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Original Article

Evaluation of the Combinatory Anti-Depressant Approach of Metformin and Quercetin in Mice Exposed to Chronic Unpredictable Mild Stress Behavorial Alteration

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ABSTRACT

Background: To develop a new drug approach, it is necessary to understand possible ways by which suppression of depressive symptoms can be achieved at the biochemical level. One such protein, which can help relieve depression when targeted, is AMP-Activated Protein Kinase (AMPK). The present *in vivo* study has focussed on AMPK and inflammatory cytokines as potential targets for improving Brain-Derived Neurotrophic Factor levels (BDNF) in depression. **Materials and Methods:** A parallel therapy was administered for standard (Fluoxetine (10 mg/kg), monotherapy (Metformin (200 mg/kg) and Quercetin (20 mg/kg) and combination of half dose (Metformin (100 mg/kg)+Quercetin (10 mg/kg) and full dose (Metformin (200 mg/kg)+Quercetin (20 mg/kg) for 3 weeks in mice. The study comprises behavorial parameters assessed using locomotor activity, Forced Swimming Test (FST), elevated plus maze, Tail Suspension Test (TST) and Sucrose Preference Test (SPT). **Results and Conclusion:** The results obtained depicted that the high-dose combination doses decreased immobility time, anhedonia and increased open-arm exploration in behavorial tests. Significant difference observed in Metformin (200 mg/kg)+Quercetin (20 mg/kg) treated group at *p<0.1 [locomotor activity, FST, EPM (dark and open arm) and SPT], ***p<0.01 (TST). Moreover, the combination lowered the number of neurodegenerative cytokines such as Tumour necrosis factor-α, Interlukin-1β, Interlukin-6 (**p<0.05), but Brain Derived Neurotropic Factor (*p<0.1) and corticosterone levels (**p<0.05) were raised indicating antidepressant effects. The studies suggested that metformin and quercetin in combination act as an antidepressant.

Keywords: Brain Derived Neurotropic Factor, Corticosterone, Depression, Interlukin-1β, Interlukin-6, Tumour necrosis factor-α.

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INTRODUCTION

Stress increases the likelihood that a variety of psychopathological diseases, such as severe depression and anxiety disorders, will manifest in people.[1] Even though stress and adversity may have a detrimental impact at any age, adolescence and childhood are perhaps the most noticeable periods, since the CNS is still evolving.[2] Selye defined stress as "a condition characterized by a particular symptom and consisting of all non-specifically caused alterations in a biologic system"[3] and later, "the nonspecific response of the body to any demand made upon it." He went on to say that "stress is not merely neurological tension; stress reactions do occur in lower creatures, which have no nervous system and even in plants."[1] One of the things that lead to depression is chronic stress, which triggers the brain's frontal cortex and hypothalamus to release a series of inflammatory cytokines. Depression causes loss of interest, anhedonia and feeling dejected. It decreases functional abilities. Around 1 million people commit suicide every year which comes to around 3000 suicides daily.[4] WHO had ranked depression as the third reason for the incumbrance of disease throughout the globe in 2008 and also envisaged that it would occupy the top spot on the list by 2030.[5] The actual cause of depression is still not known and there is research going on in this field but chronic stress has been proven to be one of the vital reasons for depression for a long time.[6]

To develop a new drug approach, it is necessary to understand possible ways by which suppression of depressive symptoms can be achieved at the biochemical level. One such protein, which can help relieve depression when targeted, is AMP-Activated Protein Kinase (AMPK).[7] Once activated, it leads to a cascade of chemical reactions which eventually leads to the formation of Brain-Derived Neurotropic factor (BDNF).[8] Under chronic stress conditions, the brain's ATP levels are depleted at an exemplary rate which leads to fatigue which is one of the symptoms of

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depression.[9] Elevated cortisol levels due to chronic stress lead to a decrease in the levels of AMPK.[10] Activation of AMPK also assists in the process of neurogenesis which has been proven to alleviate depression. Thus, activation of AMPK might help in alleviating depressive symptoms.[11] Additionally, it has been observed that depression causes levels of cytokines such as Tumour Necrosis Factor-α (TNF-α), Interlukin-1β (IL-1β) and Interlukin-6 (IL-6) to rise.[12]

Biguanide-class antihyperglycaemic medication metformin functions as an antidepressant by activating AMPK, an enzyme involved in glucose and lipid metabolism, as well as cellular and overall energy balance.[13] It does so by stimulating the phosphorylation of the key regulatory site (Thr-172) on the catalytic (α) subunit of AMPK. Once triggered by a lack of energy, it directly phosphorylates metabolic enzymes and has an impact on gene expression to activate ATP-producing catabolic pathways and inhibit ATP-consuming anabolic processes. It regulates the expression of related epigenetic enzymes and its stimulation can raise the expression of Brain-Derived Neurotrophic Factor (BDNF) which helps in maintaining synaptic plasticity.[14,15] It does so by modulating the cAMP Response Element Binding protein (CREB).[16] It also inhibits GSK3 which is involved in neurodegeneration. It may prevent the activation of NF-κB-NLRP3 inflammasome which may offer therapeutic benefit in depression.[17-19] Quercetin on the other hand is a flavonoid found in various plants and foods reported to activate AMPK.[20,21] By lowering the levels of pro-inflammatory cytokines, it has an anti-inflammatory impact. It also has an antioxidant effect and leads to a reduction in Malondialdehyde (MDA) levels.[22]

In the current research study, based on the hypothesis proposed the combination of Metformin and Quercetin was studied for Chronic Unpredictable Mild Stress (CUMS) activities. Moreover, combination studies comprise full and half doses of Metformin (100 mg/kg)+Quercetin (10 mg/kg) and Metformin (200 mg/kg)+Quercetin (20 mg/kg) respectively. The *in vivo* models were supported by studies on behavioral parameters comprising locomotor activity, Elevated Plus Maze (EPM), Tail Suspension Test (TST), Forced Swim Test (FST) and Sucrose Preference Tests (SPT). Furthermore, TNF-α, BDNF, Corticosterone, IL-1β and IL-6 levels were checked and correlated to the anti-depressant activity.

MATERIALS AND METHODS

Procurement of chemicals and mice

We received gift samples of metformin HCl and fluoxetine HCl from Abhilash Chemicals (Madurai) and Ravian Lifesciences (Hrishikesh). Otto Chemicals Pvt. Ltd., was the source of quercetin respectively (Mumbai). Male Swiss albino mice (20-25 g) were purchased from the National Institute of Biosciences, Pune, India. Mice were kept in a plastic perspex cage with a 12:12 light and dark cycle, 25±10°C and 45-55% RH. *Ad libitum* supplies of chow pellets and filtered water were provided. One week before to the start of the experiments, the mice were given time to acclimate. The Institutional Animal Ethics Committee (IAEC) of the School of Pharmacy and Technology Management at NMIMS University in Mumbai has accepted the protocol. (IAEC/P-48/CPCSEA Dt 13/10/2021). Mice were randomly divided into (n=56) groups. Prior to testing, the mice were given a 12 hr (or overnight) fast and their weights were recorded. The following were experimental groups:

Group I-Control-Vehicle (0.9 w/v saline).

Group II-Disease Control.

Group III-Standard- (10 mg/kg) Fluoxetine.

Group IV-(200 mg/kg) Metformin.

Group V-(20 mg/kg) Quercetin.

Group VI-(100 mg/kg) Metformin+(10 mg/kg) Quercetin.

Group VII-(200 mg/kg) Metformin+(20 mg/kg) Quercetin.

Table 1 includes the various stressors that were given to the mice over 5 weeks.

Behavioral parameters

After the 5th week, behavioral studies were conducted as discussed below.

Locomotor activity

In situations of CNS diseases, there is a significant reduction in motor activity. An actophotometer (INKO brand) with a photocell, light source and digital counter was used to gauge the horizontal movement of mice. Each mouse was placed in an actophotometer for 5 min, during which their initial level of activity was recorded. After each mouse received the proper therapy, the activity score was evaluated after 30 min.[23]

EPM

EPM is based on the spontaneity of the mice to explore new surroundings along with their natural tendency towards elevation. The elevated plus maze test equipment is in the shape of a+and consists of two open arms across from each other and perpendicular to two closed arms with a center platform with an open roof arrangement. Closed arms have a high wall to surround the arm. The mice were dosed and then allowed to acclimatize for 30 min. The mice were then released in the center of the configuration of the maze and were then allowed to explore the maze for 6 min each and the individual videos were recorded. The evaluation parameters comprise exploration time and time spent in either arm.[24,25]

Table 1: Stressors given to the different groups.

Time line	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1st Week	Cage tilting (45°C) for 4 hr	5 min cold swimming (4 °C)	Food deprivation (6 hr)	5 min hot water swimming (45°C)	Water deprivation (6 hr)	Disruption of l/d cycle	No stress
2nd Week	Disruption of l/d cycle	Cage tilting (45°C) for 4 hr	5 min cold swimming (4°C)	Food deprivation (6 hr)	5 min hot water swimming (45°C)	Water deprivation (6 hr)	No stress
3rd Week	Water deprivation (6 hr)	Disruption of l/d cycle	Cage tilting (45°C) for 4 hr	5 min cold swimming (4°C)	Food deprivation (6 hr)	5 min hot water swimming (45°C)	No stress
4th Week	5 min hot water swimming (45°C)	Water deprivation (6 hr)	Disruption of l/d cycle	Cage tilting (45°C) for 4 hr	5 min cold swimming (4°C)	Food deprivation (6 hr)	No stress
5th Week	Food deprivation (6 hr)	5 min hot water swimming (45°C)	Water deprivation (6 hr)	Disruption of l/d cycle	Cage tilting (45°C) for 4 hr	5 min cold swimming (4°C)	No stress

Abbreviations: l/d cycle: light/dark cycle

TST

The TST is a method of evaluating potent antidepressants. Immobility demonstrated by the mice when they undergo stress is hypothesized to suggest behavioral quail which in turn reflects disorders in humans. In the TST test, the mice were suspended in the free air on the edge of the shelf 58 cm above the table top by their tail using an adhesive tape placed 1 cm from the top. The mice were given their respective treatment doses 30 min before the test. In the hanging state, the mice were completely immobile. The test was conducted for 5 min and individual videos were recorded.[26]

FST

In this test, the mice were subjected to swimming in a restricted space from which they could not escape. Each mouse received drug treatment 30 min before the test. The mice were allowed to swim in a Plexiglas cylinder (10 cm diameter x 25 cm height) containing water (room temperature) with 18 cm depth. The mice were allowed to swim for 6 min and the video was recorded. Also, each mouse went through a pre-test before actually going through the main test. Post the test, each mouse was towel-dried before returning to cage.[27]

SPT

The SPT facilitates the observation of anhedonia in mice following depression induction. Before the test began, the mice were exposed to a variety of stressors, including overnight lights, wet bedding, food and water deprivation and tilting the cage to 45°. Before the test, each mouse was housed individually and given two bottles, one containing a 1% (w/v) solution of sucrose and the other tap water. Once the adaptation process was completed, the 24 hr fast was managed. To avoid any chance of position preference, the mice in the cage were spaced equally apart from each other and from the two bottles and their places were switched around every 12 hr. Two bottles with tap water and a 1% w/v sucrose solution were provided to the mice during the dark period (7:00 p.m. to 9:00 p.m.). The respective consumptions of the two solutions were measured after 2 hr of exposure. Using the sucrose consumption rate and the other tap water, the following formula was used to calculate Sucrose Preference (SP): SP=(sucrose intake (g)/(sucrose intake (g)+water intake (g))×100%.[27]

Biochemical analysis

Blood was withdrawn using the retro-orbital plexus puncture technique under the condition of light anesthesia. The blood samples were then centrifuged at 8000 RPM for 15 min to separate the plasma. Also, after subjecting mice to euthanasia; brains were extracted, washed with 0.9% w/v solution, weighed and stored. Furthermore, brain homogenate was prepared and cold centrifuged at 8000 RPM for 20 min at 4°C. The supernatant was separated and was later stored at -80°C. The samples were subjected to the estimation of BDNF, corticosterone, TNF-α, IL-1β and IL-6 levels.[27]

BDNF

The plate wells were filled with standard and samples that had specific antibodies. Before being incubated, a biotinylated detection antibody specific for mouse BDNF and an Avidin-Horseradish Peroxidase (HRP) combination were added to each microplate well. While being washed, free pieces were eliminated. Additionally, the substrate solution is added to every well. Only the wells containing mice BDNF, biotinylated detection

antibody and anavidin-HRP conjugate showed blue colouring. When a stop solution is applied, the enzyme-substrate reaction is stopped and the colour changes to yellow. Spectrophotometry is used to calculate the absorbance at 450 nm.[27]

Corticosterone

The kit included one micro-ELISA plate that had been pre-coated with Corticosterone (CORT). Throughout the procedure, a predetermined amount of CORT on the solid phase supporter competes with CORT from the samples and the standard for spots on the CORT-specific Biotinylated Detection Ab. The conjugate of Avidin-Horseradish Peroxidase (HRP) is added to each microplate well and the plate is then incubated. The excess conjugate, unbound samples and standard were eliminated using water. The TMB substrate solution is then added to each well. The application of a stop solution causes the colour to change from blue to yellow and stops the enzyme-substrate reaction. The Optical Density (OD) was measured spectrophotometrically at 450 nm.[27]

IL-1 β and IL-6

The micro-ELISA plate that comes with this kit has already been pre-coated with an anti-IL-1 and IL-6 mouse-specific antibody. Some standards, samples and antibodies were placed into the micro-ELISA plate wells. Then, a combination of Avidin-Horseradish Peroxidase (HRP) and a biotinylated detection antibody specific for mouse IL-1 are added to each microplate well. During washing, parts that are free are taken out. Divide the substrate solution among all of the wells. The mice's IL-1β and IL-6, biotinylated detection antibody and Avidin-HRP conjugate-containing wells were coloured blue. The enzyme-substrate reaction is halted and the colour turns yellow when a stop solution is added. The OD was measured at a wavelength of 450 nm+2 nm.[27]

TNF-α

The samples were homogenised in PBS (*p*H 7.4) after being weighed and kept at -800°C. The centrifuge run on the samples took about 15 min. Subsequently, Streptavidin-HRP and Mice TNF-α Biotin Conjugated Detection Antibody were added and the mixture was incubated to form a complex. After cleaning the microwells to remove any non-specific binding, the substrate solution (TMB) was added and colour developed in accordance with the number of mouse AS in the sample. Next, a stop solution was added to halt the development of colour. After adding the stop solution, absorbance was finally seen at 450 nm in 10 to 15 min.[27]

Statistical analysis

Results are presented using GraphPad Prism version 8.4.2 and are reported as mean+SEM. One-way Analysis of Variance (ANOVA) and Dunnett's *post hoc* test were used.

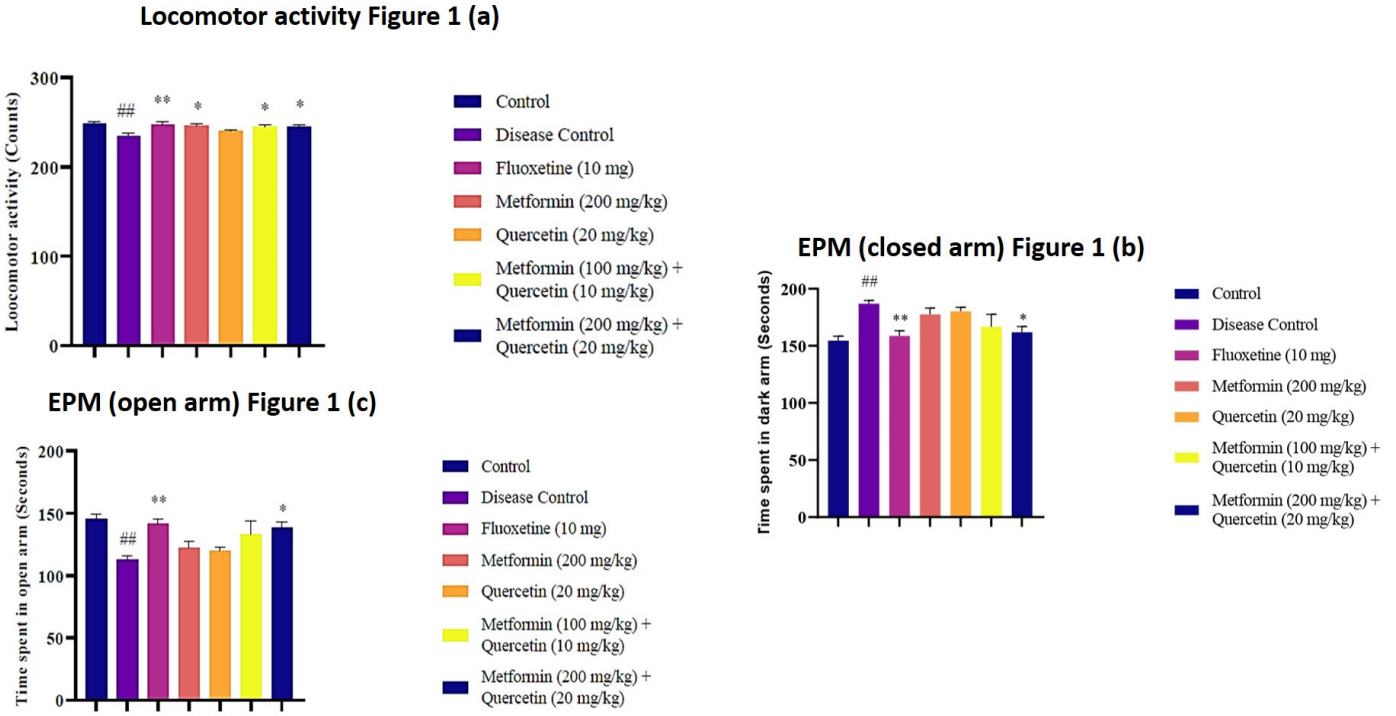


Figure 1 (a, b): Locomotor and Elevated Plus Maze (closed and dark arm) activities of experimental groups.
Values are expressed as Mean ± SEM (n=8) *$p < 0.05$, **$p < 0.01$, when compared with disease control. ##$p < 0.01$ when compared to normal control

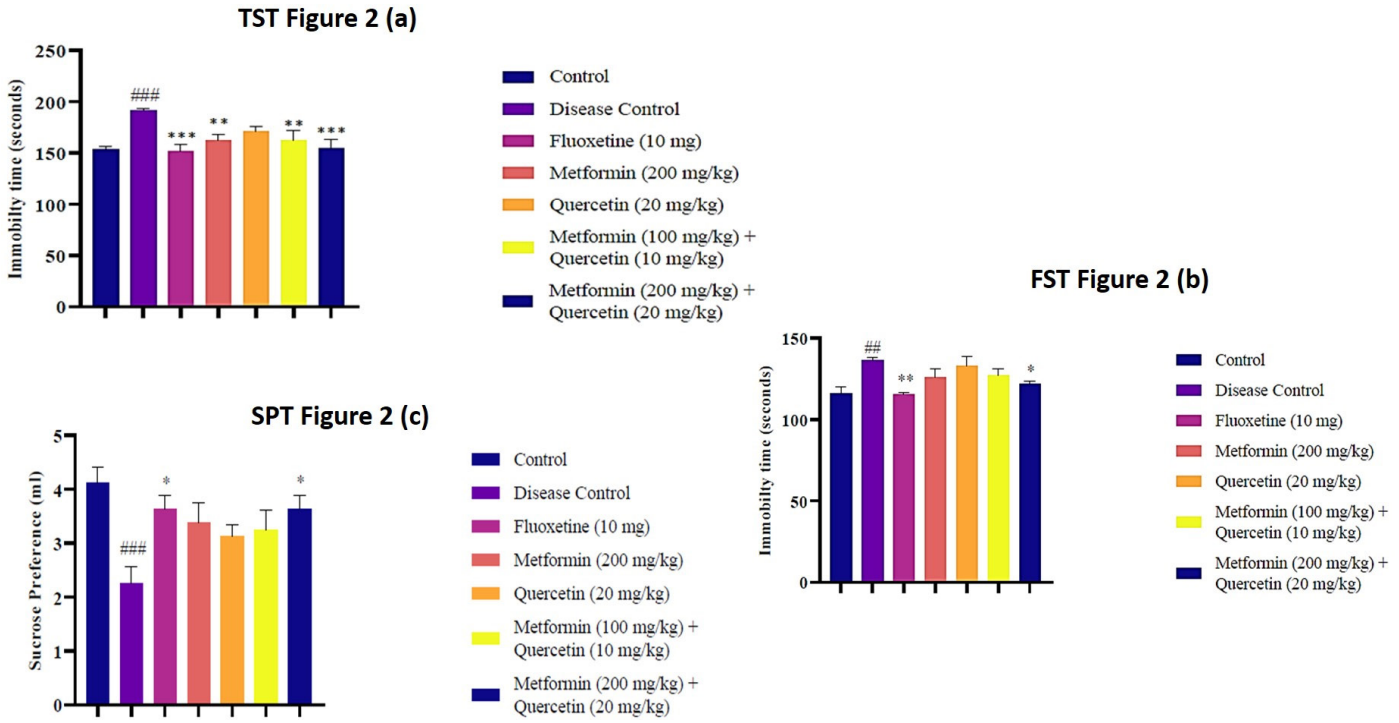


Figure 2 (a, b, c): Tail Suspension, Sucrose preference and Forced Swim tests of experimental groups. Values are expressed as Mean ± SEM (n=8) *p < 0.05, **p < 0.01, when compared with disease control. ##p < 0.01, ### p < 0.001 when compared to normal control.

RESULTS

Behavioral studies

Locomotor activity

The locomotor activity was higher in the therapy groups contrary to the disease control. A significant increase (*p < 0.05, **p < 0.01) in locomotor activity (counts) was seen in all groups, except Quercetin (20 mg/kg) group as shown in Figure 1 (a). Disease control (##p<0.01) was significant compared to control.

EPM (closed arm)

In comparison to the disease control, the exploration time on the dark arm (closed) was shorter in the therapy groups. The dark (closed) arm exploration was significantly lower in all groups contrary to the disease control (*p < 0.05, **p < 0.01) as shown in Figure 1 (b). Disease control (##p<0.01) was significant compared to control.

EPM (Open Arm)

The open-arm exploration time was longer in the therapeutic groups contrary to the disease control (*p < 0.05, **p < 0.01) as seen in Figure 1 (c). Disease control (##p<0.01) was significant compared to control.

TST

As indicated in Figure 2 (a), all groups except the Quercetin (20 mg/kg) group demonstrated a substantial reduction in immobility time contrary to the disease control (**p < 0.01, ***p < 0.001). A significant difference was observed in Metformin (200 mg/kg) + Quercetin (20 mg/kg) treated group (***p<0.001). Disease control (### p < 0.001) was significant compared to control.

FST

As demonstrated in Figure 2 (b) , all groups showed a substantial reduction in immobility time in comparison to the disease control (*p < 0.05, **p < 0.01).Disease control (##p<0.01) was significant compared to control.

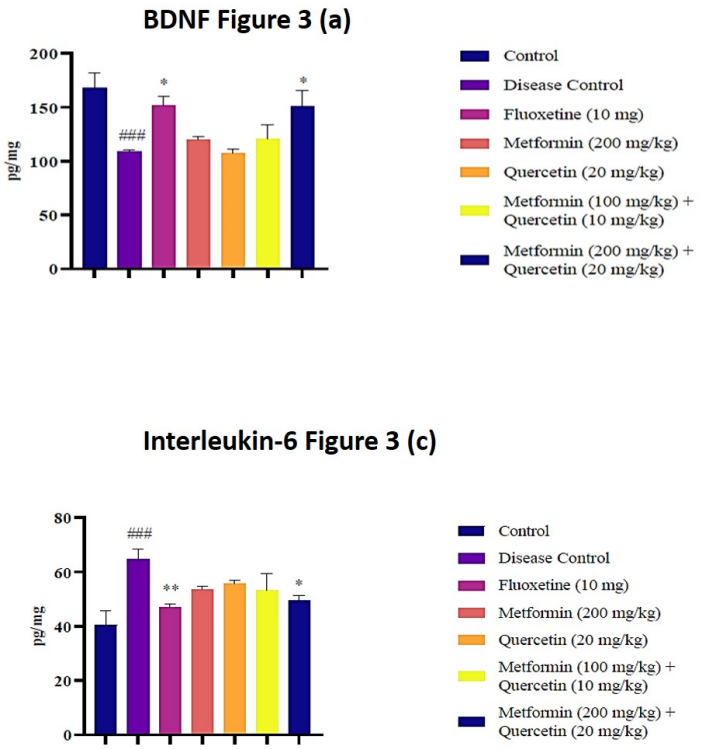
SPT

In this experiment, the treatment groups drank more sucrose on average than the disease control (*p < 0.05) as shown in Figure 2 (c). Disease control (### p < 0.001) was significant compared to control.

Biochemical analysis

BDNF

When contrary to the disease control, the levels of BDNF were higher in the treatment groups. In comparison to the disease control, all groups except those on metformin (200 mg/kg), quercetin (20 mg/kg) and metformin (100 mg/kg)+quercetin (10 mg/kg) showed a substantial increase in BDNF levels (Figure 3a, *p < 0.05). A significant difference observed in Metformin (200 mg/kg)+Quercetin (20 mg/kg) treated group was *p<0.1.



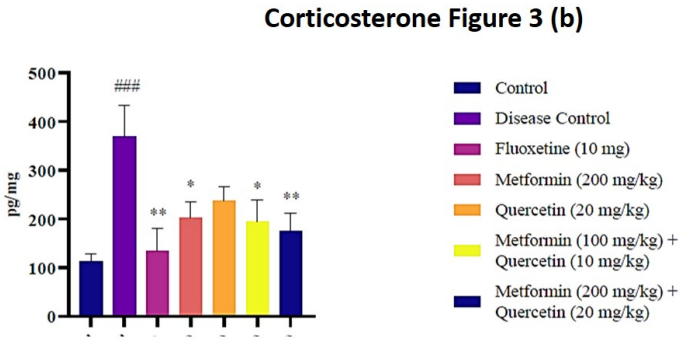
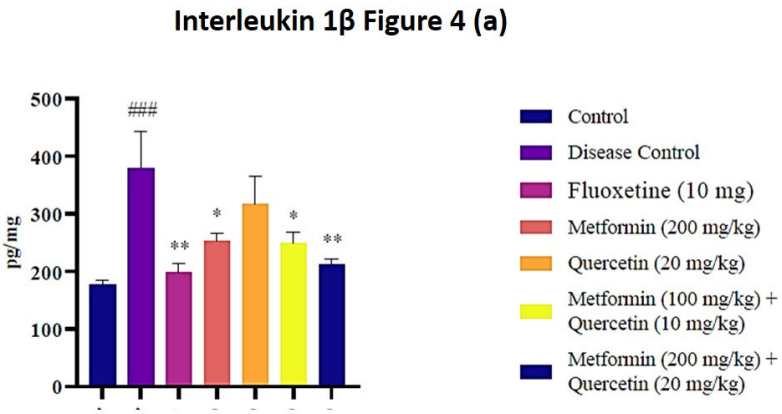


Figure 3 (a, b, c): BDNF, Interlukin-6 and Corticosterone levels of experimental groups.
Values are expressed as Mean ± SEM (n=8) *$p < 0.05$, **$p < 0.01$, when compared with disease control. ### $p < 0.001$ when compared to normal control.



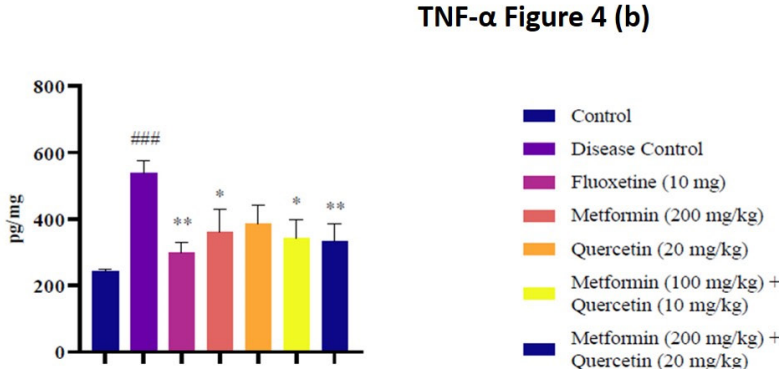


Figure 4 (a, b): Interlukin-1β and TNF-α levels of experimental groups
Values are expressed as Mean ± SEM (n=8) *$p < 0.05$, **$p < 0.01$, when compared with disease control. ### $p < 0.001$ when compared to normal control.

Corticosterone

As compared to disease control, the levels of corticosterone were lower in the therapy groups ($p < 0.05$, $^{**}p < 0.01$). (Figure 3 b). Disease control (### $p < 0.001$) was significant compared to control.

Interleukin-6

When contrary to the disease control, Interleukin-6 levels were lower in the therapy groups. A significant reduction in Interleukin-6 levels was seen in all groups when contrary to the disease control, ($p < 0.05$, $^{**}p < 0.01$, Figure 3 c). Disease control (### $p < 0.001$) was significant compared to control.

Interleukin 1β

When contrary to the disease control, Interleukin-1 levels were lower in the therapy groups. In comparison to disease control, Interlekin-1β levels significantly decreased in all groups ($^{*}p < 0.05$, $^{**}p < 0.01$) (Figure 4 a). Disease control (### $p < 0.001$) was significant compared to control.

TNF-α

In comparison to the disease control, TNF- α levels significantly decreased in all groups. ($^{*}p < 0.05$, $^{**}p < 0.01$ Figure 4 b). Disease control (### $p < 0.001$) was significant compared to control.

DISCUSSION

An array of physiological responses, including those of the neurological, endocrine and immune systems, can be elicited by psychological stress that would otherwise be harmful in some circumstances. This is because psychological stress originated as an adaptation to the fight-or-flight response.[28] Animal models may be used to study the neurobiology of psychological stress as well as mental illnesses such as depression and anxiety. Chronic stress leads to depression which is a well-established fact but the exact pathophysiology of which is not known.[29] The CUMS model was performed over 5 weeks which has been proven to be efficient in experimental studies.[30] A parallel therapy was administered for standard, monotherapy and combination groups of metformin and quercetin. half dose and full dose for 3 weeks. Metformin belongs to the biguanide class and is the first-line medicine for glycaemic management because it significantly decreases hepatic glucose synthesis and increases insulin sensitivity in people with Type 2 diabetes.[31] Whereas Quercetin leads to the reduction in levels of inflammatory cytokines, serum cortisol and reduction of oxidative markers in depression.[32] Recent research suggests that persistent stress-induced depressed behaviors may be explained by intracellular energy metabolism which can be linked to AMPK.[33] The purpose of the study was to evaluate the effect of Metformin and Quercetin combination to alleviate the symptoms of depression. The results obtained show that depression was induced in the experimental animals and was maintained throughout the study. Stress-induced depression animal model is a scientifically justified standard experimental model for the induction of depression in laboratory mice.[34,35]

Behavioral parameters like locomotor activity, EPM, TST, FST and SPT were performed. The results obtained depicted that the high-dose combination groups showed slightly higher locomotor activity when contrary to monotherapy. Whereas TST and FST it was observed that the high-dose combination group showed decreased immobility time as contrary to monotherapy groups. In EPM, mice administered with the high dose combination dose were seen to explore the open arm more as contrary to the monotherapy and disease control. ELISA immunoassays were performed to measure the levels of BDNF, corticosterone, IL-1 β, TNF-α and IL-6. Mogroside V decreases oxidative stress damage and apoptosis in hippocampal nerve cells, indicating that its antidepressant effects may be achieved by blocking the BDNF/TrkB/AKT pathway as well as inflammatory and oxidative stress pathways.[36] Additionally, Rannasangpei demonstrated stronger anti-inflammatory and antioxidant capabilities than crocin-1 in stressed mice, encouraging the development of BDNF and Neuroprotection.[37] Metformin and quercetin have been found to lower corticosterone levels in mice through different mechanisms. Metformin enhances insulin sensitivity and decreases hepatic glucose production, indirectly affecting corticosterone levels. Quercetin, a bioflavonoid with blood glucose-regulating properties, reduces corticosterone levels by modulating the hypothalamic-pituitary-adrenal axis.[38] Quercetin reduces stress-induced corticotropin-releasing factor expression in the hypothalamus, lowering plasma corticosterone levels, suggesting metformin and quercetin could effectively manage glucocorticoid-induced hyperglycemia and related complications in mice by targeting specific corticosterone regulation pathways.[39] BDNF levels were observed to be increased significantly whereas corticosterone, TNF-α, IL-1 and IL-6 were observed to be significantly reduced in the high-dose combination-treated group as contrary to disease control.

Metformin and quercetin have been shown to have superior antidepressant effects due to their synergistic actions, which modulate liver metabolism and mitigate metabolic changes caused by CUMS.[40] Quercetin has demonstrated anti-obesity and antidepressant effects in obese animals, reducing food intake, reducing body weight and enhancing psychological behavior, suggesting potential for addressing depression-related neuroendocrine imbalance.[41] Metformin and quercetin in diabetic rats showed stronger antidiabetic effects and protection against hyperglycemia-induced endothelial dysfunction, suggesting synergistic action modulates metabolic pathways and endothelial function, potentially enhancing antidepressant efficacy.[42] The combination of metformin and quercetin showed neuroprotection and increased neurogenesis in depression as evidenced by improvement in behavioral parameters and

BDNF levels and reduction in corticosterone, IL-1β, IL-6 and TNF-α levels. This improvement observed by the combination of metformin and quercetin was statistically more significant than the disease control. Findings from the study justify that a combination of metformin and quercetin may be useful for the management of depressive symptoms.

The current study has pitfalls in that, it did not evaluate long-term effects nor did it assess the reversibility of these treatments after the metformin and quercetin were stopped. Further detailed studies with metformin plus quercetin on other benefits that they offer are required. Such studies will help to determine the varied effects of this combination on depression. The exclusion of the effects of metformin and quercetin on female mice was a drawback in the study. Only male mice were utilized in this experiment since the hormonal changes in female mice can introduce additional variables and make it more difficult to interpret the data. It would be helpful for future research to include plasma/serum biochemical profiling to give a more thorough understanding of both central and peripheral changes in depressive states.

CONCLUSION

In summary, the combination of metformin and quercetin may have antidepressant potential. Further detailed studies with metformin and quercetin need to be undertaken exhaustively. The future task is to extrapolate studies that will help to determine the varied effects of this combination on depression.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CUMS: Chronic Unpredictable Mild Stress; **SPT:** Sucrose Preference Test; **TST:** Tail Suspension Test; **FST:** Forced Swim Test; **APT:** Actophotometer test; **SLA:** Spontaneous locomotor activity; **ELISA:** Enzyme-Linked Immunosorbent Assay; **IL-1β:** Interleukin- 1beta; **IL-6:** Interleukin-6; **TNF-α:** Tumor necrosis factor-alpha.

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