

Quercetin Protects Against Stress-Induced Anxiety- and Depression-Like Behavior and Improves Memory in Male Mice

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Summary

The present study evaluates the protective role of Quercetin (Quer), against immobilization stress-induced anxiety, depression and cognition alteration in mice using behavioral and biochemical parameters. 24 adult Albino mice were distributed into 2 groups vehicle (n=12; 1 ml/kg) and Quer injected (n=12; 20 mg/kg/ml). The animals received their respective treatment for 14 days. On day 15, after the drug administration, animals were sub-divided into 4 groups (n=6); (i) unstressed + vehicle; (ii) stressed + vehicle; (iii) unstressed + Quer; (iv) stressed + Quer. On day 16, 24 h after the immobilization stress behavioral activities (light-dark activity, elevated plus maze, Morris water maze, and forced swim test) monitored and then animals were decapitated 1 h after the drug administration. Brain samples were collected for biochemical (antioxidant enzymes, AChE, ACh, 5-HT and its metabolite) analysis. The present study indicates the Quer reversed the stress-induced anxiety and depression, in addition, memory performance was more enhanced in stressed group. Following the treatment of Quer, stress-induced elevation of lipid peroxidation and suppression of antioxidant enzymes were also reversed. Administration of Quer decreased AChE in unstressed, while levels of acetylcholine were increased in vehicle and Quer treated stressed animals. The metabolism of 5-HT was increased in Quer treated stressed than unstressed animals. In conclusion, the present finding showed that Quer could prevent the impairment of antioxidant enzymes and also regulate the serotonergic and cholinergic neurotransmission and produce antianxiety, antidepressant effect and enhance memory following 2 h immobilization stress in mice.

Key words

Quercetin • Immobilization stress • Antioxidant enzymes • Serotonin • Acetylcholine

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Introduction

Stress can be characterized as physical and psychological alterations that upset the homeostasis and the equilibrium of organisms. It is recognized as one of the main reasons of a number of diseases (Borys 2008). Common stress symptoms include touchiness, muscular tension, lack of concentration and a range of bodily reactions, such as headaches, increased cardiac activity, hypertension, etc. (Alves *et al.* 2007) Studies with short term exposure to stress have been shown to assist cognition both in animals (Oitzl *et al.* 2001, Samad *et al.* 2017) and humans (Cahill *et al.* 2003). This enhance in memory has been ascribed to various glands secretions (hormones) and neuronal release (neurotransmitters) under stress condition. Previously it has reported that both sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis have a vital role in memory amalgamation following the experience of stress circumstance (Joëls *et al.* 2006). Acute immobilization stress has reported to change locomotor activity and cause anxiety (Kumar *et al.* 2010, Haraguchi *et al.* 2012).

Immobilization/restraint stress is one of various models that is most commonly employed, as it effectively mimics potent physical and psychological stress (Lucas *et al.* 2007). These models are linked with increased free radical production and altered antioxidant enzyme activities (Gümüs *et al.* 2002). Restraint stress prompts cellular pathways that lead to raise production of free radicals (Tseilikman *et al.* 2009). The central nervous system has conventionally been considered as a target site for free radical damage because brain contains abundant lipid content and consumes high amount of oxygen (Halliwell and Gutteridge 1985).

The psychological deficits linked with acute stressful events may prevent using therapeutic strategies involving medicinal and dietary phyto-antioxidants. One such nutraceutical is quercetin (Quer), which is belong to flavonoids – secondary metabolites of plants. Since flavonoids are present in food and medicinal plants, they are consumed by humans. Their main sources are vegetables, fruits, tea and wine and the average daily intake of flavonoids by humans on a normal diet is 1-2 g/day (Havsteen 2002). These compounds have reported to have beneficial effects in numerous diseases in humans, including cancer and cardiovascular diseases. In addition, their antioxidant, anti-inflammatory, anti-tumoral and antiviral properties were also noted (Fernandez *et al.* 2006). The ability of flavonoids (Quer) to pass the blood-brain barrier causes numerous effects on the central nervous system, which revealed both in the *in vitro* and *in vivo* studies (Jager and Saaby 2011). Moreover, they might be ligands for benzodiazepine binding sites of the γ -aminobutyric acid type A (GABAA) receptor, which confirmed by behavioral effects in animal models of anxiety, sedation and convulsions. Flavonoids might also affect the activity of other neurotransmitter systems. It was revealed that Quer modulate activity of adenosine, serotonin, glycine and acetylcholine receptors (Lee *et al.* 2011).

Quer exhibit neuroprotective properties in animal models, i.e. it improve memory and learning abilities (Nassiri-Asl *et al.* 2010b, Nassiri-Asl *et al.* 2013a, Tongjaroenbuangam *et al.* 2011) and have antidepressant and anti-stress (Kawabata *et al.* 2010) action as compound of extracts from herbs (Herrera-Ruiz *et al.* 2011, Machado *et al.* 2008). It also improved the behavioral performance of mice fed a high-cholesterol diet both in the step-through test and the Morris water maze task. This effect seemed to be connected to inhibition of AMP-activated protein kinase (AMPK) by

Quer and might be utilized in therapy and prevention of Alzheimer's disease (Lu *et al.* 2010). Quer has been examined to protect biological membrane from peroxidative damage. The inhibition of lipid peroxidation by Quer mainly exhibited due to its scavenging ability of free reactive radical (Mercer *et al.* 2005, Nabavi *et al.* 2015, Sharma *et al.* 2015). Quer is regarded as a potent inducer of detoxifying enzymes such as super oxide dismutase, catalase and glutathione peroxidase and dissuade lipid peroxidation thereby ameliorates oxidative stress (Mercer *et al.* 2005, Haleagrahara *et al.* 2009). In several studies it has been used as an antioxidant to reduce anxiety-like behavior (Wattanathorn *et al.* 2007) and increased cognitive dysfunction in diabetic rats (Maciel *et al.* 2016) inhibits apoptosis, and improves memory function in animal model of Alzheimer's disease (Mesram *et al.* 2017) and also prevents D-galactose induced aging (Sun *et al.* 2007). It has also been reported that Quer has a role in alleviating stress and depression like symptoms by enhancing antioxidant enzyme (Yoshino *et al.* 2011, Kumar and Goyal 2008) and other neurological disorders associated with cadmium intoxication (Abdalla *et al.* 2013, Abdalla *et al.* 2014). Recently, it is reported that Quer can improves serotonergic and cholinergic functions following repeated treatment (Liaqat *et al.* 2018).

Based on the antioxidant effects of Quer, we hypothesized that, being a flavonoid, Quer may enhance the memory function, reduce anxiety and depression like behavior following 2 h immobilization stress. To elucidate this issue, the current study aimed to determine the effects of repeated administration of Quer on cognitive function, stress responses, cholinergic function and serotonin metabolism in animals following acute immobilization stress

Methods

Animals

Male Albino Wistar mice with mean weight 20 ± 5 g, bred in Animal House facility of University of Lahore, Lahore, Pakistan, were used for the experiment. Animals were caged individually in plastic cages under standard laboratory conditions and maintained on a 12 h light/dark cycle. Animals had access to cubes of standard rodent diet and tap water *ad libitum* for 3 days prior to acclimatization. The experimental protocols were approved by the institutional ethics and animal care committee and performed in strict accordance with National Institute of

Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Chemicals and reagents

Thiobarbituric acid (TBA), H₂O₂ stock (35 %) solution, nitroblue tetrazolium (NBT), trichloroacetic acid (TCA) and Dithio-bisnitrobenzoic acid (DTNB) were purchased from British Drug House (BDH, Dorset, UK). Acetylthiocholine (ATC), hydroxylamine hydrochloride and all other analytical grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment schedule

The experiment performed in a non-blind fashion as previously reported (Samad and Haleem 2009). Twenty-four animals randomly divided into two equal groups of 12 each. Test group received Quer dissolved in 25 % ethanol (20 mg/ml/kg; i.p.), while the other control group was injected with vehicle (25 % ethanol) (1 ml/kg; i.p.) as reported previously (Maciel *et al.* 2016). The animal received this treatment for 14 days.

On day 15, animals were further divided into four groups, of six each were assigned as (i) unstressed + vehicle; (ii) stressed + vehicle; (iii) unstressed + Quer (iv) stressed + Quer. The stressed group was accommodated in a separate room for immobilization stress for 2 h.

Behavioral activities [Forced swim test (FST), light dark activity (LDA), elevated plus maze (EPM) and Morris water maze (MWM) test] were monitored 24 h after the immobilization stress (on day 16). There was 60 min break between each activity in which animals were kept back in their home cages to avoid overlapping. Behavioral activities of control mice were also monitored at the same time.

On day 16, after behavioral tests animals were decapitated and their brains were collected. All brain samples were instantly kept freeze at 70 °C for biochemical assays.

Immobilization stress

The animals were restrained on wire grids of 10" × 9" fitted with a Perspex plate of 9" × 6.5". Restraining procedure was same as described earlier (Samad and Haleem 2009, Samad *et al.* 2006). Immobilization was produced by pressing the fore legs of the rats through the gaps in the metal grids and taping them together with Zinc Oxide plaster tape. Hind limbs were also taped and the head of animal rested on the Perspex plate.

Behavioral tests

Light-dark activity (LDA) test

The test was conducted in a locally-made compartment box (Samad and Haleem 2009). The compartment of equal size (26 × 26 × 26 cm), with an access (12 × 12 cm) between the compartments, differed in their sensory properties. Walls of one compartment were light (transparent) and other dark (Black). A rat placed in this box expected to pass more time in the dark compartment. To determine the activity a rat was introduced *via* the dark compartment of the box. Time spent in the light compartment was monitored with stop watch for a cut off time of 5 min.

Elevated plus maze (EPM) test

Anxiety was assessed by EPM according to the method as described by Naqvi *et al.* (2012). The apparatus used in the present study was consisted of two closed arms and two open arms with same dimensions (50 × 10 cm). Close arms were enclosed by 40 cm high walls. The arms were connected with a central square (10 × 10 cm) to give the apparatus a plus sign appearance. The maze was elevated 60 cm above the floor. To monitor the activity, mice were individually placed in the central square facing an enclosed arm and the time spent in open arm was recorded manually for a cut off time of 5 min.

Forced swimming test (FST)

The FST apparatus comprised of a glass tank with 56 cm height and 30 cm width, which contained water at the height of 22 cm and temperature of 25 °C. In this glass tank animals were individually forced to swim for 5 min. The height of water was selected so that animal was prevented from touching the bottom of the glass tank and also to prevent its escape from the glass tank. The FST is commonly used as standard pharmacological model for evaluating depression like symptoms in rat/mice (Porsolt *et al.* 1997). When the mice are placed in an inescapable chamber which is filled with water then the development of the state of immobility reflects the cessation of persistent escape directed behavior. In this test animal's swimming behavior was monitored which can be defined as movement throughout the swim chamber (glass tank). The immobility time was monitored manually. The animal is considered immobile when it makes no further attempts to escape and only tries to keep its head above the water.

Morris water maze (MWM) test

MWM test was performed to examine the effects on spatial memory as described by Morris in 1981. This is a well-known, conventional cognitive test which requires an animal to use spatial learning and memory to locate a hidden platform just below the surface of a circular pool of water and also to remember its location as in the previous trial. It is reported that the animal uses cues in order to locate the hidden platform. The maze used for rats is same as described earlier (Haider *et al.* 2011). It is a circular pool of water with a diameter of 45 cm, height of 37 cm and depth of water is 12 cm. The metal pool painted white on the inner side and a wooden escape platform with a surface diameter of 8 cm was placed 2 cm below the surface of water during water maze training. The pool was filled with milky water (23 ± 2 °C) in order to obscure the platform. In our experiment we have assessed the acquisition short-term memory and long term memory in terms of latency to locate the escape platform. The test is based upon two phases; the training phase and the test phase, while starting position was same in both phases. The cut off time was 2 min for each session. Memory functions of rats were tested by noting the retention latency. Initially the training session was performed during which each mice placed into the water facing towards the wall of the tank. After placing, 120 s were given to each animal to find and mount onto the hidden platform. If the mice located the platform it was allowed to stay on it for 10 s. If it failed to locate the platform during the allocated time then it was guided gently to the platform and that session repeated twice. The assessment of memory was performed immediately after (acquisition) and 60 min (short term memory) following training session during which escape latency was monitored manually.

Biochemical parameters

All the animals were decapitated on the same day immediately after behavioral analysis. The whole brains were removed, rinsed in isotonic saline, and weighed. A 10 % (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4) which was obtained by centrifugation at $12,000 \times g$ for 20 min at 4 °C for biochemical estimations

Determination of malondialdehyde (MDA) content

Estimation of lipid peroxidation was essentially the same as described by Chow and Tappel (1972) with

slight modifications. In a reaction mixture 300 μ l homogenate was taken and 2 ml of TCA (15 %)-TBA (0.375 %) mixture was added. The mixture was boiled for 20 min in water bath, cooled with ice cold water at 4 °C and then centrifuged at 3,500 rpm for 10 min. Supernatant of light pink color was then collected and absorbance was taken at 532 nm. Lipid peroxidation was expressed as mM of MDA/g of brain tissue.

Determination of catalase (CAT)

CAT was estimated using a previously reported method (Pari and Latha 2004). Brain homogenate (10 %) in 0.01 M phosphate buffer (pH 7.0) was prepared and filtered. Then 0.1 ml of filtrate was mixed with 1.4 ml of reaction mixture that contained 0.4 ml of 2 M hydrogen peroxide and 1 ml of same phosphate buffer. The reaction was terminated after 1 min by adding 2.0 ml of dichromate-acetic acid reagent. Blank contained distilled water in place of filtrate. The absorbance of both test and blank were measured at 620 nm to calculate percent inhibition of CAT.

Determination of glutathione peroxidase (GPx) activity

GPx activity was measured by the procedure of Flohe and Gunzler (1984). One ml of reaction mixture was prepared which contained 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of reduced glutathione (2 mM), 0.1 ml of sodium azide (10 mM), 0.1 ml of H₂O₂ (1 mM), and 0.3 ml of brain supernatant. After incubation at 37 °C for 15 min, reaction was terminated by addition of 0.5 ml 5 % TCA. Tubes were centrifuged at $1,500 \times g$ for 5 min, and supernatant was collected. Phosphate buffer 0.2 ml (0.1 M, pH 7.4) and DTNB 0.7 ml (0.4 mg/ml) were added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm. Activity of GPx was expressed as μ mol/min/g of brain.

Determination of superoxide dismutase (SOD)

The SOD was estimated by the method (Naskar *et al.* 2010). An aliquot of brain homogenate (10 %) was treated with 0.75 ml of ethanol and 0.15 ml of ice chilled chloroform then centrifuged. Then 0.5 ml of EDTA (0.6 mM) and 1.0 ml of carbonate-bicarbonate (0.1 M; pH 10.2) buffer was added in 0.5 ml of supernatant. The reaction was started by adding 0.5 ml of epinephrine (1.8 mM) and the absorbance was measured for 3 min at 480 nm. Blank contained all reagents except supernatant. Finally, percent inhibition of SOD was calculated.

Determination of brain acetylcholine (ACh) content

The tissue acetylcholine content was estimated by the method of Hestrin (1949) as described by Augustinsson (1957). The tissue sample was boiled to inactivate the enzyme and release the bound ACh which reacts with ferric chloride and the brown color developed was read at 540 nm against the reagent blank. The concentration of ACh was expressed as $\mu\text{mol/g}$ of brain tissue.

Determination of acetylcholinesterase (AChE) activity

Activity of AChE in homogenate was determined according to the method of Ellman *et al.* (1961) using ATC as substrate. The reaction mixture contained 0.4 ml brain homogenate (0.02 g/ml), 2.6 ml phosphate buffer (0.1 M, pH 8.0), 100 μl DTNB. The reaction mixture was mixed by bubbling air, and placed in the spectrophotometer. Once the reaction content was stable, the absorbance was noted at 412 nm for the basal reading followed by addition of 5.2 μl of ATC to this cuvette. Any change in absorbance was recorded from zero time followed by 10 min at 25 °C. The activity of AChE was expressed as $\mu\text{mol/min/g}$ of brain tissue.

Determination of 5-HT and 5-HIAA

For determination of 5-HT and 5-HIAA homogenization of frozen brains was carried out in an extraction medium using an electrical homogenizer (Polytron; Kinematica). The neurochemical analysis was done to assess concentrations of 5-hydroxytryptamine (5-HT), and their metabolites 5-hydroxyindoleacetic acid (5-HIAA) brain as described by Samad and Haleem (2009). Reversed-phase High Performance Liquid Chromatography (HPLC) with an electrochemical detector (Shimadzu LEC 6A detector) was performed to detect levels of biogenic amines in brain samples. The EC detector was operated at a potential of +0.8 V. The stationary phase used for separation is a 5- μ Shim-pack ODS column having an internal diameter of 4.0 mm and a length of 150 mm. The mobile phase that passes through a column with a pump pressure of 2,000-3,000 psi contains octyl sodium sulfate (0.023 %) in 0.1 M phosphate buffer at pH 2.9.

Statistical analysis

The results are presented as mean \pm SD for six animals in each group. The statistical significant differences were evaluated by Tukey's test following two-way ANOVA using SPSS version 20. Value of $p < 0.05$ was considered as a significant difference.

Results

Effects of pre-administration of Quer on anxiety profile in unstressed and stressed mice observed in light dark activity box

Figure 1 shows the effects of pre-administration of Quer on anxiety profile in unstressed and stressed mice observed in light dark box. Data for time spent in light box analyzed by two-way ANOVA revealed that the effect of stress [F (1,20)=34.80, $p < 0.01$], Quer [F(1,20)=215.53, $p < 0.01$], and interaction of stress \times Quer interaction [F(1,20)=19.68, $p < 0.01$] were significant. *Post-hoc* analysis by Tukey's test showed that 2 h immobilization stress significantly decreased ($p < 0.01$) time spent in light box in vehicle treated mice as compared to unstressed animals. Time spent in light box significantly increased ($p < 0.01$) in Quer treated unstressed and stressed mice as compared to controls.

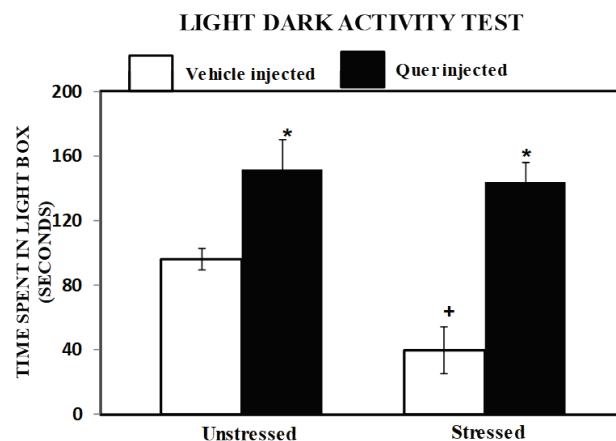


Fig. 1. Time spent in light box in light dark box test for the vehicle and Quer treated unstressed and stressed groups following single 2 h immobilization stress. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p < 0.05$ versus respective control and + $p < 0.05$ vs. unstressed groups.

Effects of pre-administration of Quer on anxiety profile in unstressed and stressed mice observed in EPM

Figure 2 shows the effects of pre-administration of Quer on anxiety profile in unstressed and stressed mice observed in EPM. Data for time spent in open arm analyzed by two-way ANOVA revealed that the effect of stress [F(1,20)=39.95, $p < 0.01$], Quer [F(1,20)=149.60, $p < 0.01$], and interaction of stress \times Quer interaction [F(1,20)=24.62, $p < 0.01$] were all significant. *Post hoc* analysis by Tukey's test showed that 2 h immobilization stress significantly decreased ($p < 0.01$) time spent in open

arm in vehicle treated mice as compared to unstressed controls. Time spent in open arm significantly increased ($p < 0.01$) in Quer treated unstressed and stressed mice as compared to controls.

Effects of Quer administration following acute immobilization stress on immobility in FST

The effects of Quer administration following acute immobilization stress on immobility in FST is shown in Figure 3. In FST the effect of stress [$F(1,20)=73.38$, $p < 0.01$], Quer [$F(1,20)=429.31$, $p < 0.01$] and stress \times Quer interaction [$F(1,20)=106.57$, $p < 0.01$] were significant following two-way ANOVA. *Post hoc* analysis by Tukey's test found that repeated pre-administration of Quer significantly decreased immobility time in unstressed ($p < 0.01$) and stressed mice ($p < 0.01$). On the other hand, immobility time in FST was increased in vehicle+stressed animal.

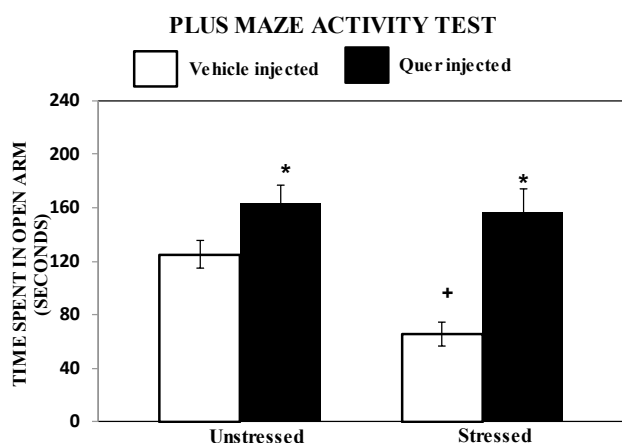


Fig. 2. Time spent in open arm in elevated plus maze test for the vehicle and Quer treated unstressed and stressed groups following single 2 h immobilization stress. Values are mean \pm SD ($n=6$). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p < 0.05$ versus respective control and + $p < 0.05$ versus unstressed groups.

Effects of pre-administration of Quer on memory function in vehicle and Quer treated stressed and unstressed mice

Figure 4 shows memory function that was determined by performing MWM activity in vehicle and Quer treated stressed and unstressed mice. The MWM activity is expressed as time to find the hidden platform performed immediately after training (acquisition) and 1 h (short term memory).

Results of acquisition analyzed by two-way ANOVA showed effects of stress [$F(1,20)=2.29$, $p=0.14$], Quer [$F(1,20)=0.13$, $p=0.71$] and interaction between the two factors [$F(1,20)=0.59$, $p=0.44$] were not significant.

Two-way ANOVA for short term memory showed that significant effects of stress [$F(1,20)=22.94$, $p < 0.01$] and Quer [$F(1,20)=34.27$, $p < 0.01$], while interaction [$F(1,20)=0.05$, $p=0.82$] was not significant. *Post hoc* analysis by Tukey's test demonstrated that pre administration of Quer produced non-significant effects on memory during acquisition. Whereas analysis of short term memory revealed that Quer significantly decrease the time to reach the hidden platform in stressed as well as unstressed mice. Similarly immobilization stress also decreased the time during analysis of short term memory.

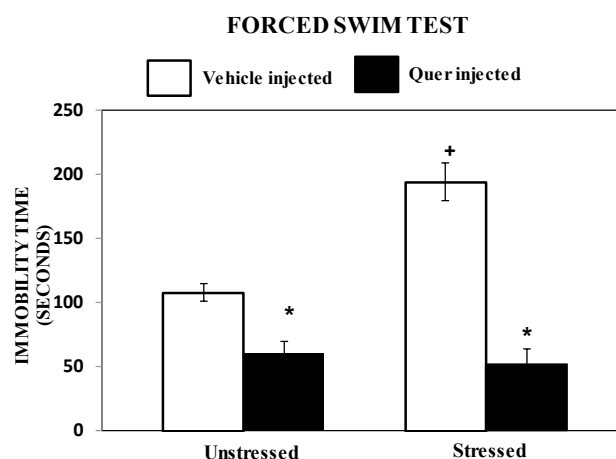


Fig. 3. Effect of Quer on depression like symptoms assessed by forced swim test in terms of immobility time (s) following single 2 h immobilization stress. Values are mean \pm SD ($n=6$). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p < 0.05$ versus respective control and + $p < 0.05$ versus unstressed groups.

Effects of Quer administration following single 2-h immobilization stress on brain MDA activity

Figure 5 shows the effects of Quer following immobilization stress on MDA levels. Data was analyzed by two-way ANOVA exhibited a significant effect of Quer [$F(1,20)=12.79$, $p < 0.01$] and interaction between stress and Quer [$F(1,20)=12.27$, $p < 0.01$]. While, effect of stress [$F(1,20)=0.007$, $p > 0.05$] was not significant. *Post hoc* analysis by Tukey's test showed that 2-h immobilization increased MDA levels in vehicle+stressed animals. On the other hand, administration of Quer decreased lipid peroxidation in stressed than vehicle treated animals.

Effects of Quer administration following single 2-h immobilization stress on brain antioxidant enzyme activity

Figure 6 shows the effects of pre-administration of Quer on antioxidant enzyme in unstressed and stressed

animals. Data on the activity of SOD was analyzed by two-way ANOVA exhibited a significant effect of Quer [F(1,20)=61.10, $p<0.01$], stress [F(1,20)=5.33, $p<0.05$] and interaction between stress and Quer [F(1,20)=16.94, $p<0.01$]. *Post hoc* analysis by Tukey's test showed that stress decreased the activity of SOD in vehicle treated animals. On the contrary, administration of Quer increased the activity of SOD in stressed than vehicle treated animals.

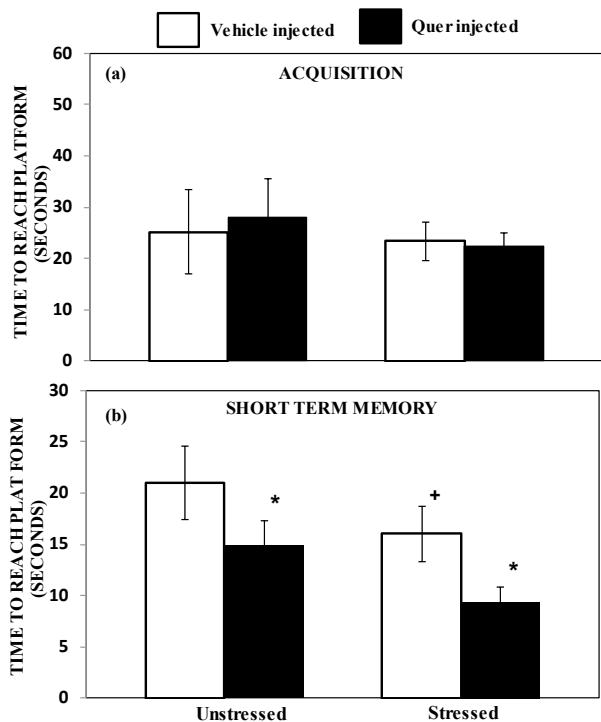


Fig. 4. Effect of Quer administration following single 2 h immobilization stress on (a) acquisition and (b) short term memory in terms of escape latency (s) assessed by Morris water maze. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p<0.05$ versus respective control and + $p<0.05$ versus unstressed groups.

Data on the activity of CAT was analyzed by two-way ANOVA exhibited a significant effect of Quer [F(1,20)=28.42, $p<0.01$], and interaction between the two factors [F(1,20)=21.34, $p<0.01$]. Effect of stress [F(1,20)=3.17, $p=0.09$] was not significant. *Post hoc* analysis by Tukey's test showed pre-administration of Quer increased the activity of CAT in stressed than vehicle + stressed animals. In addition activity of CAT was also increased in Quer treated stressed than unstressed animals. The data indicating that pre-administration of Quer increased the activity of antioxidant enzymes in stressed mice.

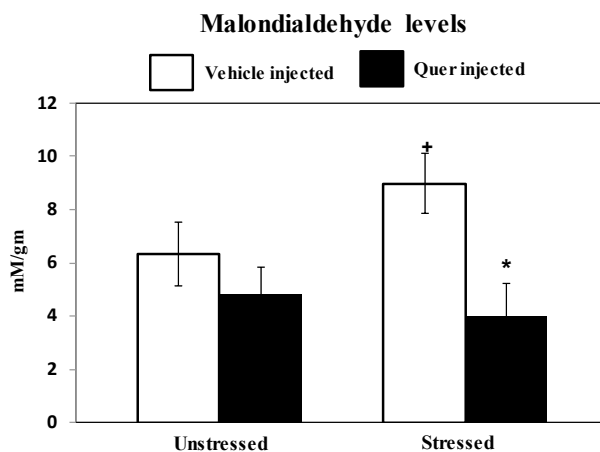


Fig. 5. Effect of Quer administration following single 2 h immobilization stress on brain MDA activity. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p<0.05$ versus respective control and + $p<0.05$ versus unstressed groups.

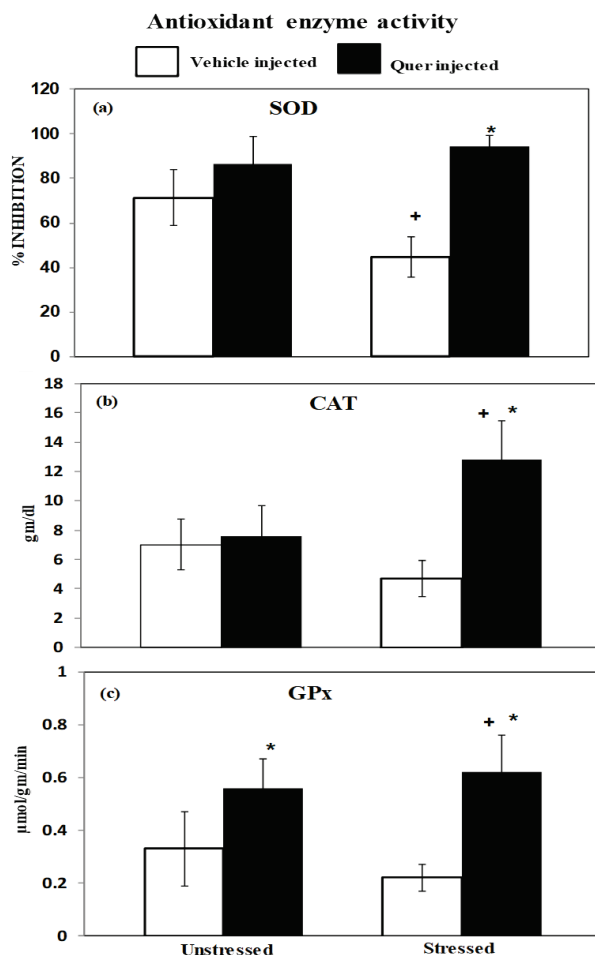


Fig. 6. Effect of Quer administration following single 2 h immobilization stress on brain SOD (a), CAT (b) and GPx (c) activity. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p<0.05$ versus respective control and + $p<0.05$ versus unstressed groups.

Data on the activity of GPx was analyzed by two-way ANOVA exhibited a significant effect of Quer [F(1,20)=41.03, $p<0.01$]. Effect of stress [F(1,20)=0.27, $p=0.60$] and interaction between stress and Quer [F(1,20)=3.03, $p=0.09$] were not significant. *Post hoc* analysis by Tukey's test showed that administration of Quer significantly increased the activity of GPx in stressed and unstressed than their counterparts. Similarly, Quer administration also increases GPx activity in stressed animals as compare to unstressed animals.

Effects of Quer administration following single 2-h immobilization stress on brain AChE activity

Figure 7 shows the brain AChE activity ($\mu\text{mol}/\text{min}/\text{g}$) that was assayed to find out the cholinergic function. Two way ANOVA for the attained data validated a significant effect of Quer [F(1,20)=12.09, $p<0.05$] and interaction between stress x Quer [F(1,20)=20.80, $p<0.01$] whereas, stress [F(1,20)=3.12, $p=0.09$] produced a significant effect. *Post hoc* analysis by Tukey's test showed pre-administration of Quer significantly decreased ($p<0.01$) the activity of AChE in unstressed than vehicle. The levels of AChE activity was also decreased in vehicle + stressed animals.

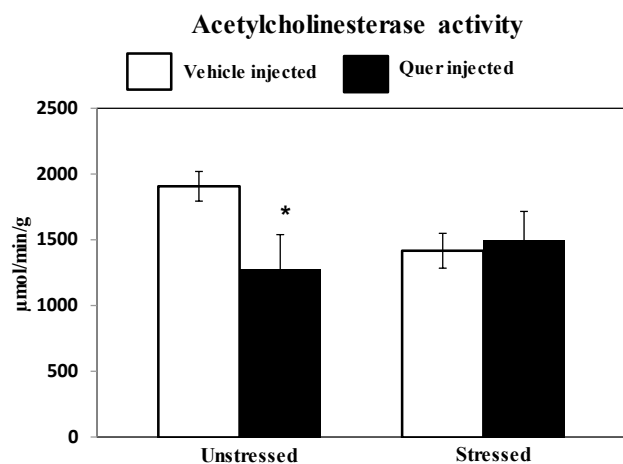


Fig. 7. Effect of Quer administration following single 2 h immobilization stress on brain AChE activity. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p<0.05$ versus respective control and + $p<0.05$ versus unstressed groups.

Effects of Quer administration following single 2-h immobilization stress on brain ACh levels

Figure 8 shows the effects of administration of Quer on brain ACh level in unstressed and stressed animals. Two way ANOVA for the attained data validated a significant effect of stress [F(1,20)=22.09,

$p<0.01$], and Quer [F(1,20)=11.12, $p<0.01$]. While interaction between two factors [F(1,20)=0.04, $p=0.83$] was not significant. *Post hoc* analysis by Tukey's test showed 2-h immobilization stress significantly increased Ach levels in vehicle stressed than unstressed animals. Furthermore administration of Quer also significantly increased Ach levels in the brain of stressed than unstressed mice.

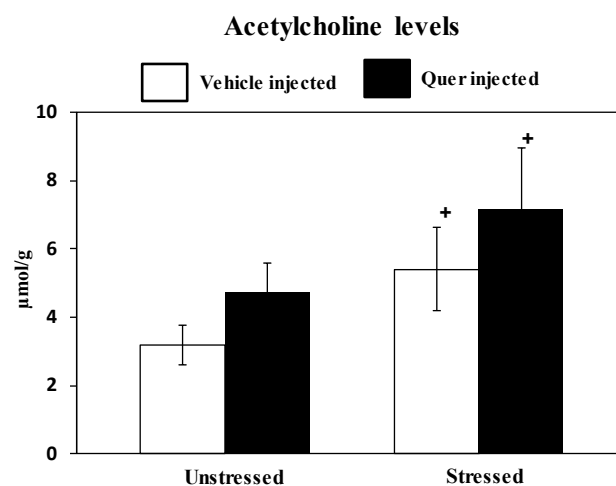


Fig. 8. Effect of Quer administration following single 2 h immobilization stress on brain ACh Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as * $p<0.05$ versus respective control and + $p<0.05$ versus unstressed groups.

Effects of Quer following immobilization stress on brain 5-HT and 5-HIAA levels

Figure 9 shows the effects of Quer following immobilization stress on brain 5-HT and 5-HIAA levels. Data on 5-HT levels was analyzed by two-way ANOVA exhibited a significant effect of Quer [F(1,20)=8.06, $p<0.05$] stress [F(1,20)=26.80, $p<0.01$]. Interaction between the two factors [F(1,20)=0.355, $p=0.55$] was not significant. *Post hoc* analysis by Tukey's test showed that 2-h immobilization increased 5-HT levels in vehicle and Quer treated stressed animals.

Data on 5-HIAA levels was analyzed by two-way ANOVA exhibited a significant effect of Quer [F(1,20)=17.8, $p<0.01$], and stress [F(1,20)=29.5, $p<0.01$]. Interaction between stress x Quer [F(1,20)=0.19, $p=0.66$] was not significant. *Post hoc* analysis by Tukey's test showed that 2-h immobilization increased 5-HIAA levels in vehicle and Quer treated stressed animals. On the other hand immobilization-induced increases of 5-HIAA levels were decreased in Quer treated stressed animals.

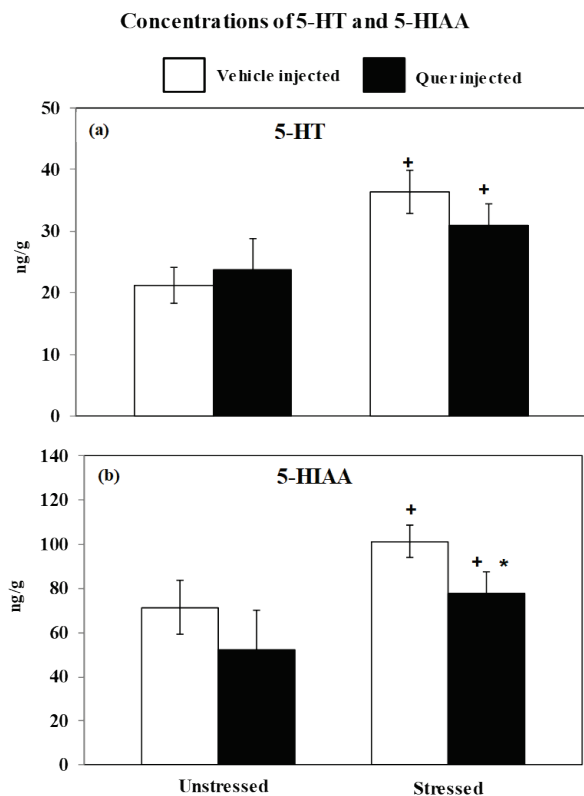


Fig. 9. Effect of Quer administration following single 2 h immobilization stress on (a) brain 5-HT and (b) 5-HIAA. Values are mean \pm SD (n=6). Data was analyzed by Tukey's test following two-way ANOVA. Statistical difference is represented as *p<0.05 versus respective control and +p<0.05 versus unstressed groups.

Discussion

Research works on experimental animals demonstrate that an uncontrollable stress situation produced neurochemical changes and behavioral deficits (Samad and Haleem 2009). Stress induced behavioral deficits in experimental animals are generally used as animal model of depression (Samad *et al.* 2006). Previously, it has been reported that Quer has antioxidant potential (Nayabi *et al.* 2015), antidepressant (Demir *et al.* 2016, Holzmann *et al.* 2015) and memory enhancing effects (Mereci *et al.* 2016, Islam *et al.* 2013). Acute stress also boosts memory functions. Our results show that 2-h immobilization stress produced anxiety like behavior in LDA (Fig. 1) and EPM (Fig. 2), and depression-like behavior in FST (Fig. 3). Both anxiety- and depression-like behavior were reversed by prior administration of Quer (Figs 1, 2 and 3) due to antioxidant potential. It has been extensively reported that stress situation involved in generation of free radicals (Sharma *et al.* 2015) and suppress antioxidant mechanism

of the body (Nayabi *et al.* 2015). The natural cellular antioxidant enzymes include SOD, which scavenges the superoxide ion by speeding up its dismutation; CAT, a heme containing enzyme which removes hydrogen peroxide; and GPx, a selenium-containing enzyme, which scavenges hydrogen peroxide and other peroxides (Blake *et al.* 1987). In our study the levels of MDA (Fig. 5) and SOD (Fig. 6) were significantly altered while CAT and GPx were non-significantly decreased, suggesting increased oxidative stress produced an inhibitory effect on SOD levels in these animals. The significant correlation between MDA and SOD in vehicle treated unstressed and stressed animals may be attributed the immobilization stressed induced oxidative stress which may involved in alteration of antioxidant enzymes activities. Quer is a powerful antioxidant as extensively reported and possessing reactive oxygen species scavenging activity (Nayabi *et al.* 2015, Sharma *et al.* 2015). It is reported that Quer enhances the antioxidant enzymes activity and diminishes the lipid peroxidation (Mercer *et al.* 2005, Haleagrahara *et al.* 2009). As observed in the present study due to antioxidant potential Quer attenuates the behavioral deficits following 2-h immobilization stress and also normalizes the SOD, CAT and GPx activity with reduction in oxidative stress. The aforementioned results suggest that protecting effects of Quer may be at least being in a part due to its antioxidant effect.

5-HT levels have been reported to increase following immobilization stress in whole brain (Samad *et al.* 2006) and various brain regions (Haleem and Parveen 1994, Moa *et al.* 2008) of rats. An ample evidence indicate that dysfunction of serotonergic neurotransmission in CNS is involved in the development of depression, anxiety and memory disorders (Hugus *et al.* 2002, Naughten *et al.* 2000). Increased level of brain 5-HT enhances memory (Haider *et al.* 2006) and produced antidepressant effects whereas decreased level of brain 5-HT impairs cognitive performance (Porter *et al.* 2003) and produced antidepressant effects. It was observed in this study that administration of Quer increases 5-HT levels in stressed than vehicle treated animals consistent with the recent study (Liaqat *et al.* 2018). Hence, it can be suggested that administration of Quer increases cognitive performance and antidepressant due to increase in 5-HT levels. Recently, it has been studied that Quer improves serotonergic functions (Liaqat *et al.* 2018). Earlier studies have also been shown that catabolism of 5-HT by MAO involved in the production

of free radicals production (Bianchi *et al.* 2005, Antkiewicz-Michaluk *et al.* 2014) which produces oxidative stress and associated with behavioral deficits development following immobilization stress. Quer which is reported as MAO inhibitor (Bandaruk *et al.* 2012) may also be involved in the decreasing catabolism of 5-HT (Fig. 9), which could be reduced the free radical generation and decreased the oxidative stress-induced (Figs 5, 6) behavioral alteration (Figs 1, 2, 3 and 4) following immobilization stress. Though, under immobilization stress principal actions of repeated administration of Quer on 5-HT may be presynaptic, which inhibits MAO (Bandaruk *et al.* 2012) and increase the availability of 5-HT in the synapse. This may stimulate the regulation of 5-HT release *via* presynaptic 5-HT auto-receptors in mice hippocampus causing the decreased release of 5-HT. These actions of Quer may serve to correct 5-HT function abnormalities under stress.

ACh is also one of the key neurotransmitter concerned in cognition. Previously it has reported that acute stress increases the release of acetylcholine in the hippocampus (Imperato *et al.* 1991) and modulates the genes that regulate acetylcholine availability after stress and blockade of AChE (Kafur *et al.* 1998). AChE is one of the important enzymes to determine the cholinergic function (Papandreou *et al.* 2011). Extensive research studies have been reported the association between AChE and memory performance, but still there is need of more studies because definite pattern for this association is not clear. The altered memory function following neonatal iron exposure (Perez *et al.* 2010) and acute and immobilization stress (Das *et al.* 2000) decreased AChE activity. A pronounced increase in transfer latency time was observed in passive avoidance test as compared to that of control and chronically stressed groups, indicating a better cognitive ability in these rats (Das *et al.* 2000). The result on AChE activity in the present study are also

in agreement with the previous (Fig. 7), suggesting that Quer which known as inhibitor of AChE activity (Islam *et al.* 2013) and improves cholinergic function (Liaqat *et al.* 2018) may be involved in availability of ACh (Fig. 8) to strengthen the memory function. Quer and acute stress have demonstrated to enhance the memory performance, which is also observed in the present study (Fig. 4); however, a novel finding is that Quer may enhance acute stress-induced increase in memory function.

In addition, it can be postulated that Quer which is involved in normalization of brain serotonergic function, thereby improving memory function in mice. The memory enhancing effects of acute stress with prior administration of Quer may also be explainable in the same milieu as increased cholinergic function (Fig. 8) may also activate serotonergic system (Fig. 9) to boosts up the cognition.

In conclusion, we evaluated the anxiolytic, antidepressant and memory enhancing effects of Quer following 2-h immobilization stress in mice. The results revealed that pre-administration of Quer attenuated stress-induced behavioral deficits strongly by its antioxidant potential. Furthermore, Quer also involves in the regulation of serotonergic and cholinergic functions, produces antidepressant and anxiolytic effect, and boosts memory performance. The present study therefore, emphasizes the use of dietary sources rich in Quer contents and/or supplementation of Quer as an effective remedy for daily stress life events.

Conflict of Interest

There is no conflict of interest.

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