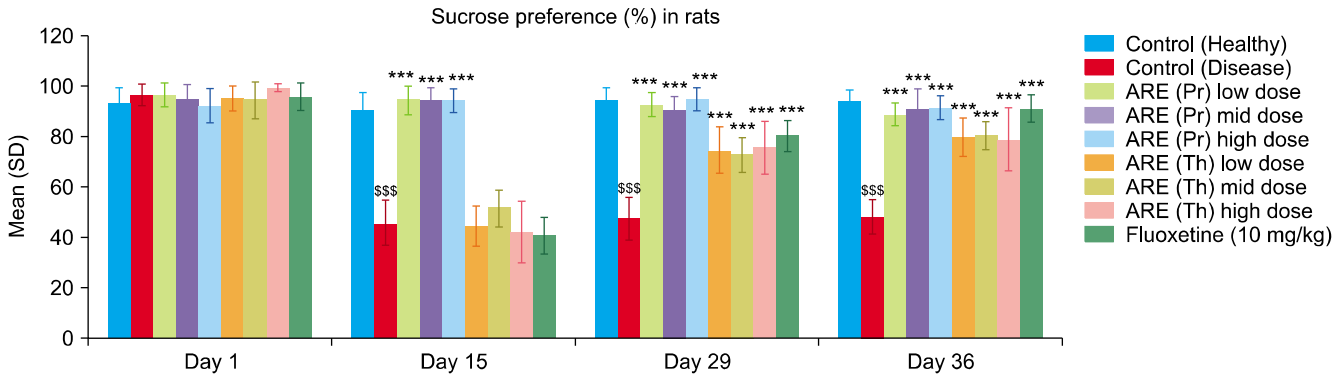

Fig. 2. Effects of ARE on the sucrose preference percentage. Values are presented as mean±SD (n=8). Analysis of variance followed by Dunnett's multiple comparisons test was used. $^{\$\$\$}P<0.001$ in comparison with the control group. $^{***}P<0.001$ in comparison with the disease control group. ARE, Ashwagandha root extract; Pr, prophylactic; Th, therapeutic.

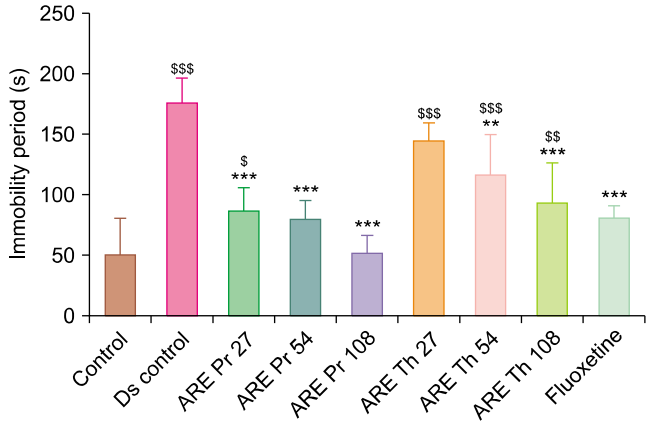

Fig. 3. Effects of ARE on the immobility time of rats in the FST on day 36. Values are presented as mean±SD (n=8). Analysis of variance followed by Tukey's and Dunnett's multiple comparisons test were used. $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ in comparison with the control group. $^{**}P<0.01$ and $^{***}P<0.001$ in comparison with the disease control group. ARE, Ashwagandha root extract; FST, forced swim test; Ds, disease; Pr, prophylactic; Th, therapeutic.

in the closed arms compared with the disease control group. On day 29, the ARE Pr-groups (MD, $P<0.05$; HD, $P<0.05$) and Th-groups (HD, $P<0.05$) showed a significant increase in time spent in the open arms, and these were comparable to those in the fluoxetine group. On day 36, the time spent in the open arms continued to be higher in the ARE Th- and Pr-groups, but the difference was not statistically significant. Moreover, the number of entries into the open arms tended to be higher in the ARE-treated groups than in the disease control group, further supporting the anxiolytic effect of ARE treatment (Table 2).

Effects of the prophylactic and therapeutic administration of Ashwagandha root extract on stress-related biochemical markers

On day 36, MAO-A and MAO-B levels were significantly increased ($P<0.001$) in the disease control group than in the healthy control group. Prophylactic and therapeutic ARE treatments significantly decreased MAO-A and MAO-B levels compared with the disease control group. Furthermore, brain tissue serotonin levels showed a dose-dependent increase ($P<0.001$) in the ARE-treated groups than in the disease control group, and the prophylactic MD and HD groups showed comparable brain tissue serotonin levels to the healthy control group.

Prophylactic and therapeutic treatments resulted in significant decreases in stress- and inflammation-related biomarkers, including CRH, ACTH, cortisol, IL-6, and TNF-α, compared with the disease control group. Moreover, serotonin (brain tissue and serum) and BDNF levels in the Pr- and Th-groups were significantly increased compared with those in the disease control group (Fig. 4). These results indicated that the prophylactic and therapeutic administration of ARE significantly prevented and alleviated the stress-induced alteration of biochemical markers to a similar extent to fluoxetine.

Effects of the prophylactic and therapeutic administration of Ashwagandha root extract on the organ weight of the spleen and adrenal glands

No significant changes in the organ weights of the spleen and adrenal gland were observed in any of the ARE-treated groups compared with the control group, suggesting that ARE does not adversely affect these organs.

DISCUSSION

The present study revealed that the prophylactic and therapeutic administration of ARE could normalize and suppress behavioral and biochemical alterations induced by unpredictable chronic stress in rats. We found that ARE pretreatment for 15 days suppressed the UCMS-induced decrease of sucrose preference and the time spent in the open arm, as well as the increase in immobility time, suggesting that ARE has a protective effect on chronic stress-induced anxiety and depression-like behaviors. In

Table 2. Time spent in the open and closed arms in the elevated plus maze test

	Day 0	Day 15	Day 29	Day 36
Control (healthy)				
Open	111.31±33.65	99.13±31.78	139.63±35.38	98.75±40.39
Closed	130.19±24.18	166.06±28.17	96.25±27.47	101.38±30.58
Control (disease)				
Open	107.50±23.31	82.88±12.55	73.63±21.00	76.06±35.58
Closed	98.00±12.67	128.25±22.24	118.06±29.63	144.81±31.82
ARE (Pr) LD				
Open	123.00±23.63	201.00±39.45*$	137.00±24.70	106.69±35.68
Closed	99.69±15.56	73.06±29.64	71.50±19.78	88.81±31.54
ARE (Pr) MD				
Open	130.00±33.37	200.38±19.60*$	169.50±35.71*	162.56±38.18
Closed	122.38±30.77	75.06±15.15	59.25±20.99	87.63±32.95
ARE (Pr) HD				
Open	127.69±29.67	180.63±29.33**	158.00±20.58*	140.31±37.51
Closed	124.50±38.82	51.50±19.56	52.69±13.97	41.75±16.48
ARE (Th) LD				
Open	120.38±31.76	121.31±14.21	126.94±16.66	141.06±34.49
Closed	125.31±32.41	116.56±18.31	76.38±17.02	73.88±23.72
ARE (Th) MD				
Open	113.19±10.18	113.13±35.48	118.38±30.90	123.75±37.51
Closed	115.94±12.67	109.25±28.95	71.25±14.16	50.63±10.93
ARE (Th) HD				
Open	117.69±26.85	132.13±33.18	177.25±30.22*	169.00±37.42
Closed	135.06±31.22	118.88±24.70	62.00±25.24	55.19±25.28
Fluoxetine (10 mg/kg)				
Open	116.81±32.29	146.38±38.33	129.94±38.33	160.88±31.13
Closed	135.94±38.29	120.69±35.95	72.56±27.20	79.06±28.00

Values are presented as mean±SD (n=8).
Analysis of variance followed by Sidak's and Dunnett's multiple comparisons test were used. *P<0.05, **P<0.01 in comparison with the closed arm group. $^{\$}P$<0.05 in comparison with the disease control group.
ARE, Ashwagandha root extract; Pr, prophylactic; Th, therapeutic; LD, low dose; MD, medium dose; HD, high dose.

addition, posttreatment of ARE after 15 days of stress effectively normalized the UCMS-induced anxiety and depression-like behaviors, implying that ARE could serve as a viable option for managing anxiety and depression.

Anhedonia is a fundamental symptom of major depressive disorder, characterized by a diminished ability to engage in activities that are pleasurable and rewarding (Primo et al., 2023). The results of the SPT indicate that therapeutic ARE treatment significantly increased interest in pleasurable activities to a level comparable to that of the fluoxetine group. Moreover, normal sucrose preference was maintained after the 15-day prophylactic ARE administration, even in stress conditions. This finding implies that ARE can prevent the onset of depression, which has been less emphasized in previous studies. In the FST, prophylactic and therapeutic ARE treatment demonstrated a dose-dependent decrease in the immobility time, which reflects a reduction in behavioral despair. Prophylactic use seemed to be more effective than therapeutic use, significantly inhibiting the increase in the immobility time, which is a core measure in depression research (Lucki, 1997). Furthermore, the results of the EPM test confirmed these findings by showing that preventive and therapeutic ARE treatment enhanced the duration and frequency of entries to the open arms of the EPM, indicating decreased anxiety levels. This effect was particularly pronounced in the Pr-groups, aligning with the results of the meta-analysis by Durg et al. (2015), which reported increased open arm entries and duration with ARE (100 and 200 mg/kg) pretreatment.

Various biomarkers were studied to obtain insights into the mechanism behind the anxiolytic and antidepressant effects of Ashwagandha. Our study showed a significant reduction in MAO-A and MAO-B levels in the ARE-treated groups. This finding is important because elevated MAO levels are associated with increased degradation of monoamines, contributing to depression (Duncan et al., 2012). In addition, serotonin levels were increased in the ARE-treated groups compared with the disease control group, further supporting the antidepressant effect of ARE.

The dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is commonly observed in major depression, with increased plasma cortisol being a notable finding (Heuser, 1998). Central HPA axis overdrive has been observed in severe depression (Carroll et al., 2007). Our

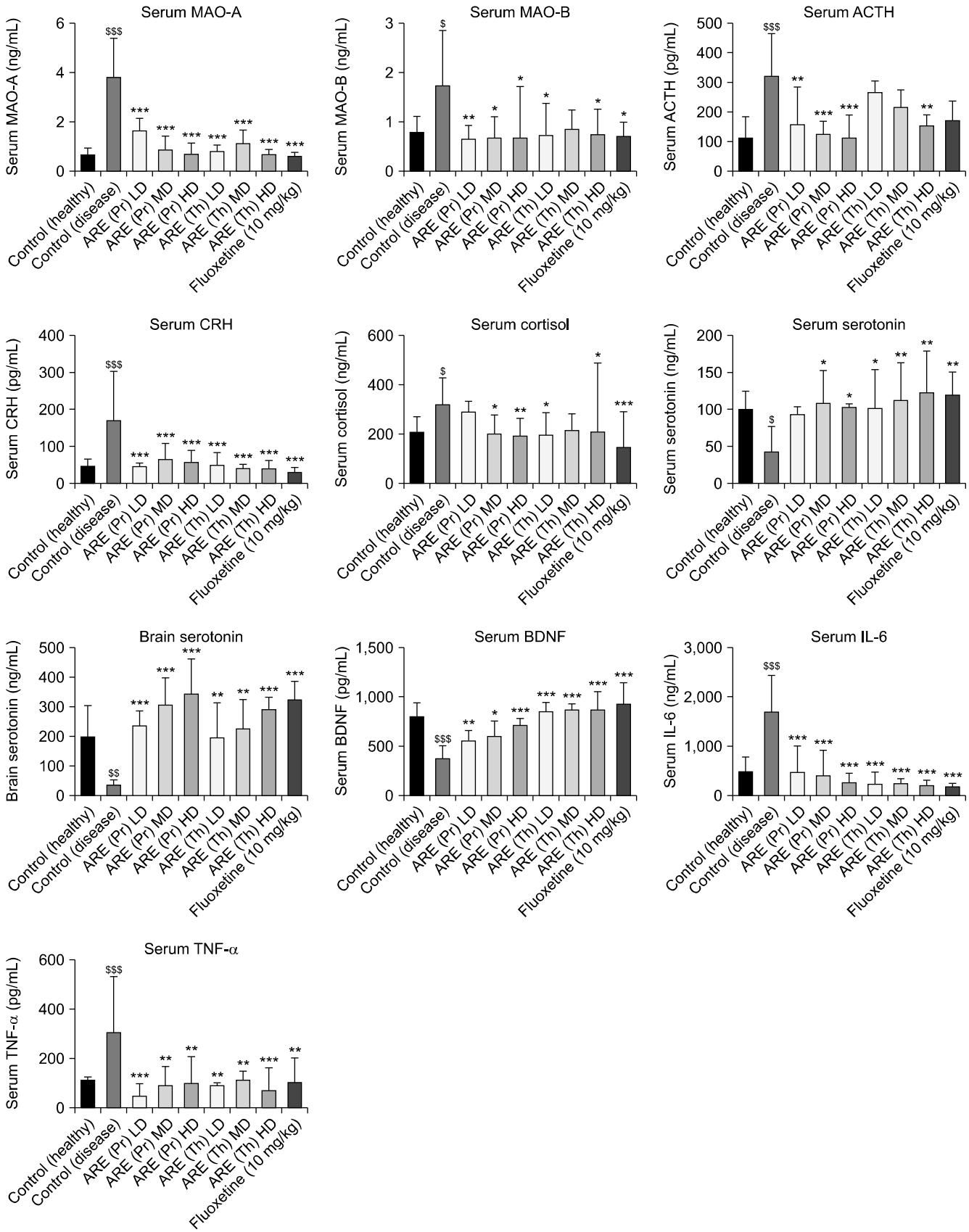

Fig. 4. Effects of ARE on different biochemical parameters on day 36. Values are expressed as mean±SD (n=8). Analysis of variance followed by Tukey's and Dunnett's multiple comparisons test were used. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ in comparison with the disease control group. $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ in comparison with the control group. MAO, monoamine oxidases; ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; BDNF, brain-derived neurotrophic factor; IL, interleukin; TNF, tumor necrosis factor; ARE, Ashwagandha root extract; Pr, prophylactic; Th, therapeutic; LD, low dose; MD, medium dose; HD, high dose.

findings indicate that ARE treatment modulated the HPA axis. The prophylactic treatment with ARE prevented the increase in cortisol and ACTH levels, similar to those of fluoxetine. The Th-groups also showed reduced levels of these markers, although to a slightly lesser extent. Moreover, the role of proinflammatory cytokines in activating the HPA axis and worsening depressive symptoms is well established (Cattaneo et al., 2015). Elevated levels of cytokines, including IL-1β, IL-6, C-reactive protein, TNF-α, and interferon (INF)-α, are known to increase cortisol secretion and decrease BDNF levels, contributing to the pathophysiology of depression and anxiety (Young et al., 2014). In our study, prophylactic and therapeutic ARE treatments resulted in reduced levels of IL-6 and TNF-α, which are consistent with the findings of Verma et al. (2023). The significant reductions in cortisol, ACTH, CRH, IL-6, and TNF-α levels in the ARE-treated groups highlight its role in modulating the HPA axis and reducing neuroinflammation.

The increase in BDNF levels in the ARE-treated groups is particularly noteworthy, indicating a neuroprotective and potential antidepressant effect (Angelucci et al., 2000; Mondal and Fatima, 2019). The "neurotrophic hypothesis of depression" is largely based on observations that a decrease in hippocampal BDNF levels is correlated with stress-induced depressive behavior (Duman and Monteggia, 2006; Martinowich et al., 2007).

Stress can lead to adrenal hypertrophy and spleen atrophy because of the release of corticosteroids and increased T-cell proliferation (Hara et al., 1981). Rogóz et al. (2005) reported that treatment with imipramine and metyrapone reduced spleen weight, potentially due to erythrocyte redistribution. ARE treatment did not change the spleen and adrenal gland weights, indicating that it may mitigate T-cell proliferation and reduce cortisol, ACTH, and CRH levels even under stress (Rogóz et al., 2005; Meera and Nagarjuna, 2009).

This study provided evidence that the preventive and therapeutic administration of ARE at doses of 27, 54, and 108 mg/kg significantly reduced anxiety and depression induced by unpredictable chronic stress in rats. The preventive and therapeutic treatment of ARE significantly inhibited the decrease in sucrose preference and immobility time of FST under stress condition. Furthermore, it suppressed the increase in stress-induced biochemical markers (e.g., MAO-A/B, cortisol, ACTH, CRH) and inflammatory cytokines (IL-6, TNF-α) while retaining comparable serotonin and BDNF levels to those of the healthy control and fluoxetine groups. These findings suggest that ARE effectively prevents the onset of anxiety and depression-like behaviors, as well as stress-induced biochemical changes. Our research suggests that ARE could be a viable option for preventing and managing anxiety and depression.

FUNDING

This research was supported by Kolmar BNH, Seoul, Republic of Korea.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: JD, PD. Analysis and interpretation: DL. Data collection: JD, PD. Writing the article: DL. Critical revision of the article: SS, HH, SBK. Final approval of the article: all authors.

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