# Neuroprotective Effects of Rhamnazin as a Flavonoid on Chronic Stress-Induced Cognitive Impairment

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**Abstract**: The aim of the present examination was to research the conceivable impacts of chronic administration of rhamnazin, on constant stress instigated learning and memory impairment in rodent. The rats were controlled every day 4 h/day for 30 days in very much ventilated plexiglass tubes without access to nourishment and water. These creatures were infused with rhamnazin or vehicle worry over a time of 30 days. At that point, spatial learning and memory of the rodent were assessed by the Morris water maze. We didn't watch a noteworthy contrast in the escape inactivity in rodent subjected to rehashed restriction push, which shows that learning capacity was not influenced by limitation stress. Be that as it may, the spatial memory capacity was essentially disabled in the over and again limited rodent. Rhamnazin administration particularly expanded the time spent in the objective quadrant in rodent presented to chronic stress in the probe trial as tried in the Morris water maze. Additionally contemplates demonstrated that rhamnazin treatment diminished plasma adrenocorticotrophic hormone levels (ACTH) of those rats subjected to rehashed restriction push. The impact of rhamnazin on the levels of mind inferred neurotrophic factor (BDNF) in hippocampus was likewise researched. The outcome demonstrated that rhamnazin standardized the diminished BDNF levels in rodent subjected to rehashed limitation push. These discoveries give more confirmation that ceaseless administration of rhamnazin enhances spatial memory in over and over controlled rodent and BDNF motioning in the hippocampus might be included in the defensive impacts of rhamnazin.

**Keywords**: Flavonoids, Stress, Learning, Memory, Neuroprotective.

## INTRODUCTION

Chronic exposure to stressful life events is a well-established and significant risk factor for the development and maintenance of cognitive deficits. Chronic stress can trigger or exacerbate disruption in the activity of the hypothalamic–pituitary–adrenal (HPA) axis and increases circulating levels of the stress hormones glucocorticoid (GC) [1, 2]. The hippocampus, which is intrinsically linked to both learning and memory, is particularly vulnerable to GC as it contains the highest density of GC target receptors within the brain [3, 4]. Long-term exposure to stress or GC leads to numerous changes in the hippocampus, such as altered excitability, neuronal morphology, and even cell death [5, 6]. As the hippocampus plays a key role in processing new information leading to learning and memory, chronic stress can be associated with cognitive deficits in animals that can be reflected in several tasks, such as the Morris water maze and so on [7]. Brain-derived neurotrophic factor (BDNF) is by all accounts required in cognitive hindrance show in chronic stress. It is realized that BDNF is exceedingly communicated in the hippocampus. In addition, investigations about completed with particular erasure have demonstrated that hippocampus-confined BDNF mutant rodent indicate particular cognitive impairment in behavioral errands related with learning and memory [8]. It is very much demonstrated that long-haul presentation to stress may diminish BDNF articulation in the brain [9] Likewise, BDNF is known to contribute toward neuronal insurance in brain amid the maturing procedure and also in neurodegenerative injures, for example, Alzheimer's dementia and Parkinson's diseases portrayed by dynamic cognitive deficits [10, 11]. To date, the constant stress instigated debilitation in learning and memory has been contemplated broadly; notwithstanding, just a few of studies have investigated conceivable methods for keeping these stress initiated deficits. As of late, various investigations detailed that some regular flavonoids may enhance cognitive deficiencies by lessening of oxidative stress and tweak of BDNF levels [12, 13]. administration of Baicalin prior to high-dose exogenous corticosterone resulted in significant neuroprotective activity against neuronal impairment and memory dysfunction in rats and significantly alleviated memory-associated decreases in the expression levels of BDNF and CREB proteins and mRNAs in the hippocampus [14], while flavonoid chrysin counteracts age-related cognitive decay by regulation of BDNF levels in matured rat brain [9].

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Quercetin, which has a structure like that of rhamnazin, likewise averts spatial learning and memory shortages instigated by interminable chronic stress in rats by lessening of oxidative stress [15].

Rhamnazin, which belongs to the large natural group of flavonols, is presented widely in habitual food, teas, fruits, and medical herbs. It was reported that intake of flavonols including quercetin, rhamnazin, and kaempferol has favorable effects on cognitive performance [18]. Previous study has shown that rhamnazin could inhibit angiogenesis and may be a promising anticancer drug candidate [16]. Although, anti-inflammatory [17] and potent antiangiogenic [16] effects of rhamnazin have been well established, no clear evidence has shown the potential effects of rhamnazin on the protection of cognitive deficits. Therefore, in this study, we investigated the effects of rhamnazin administration on spatial learning and memory of rat subjected to repeated restraint stress and explored the possible mechanisms involved.

## MATERIALS AND METHODS

### Animals

All procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by Research and Ethics Committee of Islamic Azad University. Adult male wistar rat weighing 220–250 g were obtained from Pasture institute (Tehran, Iran) and housed under a 12 h light/dark cycle (6:00–18:00) and free access to food and water. After habituation for 2 weeks, the rats were divided into four groups of 6 animals each. Rat receiving chronic stress stimuli were restrained daily for 4 h/day (from 14:30 to 18:30) for consecutive 30 days in well-ventilated plexiglass tubes without access to food and water. Control animals were housed in their home cage without disturbance. The rat were divided as follows: chronic stress stimuli with rhamnazin injection (stress+ rhamnazin) group, chronic stress stimuli with vehicle injection (stress + vehicle) group, and controls treated with rhamnazin (control+ rhamnazin) group or vehicle (control+vehicle) group. Rhamnazin at a dose of 50 mg/kg was injected intraperitoneally at 40 min before daily restraint for consecutive 30 days. Rhamnazin used in this experiment was purchased from Sigma-Aldrich (St Louis, Missouri, USA), which was dissolved in dimethyl sulfoxide (DMSO) and diluted in a 0.9% NaCl solution.

### Morris Water Maze Test

The rat in each group was subjected to water maze training after a chronic stress procedure and repeated drug administration for consecutive 30 days. The Morris water maze test was carried out as described previously [18]. The circular water maze pool (120 cm in diameter) was located in a dimly lit room. The water temperature was maintained at 25 ± 1°C by a heating pad located beneath the pool. The circular pool was filled with water that had been made opaque by adding a white pigment and was divided into four quadrants. The maze was located in a room containing many extramaze cues (*e.g*. bookshelves, refrigerator, and posters). An escape platform (10 cm in diameter) was submerged about 0.5 cm below the water surface in a fixed position in the training quadrant. Rat in each group were handled for 2 min/day over 6 consecutive days before the start of training. The rats were subjected to six training trials per day for 6 consecutive days. On each trial, the rats were placed into the pool, facing the wall. The trial was complete once the rat found the platform or 60 s had elapsed. If the rat failed to find the platform on a given trial, the experimenter guided the rat onto the platform. One average data were collected daily for each rat and recorded in total for 6 consecutive days. The animals were allowed to rest for 30 s on the platform between trials. The spatial memory was evaluated using a probe test on days 5 and 7 of the training trials. The pool was divided into four quadrants: training (target), adjacent left, adjacent right, and opposite. The probe test on day 5 was performed 6 h after the last acquisition trial. The platform was removed and the rat was placed in the opposite quadrant and was allowed to search for 60 s in the pool. The relative time (%) spent in all the four quadrants was recorded. Behavioral data from the training and the probe tests were acquired and analyzed using an automated tracking system.

### Adrenocorticotrophic (ACTH) Hormone Analysis

It is known that exposure to chronic stress may disrupt the HPA axis. We next measured the ACTH levels in plasma of the rat. Rat in each group were killed by decapitation immediately after the last day of restraint and their trunk blood was collected in tubes with EDTA. The blood was centrifuged at 1000 rpm/min for 15 min at 4°C and the plasma was stored at −70°C until used. Plasma adrenocorticotrophic hormone (ACTH) was determined using an enzyme-linked immunosorbent assay.

### BDNF Levels Analysis in Hippocampus

Rat in each group were killed by decapitation immediately after the last day of restraint and the hippocampus was isolated for western blotting test. The protein concentration was determined using a bicinchoninic acid assay. Total proteins (20 µg) were separated by 12% SDSPAGE and transferred to PDVF membranes by electroblotting. Following blocking in 1% BSA for 2 h, the protein membrane was incubated in the primary antibody (AB1543; Millipore, Billerica, Massachusetts, USA), rabbit anti-BDNF (1 : 1000), at 4°C overnight and then in the secondary antibody, HRP-conjugated goat anti-rabbit IgG (1 : 5000), for 1 h. Bound antibodies were detected by an enhanced chemiluminescence detection reagent. Band intensities were quantified using the Image Quant software.

### Statistics

The results were represented as mean ± SEM. The data for escape latency during training of the Morris water maze were analyzed by repeated-measures analysis of variance. Other data were analyzed by one-way analysis of variance, followed by the Newman–Keuls test using SPSS 18 software. P value less than 0.05 was considered to be significant.

## RESULTS

### Effects of Rhamnazin on Spatial Learning at the Training Session

We did not observe a significant difference in the latency to escape on the hidden platform between restraint rat and vehicle, although there was a tendency of the latency to escape to be delayed in the restraint rat. We also could not detect a significant difference in rhamnazin on the escape latency on the hidden platform between restraint rat and rhamnazin-treated rat (P> 0.05, n=6) (Figure **1**).

Effects of rhamnazin on spatial memory in the probe session

As shown in Figure **2**, rat in the chronic stress treatment group showed no spatial bias for the target quadrant where the platform was located during training compared with that of the adjacent left quadrant (q=2.166, P>0.05).

Treatment with rhamnazin significantly prevented the memory impairment indicated by the increase in the time spent in the target quadrant (q=3.633, p<0.05 compared with the stress group) (Figure **3b**). Also, the rhamnazin-alone treatment group spent more time in the target quadrant similar to the vehicle group. There were no variations in the average speed [F(3,11)=0.416, P>0.05, n=6] and travel distance [F(3,24)=0.373,P>0.05,n=6] in the probe trials (Figure **3c-d**).

### Effects of Rhamnazin on Plasma ACTH Levels

We found that the ACTH content in the plasma of the restraint rat was significantly elevated compared with the vehicles (q=12.09,P<0.001;n=6 in the vehicle group,n=6 in the chronic stress group). More importantly, our results showed that administration of

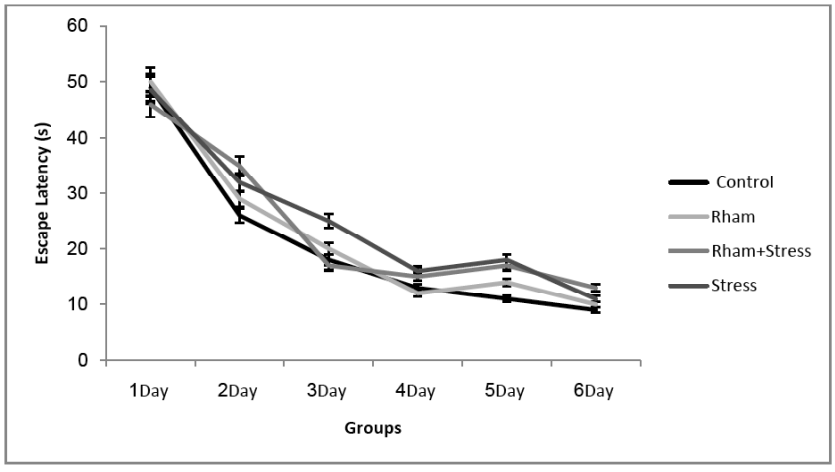


**Figure 1:** Effects of rhamnazin administration on escape latency in the training session. Latency curve in the water maze task showing that rat that received repeated restraint stress for 30 days had no spatial learning deficits, although there was a tendency of the latency to escape to be delayed in the restraint rat. The escape latency on the hidden platform was also not affected by rhamnazin treatment (P>0.05, n=6). Rham, rhamnazin.

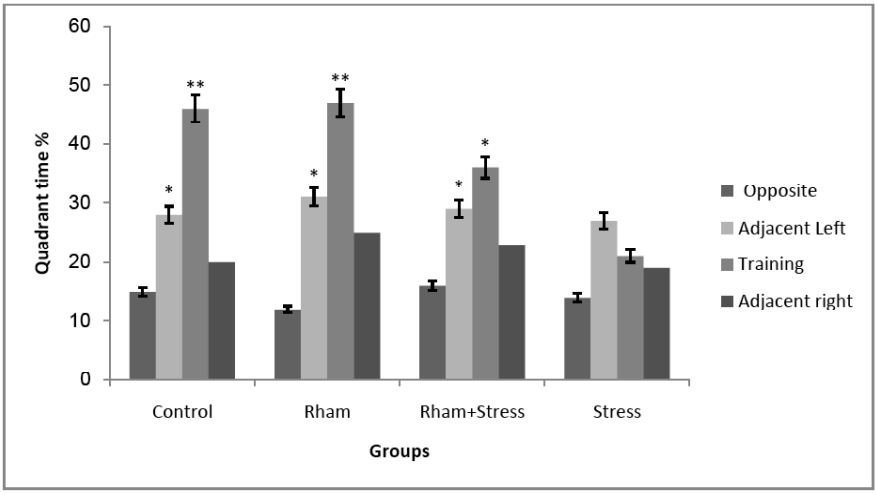


**Figure 2:** Effects of rhamnazin administration on spatial learning and memory at the probe session on day 5. Rat in the chronic stress group spent less time in the target quadrant compared with the control group. The rat also showed no spatial bias for the target quadrant compared with the adjacent left quadrant (q=2.126,P > 0.05). Rat in the rhamnazin-alone treatment group and the rhamnazin + stress group spent more time in the target quadrant than stressed rat. Rat in the vehicle group [**P<0.01;n=6], the rhamnazin group [**P < 0.01; n=6], and the rhamnazin + stress group [**P<0.01;n=6] showed strong spatial bias for the target quadrant compared with the other quadrants. Rham, rhamnazin.

rhamnazin significantly decreased the ACTH levels compared with the chronic stressed rat (q=10.16, P<0.001, n=6 in the rhamnazin with chronic stress treatment group) (Figure **4**). Again, rhamnazin alone had no detectable effect on the plasma ACTH content compared with vehicles.

### Effects of Rhamnazin on the Expression of BDNF Protein in the Hippocampus

As shown in Figure **5**, statistical analysis of the expression of BDNF protein in the hippocampus showed a significant difference among the experimental groups. Rat that received repeated restraint stress showed a significant reduction in BDNF protein in the hippocampus in comparison with the control group (q=5.741,P<0.01, n=6 in each group). This effect was clearly reversed by the chronic administration of rhamnazin at a dosage of 50 mg/kg (q=4.293,P<0.05, compared with that of the chronic stress treatment group). Interestingly, the expression of BDNF protein in the rhamnazin-alone treatment group showed a tendency to increase compared with that of the control group; however, no significant difference was detected.

## DISCUSSION

In the present investigation, we found that chronic stress weakens spatial memory and declines the outflow of BDNF protein in the hippocampus. Administration of rhamnazin could enhance cognitive impairment induced by stress in retention session. The chronic stress model that we chose in this investigation is a repeated restraint stress model. It has been accounted for that incessant restriction worry with a wire mesh is a proficient technique to create mental stress and to actuate spatial memory shortfalls in rats (19). Rats were put in straightforward plastic tubes (immobilization packs) every day 4h/day for 30 continuous days; this likewise created mental stress and HPA hub actuation [19].

To evaluate the effect of chronic stress on learning and memory, rat were restrained in plexiglass tubes. Results indicated that chronic stress impaired spatial learning and memory as the stressed rat showed no spatial bias for the target quadrant in the probe test on day 5 and spent less time in the target quadrant in the probe test on day 7 compared with vehicles. In the present study, the Morris test was performed after chronic stress and repeated drug administrations were completed. This means that the effects of rhamnazin were evaluated in animals that were in a state of withdrawal from stress and treatment. We also observed that rhamnazin at the dosage of 50 mg/kg reversed cognitive dysfunction in chronic stressed rat as the rhamnazin-treated rat spent more time in the target quadrant in the probe test on days 5 and 7. In addition, we did not detect a significant difference in the total travel distance and swimming speed among the

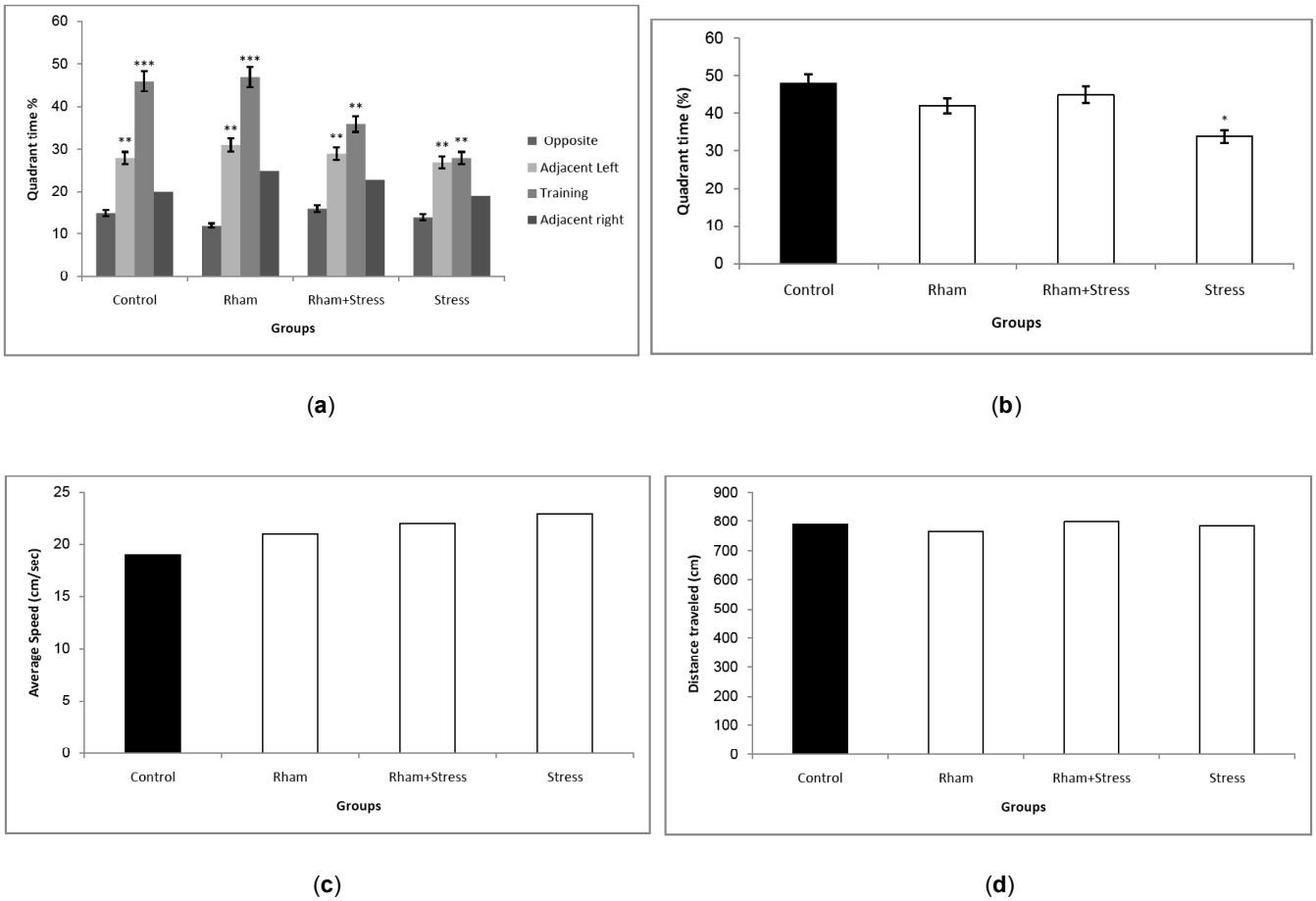


**Figure 3:** Effects of rhamnazin administration on spatial learning and memory during the probe session on day 7. (**a**) Time spent in different quadrants showing that all the rat have a spatial bias for the target quadrant after training for 6 days [P<0.001 in the vehicle group,n=6, ***P<0.001 in the chronic stress group,n=6; P<0.001 in the rhamnazin + stress group,n=6, ***P<0.001 in the rhamnazin group;n=6]. (**b**) Time spent in target quadrants showing that rat received chronic stress spent less time in the target quadrant compared with the control group (q=4.222,P<0.05; n=6). Rhamnazin treatment could prevent stress-induced impairment in memory retention (q=3.934, *P<0.05; n=6 compared to stress group). (**c**) Neither the average speed [F(3,24)=0.517, *P>0.05; n=6] nor the total distance traveled [F(3,24)=0.373,P>0.05;n=6] (**d**) was significantly different at the probe test on day 7 among the animals. Rham, rhamnazin.

four groups, which indicates that the effects of chronic stress and rhamnazin on spatial learning and memory are not because of any nonspecific changes in motor activity and motivational state. In the preliminary experiment, we used doses of 5, 25, and 50 mg/kg to determine the effect of rhamnazin; however, only 50 mg/kg rhamnazin treatment led to an improvement in cognitive behavior. To minimize the usage of laboratory animals, we chose a dose of 50 mg/kg rhamnazin in this study. The hippocampus plays a critical role in spatial memory ability. Damage to the hippocampus may induce spatial memory impairment in both animals and humans [20, 21]. The hippocampus is susceptible to stress [22]. Impairments of chronic stress on learning and memory are mainly mediated by activated HPA axis and elevated GC levels [23]. So far, several studies have investigated the modulating effects of dietary components and herbal medicines on HPA axis activation and showed a subsequent improvement in stress-related behavioral responses [24, 25]. However, the mechanisms of these actions are still poorly understood. In support of a previous study showing that chronic stress may disrupt the HPA axis, we showed that ACTH levels increased in the plasma of repeated restraint rat. Rhamnazin partially reversed the increased ACTH, which may have contributed toward rhamnazin-mediated improvement in cognitive dysfunction.

BDNF is the most prevalent growth factor that plays critical roles in the survival and growth of the neurons. It is a neurotrophin that is involved in hippocampal

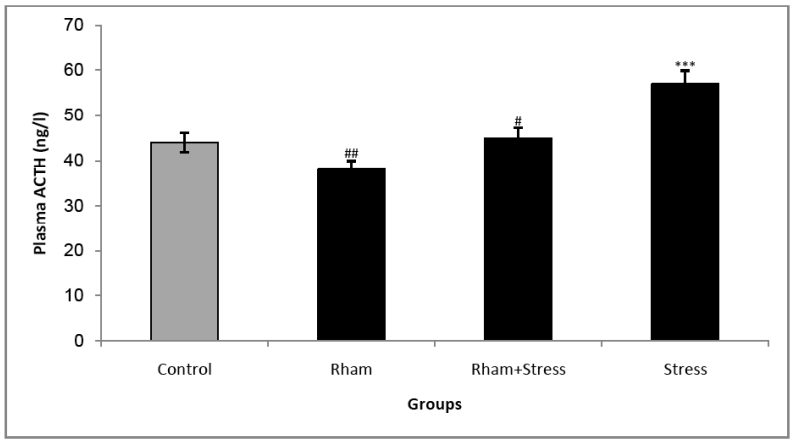


**Figure 4:** Mean plasma ACTH levels in the experimental groups. The plasma ACTH levels were significantly increased in the stressed rat compared with the control group (***P<0.001; n=6). Rhamnazin treatment reversed the stress-induced increase in plasma ACTH levels (***P<0.001; n=6). There was no difference in ACTH levels between the control and the rhamnazin-alone treatment rat. ACTH, adrenocorticotrophic hormone; Rham, rhamnazin.

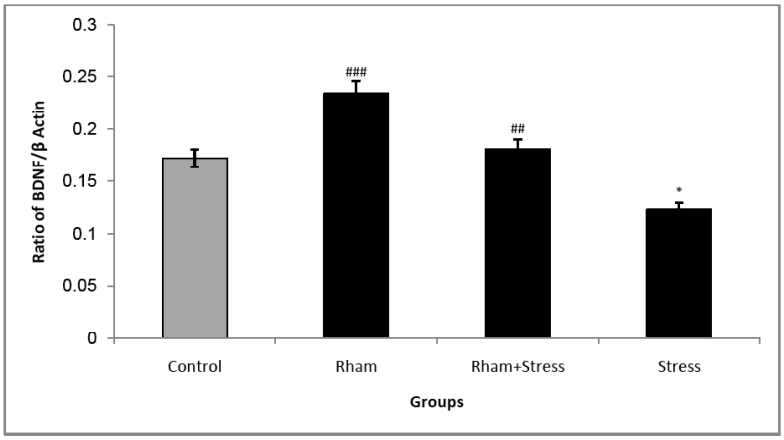


**Figure 5:** Effects of rhamnazin administration on BDNF levels in the hippocampus of rat. (**a**) Original figures showing that the expression of BDNF protein was decreased in the hippocampus of stressed rat. Co-treatment with rhamnazin partly inhibited stress-induced diminished in hippocampal BDNF protein. *P<0.05 compared with the control group, $^{\#}$P<0.01, $^{\#}$P<0.001 compared with the stress group]. (**b**) Summarized data. Data were presented as the ratio of BDNF to β-actin. BDNF, brain-derived neurotrophic factor; Rham, rhamnazin.

neurogenesis, synaptic plasticity, and learning and memory process [26]. It has been reported that plasma BDNF levels attenuated significantly in elderly humans [27].

Down-regulation of BDNF expression was also found in the hippocampus of old rodents and chronic stressed rats [28]. In the present study, repeated restraint stress was associated with deceased expression of BDNF in the hippocampus of rat. We also found that administration of rhamnazin restored hippocampal BDNF protein levels in rat subjected to repeated restraint stress, which may be one of the underlying mechanisms of the cognition improvement effects of rhamnazin. We have not investigated the possible molecular mechanisms by which rhamnazin induced restoration of BDNF. It has been reported that ginsenoside Rg1 ameliorates chronic stress-induced depression-like behaviors by a CREB-regulated increase of BDNF expression in the amygdala of rats [29, 30]. The natural flavonoid luteolin also rescues pentylenetetrazole-induced cognitive impairment in epileptic rats by reducing oxidative stress and activating PKA/CREB/BDNF signaling [31]. Previous studies also showed that rhamnazin may protect against D-galactose induced cognitive deficit by regulation of the expression of ERK–CREB pathway in rat [32]. We hypothesized that the amelioration of cognitive deficits by rhamnazin, at least in part, occurred by a CREB-regulated increase in BDNF

expression in the hippocampus of rat. To test this hypothesis, specific blockers for this signaling pathway should be used in the ongoing works.

The limitation of the present study was the high cost of the material used.

## CONCLUSION

In summary, our findings indicate that administration of rhamnazin attenuates the cognitive deficits in rat exposed to repeated restraint stress.

## ACKNOWLEDGEMENTS

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## CONFLICTS OF INTEREST

There are no conflicts of interest.

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