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Ameliorative Effect of Rutin Supplement on Chronic Unpredictable Mild Stress-Induced Depressive Phenotypes in Mice

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ABSTRACT

Depression is a severe disorder that results in poor quality of life and affects hundreds of millions worldwide. Research on the relationship between depression and oxidative stress has shown important biochemical aspects in disease development. Flavonoids are a class of natural products that exhibit several pharmacological properties, including antidepressant-like activity and hence, suggested by studies to be exciting prototypes for new antidepressant drugs. Rutin is a flavonoid glycoside abundantly found in plants. The study evaluated the effect of rutin supplementation on depression using the chronic unpredictable mild stress depression mice models. Twenty-five mice were grouped into 5 (n = 5) and subjected to chronic unpredictable mild stressors. Groups 1-5 were orally served distilled water (no treatment), Rutin 30 mg/kg, 60 mg/kg, 120 mg/kg, and fluoxetine 20 mg/kg respectively. The study was conducted in the span of 30 days. Administration of rutin produced a reduction in immobility time at a dose of 120 mg/kg, a significant ($p<0.05$) decrease in the concentration of serum superoxide dismutase at doses of 30 and 60 mg/kg, and showed no significant difference on catalase and malondialdehyde levels. Rutin supplementation showed the potential of positively reversing behavioural despair. Thus, may be considered a possible treatment option for depressive symptoms in view of it attaining lesser side effects compared to orthodox treatments.

Keywords: *Oxidative Stress, Chronic Unpredictable Mild Stress, Rutin, Superoxide dismutase, Catalase and Malondialdehyde*

INTRODUCTION

Depression is a principal cause of illness and disability worldwide (Bernaras et al. 2019) and one of the most widespread forms of psychiatric pathology (Shadrina et al. 2018). According to World Health Organization (WHO 2018), depression is a common illness affecting more than 300 million people worldwide. When prolonged, with moderate to severe intensity, depression may become a serious health challenge, making the affected persons suffer greatly and function poorly at work, school, and in the family, and can lead to suicide.

The close association between oxidative stress and lifestyle-related diseases such as Parkinson's and Alzheimer's has become well known (Yoshikawa and Naito 2002). With its high oxygen consumption and lipid-rich environment, the brain is considered highly susceptible to oxidative stress or redox imbalances. Therefore, the fact that oxidative stress is implicated in several mental disorders, including depression, anxiety disorders, schizophrenia and bipolar disorder,

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is not surprising (Salim 2014).

Superoxide dismutase (SOD) and its cofactors, zinc and copper, catalyze the breakdown of superoxide to oxygen and, paradoxically, to hydrogen peroxide, which catalase decomposes in physiological conditions. An elevated level of SOD is observed as the organism responds to high concentrations of superoxide. In the liver, catalase can function in two ways- firstly, to convert hydrogen peroxide to water and molecular oxygen, then secondly, uses hydrogen peroxide to oxidize formate, nitrate and simple alcohols. Catalase is primarily located within the peroxisome, constituting approximately 50% of the protein in the organelle (Loew 1900). The possible consequence of free radical damage is emphasized in lipid peroxidation. In lipid peroxidation, oxidative damage to the lipid moieties in cellular membranes, lipoproteins, and other lipid-rich molecules exists. Cellular membrane lipids represent most often substrates of oxidative attack (Nawrot et al. 2008).

Medicinal plants have been integral to traditional medicine since the ancient era. Thus, the contemporary scientific community has presently recognized flavonoids to be a unique class of therapeutic molecules due to their diverse medicinal properties (Ganeshpurkar and Saluja 2017). Flavonoids have been demonstrated to possess an anti-depressant and fewer adverse effects than tricyclic anti-depressants, and for this reason, flavonoids in natural products have attracted growing attention (Yusha'u et al. 2017). Rutin, a flavonoid compound, was discovered in 1842 and has been used in traditional medicine to treat vascular disorders related to capillary permeability and fragility as it is one of the most common quercetin glycosides found in several plant sources, mainly in different parts of the plants such as the fruit skins, leaves, flowers, and roots (José et al. 2019).

Rutin has significant antidepressant, antioxidant, anti-inflammatory, chelating of reactive oxygen species, antimicrobial properties, and many beneficial health effects (José et al. 2019). A study by Machado et al. (2008) demonstrated the antidepressant-like effect of Rutin mediated due to increasing serotonin and noradrenaline in the synaptic cleft. Among the previously mentioned health properties of rutin, the neuroprotective effect of this flavonoid is one of the most studied because of the increasing prevalence of neurological disorders and brain pathologies. A review by Ganeshpurkar and Saluja (2017) pointed out the pharmacological potentials of rutin, among which was its antidepressant effect. Furthermore, a study by Yusha'u et al. (2017) indicates that chronic administration of rutin supplements produced an antidepressant effect in an open space forced swim test model of depression. Hence, the study aimed to evaluate the impact of rutin supplementation on depression using the chronic mild stress mouse model.

MATERIALS AND METHODS

Experimental Animals

Twenty-five Swiss mice of 18-24 g weight were obtained from the Animal House of the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria. Animals were housed in plastic cages containing sawdust bedding and fed with pellets made from Vital feed finisher, maize offal as a binder in a ratio of 3:1 and water *ad libitum*. Each mouse was kept in a separate cage throughout the experimental period. Animals were handled humanely by their tails to avoid any pain, and regular changing of the bedding was adopted to prevent infections. Institutional ethical approval was obtained from the ABU Committee on Animal Use and Care with the approval No: ABUCAUC/2022/002.

Experimental Design

The animals were grouped into five, with each consisting of five mice: Control (distilled water, 10 ml/kg and were exposed to chronic mild stressors); Fluoxetine (20 mg/kg orally), Bing et al. (2016); Rutin groups (30, 60 and 120 mg/kg orally), Yusha'u et al. (2017)

A tail suspension test (TST1) was conducted first, which served as the basis for grouping the animals. Thereafter, chronic mild stressors (Table 1) were applied for two weeks, and a second TST (TST2) was followed to determine the level of behavioural despair. Chronic mild stressors were continued together with treatment with Rutin and Fluoxetine after the second TST for another two weeks. Lastly, a third TST (TST3) was conducted to assess the effect of the treatment.

Animal Sacrifice

Ketamine (50 mg/kg) was administered intraperitone-

Table 1: Stressors used in the Chronic Unpredictable Mild Stress

Stressors	Description
Damp bedding	Slight wetness on the saw dust
Empty cage	Removal of bedding
Tilted cage	Tilting of cages at approximately 45°
Overnight illumination	Alteration of normal 12 h light/dark cycle
Social stress	Transferring each animal from its home cage to that of the cage of a neighboring animal
Empty cage + water	Removal of bedding and adding water
Meowing sound	Exposing animals to predator sounds
White noise	Exposing animals to growling noises
New clean cage	Placing animal into clean cages

Willner et al. (1992), Mineur et al. (2006), Frisbee et al. (2015), Willner (2017) and Burstein and Doron (2018)

ally (i.p) to anaesthetize the animals before the blood sample was obtained according to the method of the Institute for Animal Studies (2019).

Blood Collection
Blood samples were drawn by cardiac puncture and collected in plain tubes. The serum was used to assess superoxide dismutase (SOD), catalase and malondialdehyde concentrations.

Drug Preparation
Rutin (0.12 g, #207671-50-9, Sigma-Aldrich Chemie GmbH Bavaria, Germany) was dissolved in 10 mL of distilled water to form a dose of 120 mg/kg. Thereafter, a serial dilution was conducted to form Rutin doses of 60 and 30 mg/kg, respectively. Fluoxetine (0.02 g, Flutex 20mg, #F011007, V.S. International Pvt. Ltd, India and NAFDAC no: 04-881) was dissolved in 10 mL of distilled water to constitute a dose of 20 mg/kg. The drugs were reconstituted daily prior to oral administration to the mice.

Neurobehavioural Assay
Chronic Unpredictable Mild Stress (CUPMS): This model is based on the fundamental concept that chronic exposure to stressors disrupts stress response systems and ultimately leads to the development of depressive disorders. Hence, during the CUPMS protocol, animals were exposed to a randomized series of mild environmental and social stressors daily (Table 2).
Animals were exposed to 1 or 2 of the stressors as listed in Table 1, each day at a randomized schedule for four weeks, stressors without treatment were applied in the first two weeks of experiment and thereafter, stressors were applied concurrently with treatment for the last two weeks

Tail Suspension Test (TST): The tail suspension test is conducted to ascertain the level of behavioural despair. The TST was conducted according to the protocol adopted by Adem et al. (2012) and Can et al. (2012). Animals were suspended in the air using a small metal hook fixed by adhesive tape of 17 cm wrapped around the animal's tail. The latency to the first bout of immobility and the time spent immobile during the 6 min testing period was recorded (Mineur et al. 2006). The animals were prevented from climbing their tail by passing the tail through a cylindrical plastic tube of about 4 cm. Mice were considered immobile only when they hang passively and completely motionless (Frisbee et al. 2015). The walls of the apparatus were cleaned with 70% ethanol after each trial to prevent any olfactory cue.

Serum Superoxide Dismutase (SOD) Assay
Superoxide dismutase (SOD) activity was determined in serum using the spectrophotometry method as stated in the enzyme assay kit protocol. The enzyme assay (#CK-bio-15885, Shanghai Coon Koon Biotech Co., Ltd, China) was carried out in the Histology Laboratory, Department of Human Anatomy, Faculty of Basic Medical Sciences of Ahmadu Bello University, using direct detection method and the stepwise procedures of the enzyme assay kit protocol.
The superoxide anion (O_2^-) is produced by the xanthine and xanthine oxidase reaction system, and the O_2^- is reducible by nitroblue tetrazolium, to form blue formazan which is absorbed at 560 nm; SOD can remove O_2^-, thereby inhibiting the formation of formazan; the deeper the blue of the reaction liquid, the lower the SOD activity.
SOD activity was calculated using the following formulae;
1. Calculation of percentage of inhibition

$$Percentage\ of\ inhibition = \frac{(A\ control\ tube - A\ measuring\ tube)}{A\ control\ tube} \times 100\%$$

2. Calculation of SOD enzyme activity in serum

$$Serum\ (plasma) SOD\ activity\ (U/mL)$$

$$= \frac{\left[\frac{Percentage\ inhibition}{(1 - Persentage\ inhibition)} \times V\ reaction\ total\right]}{V\ sample \times sample\ dilution\ factor}$$

$$= \frac{11.4 \times percentage\ of\ inhibition}{(1 - percentage\ of\ inhibition) \times Sample\ dilution\ factor}$$

where V reaction total = volume of the reaction system, 1.026 mL; V sample = sample volume added to the reaction system, 0.09 mL

Catalase Enzymes Activity Determination
The catalase (CAT) activity was measured using the method of Aebi (1974). Exactly 10 µl of serum was

Table 2: Chronic Unpredictable Mild Stress Schedule

Days	Sun 1	Mon 2	Tue 3	Wed 4	Thu 5	Fri 6	Sat 7	Sun 8	Mon 9	Tue 10	Wed 11	Thu 12	Fri 13	Sat 14
Morning session	TC	DB	EC	EW	NC	DB	TC	EC	EW	NC	DB	TC	EC	EW
Evening session	WN	OI	MS	SS	WN	OI	WN	MS	SS	MS	OI	WN	MS	SS

TC: Tilted cage, WN: White noise, DB: Damp Bedding, OI: Overnight illumination, EC: Empty cage, MS: Meowing sound, EW: Empty cage + water, SS: Social stress, NC: New clean cage

added to the test tube containing 2.8 mL of 50 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 mL of freshly prepared 30 mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 300 nm for 5 min on a spectrophotometer. A molar extinction coefficient (E) of 0.041 mM^{-1} _ cmM^{-1} was used to calculate the catalase activity in µmol/min.

$$\text{Catalase concentration} = \text{Absorbance/E}$$

Malondialdehyde (MDA) Determination

The plasma MDA level was assayed using thiobarbituric acid (TBA) spectrometric. TBA reacts with some products of lipid peroxidation to form a red product with a maximum absorption peak at 532 nm. After colorimetry, the content of lipid peroxide in the sample was estimated; and the same time absorbance at 600 nm was measured. The amount of MDA was calculated using the difference in absorbance at 532 and 600 nm.

Statistical Analysis

Results were presented as mean ± standard error of mean (SEM). All analyses were done using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons, except comparison between TST 1 and TST2, which was analysed using unpaired sample t-test. GraphPad Prism version 8.0.2 was used for all the analyses. Values with $p<0.05$ were considered statistically significant.

RESULTS

Assessment of Immobility Time in Mice Using Tail Suspension Test before Applying Chronic Mild Stressors and Treatment (TST 1)

Multiple comparisons of immobility time using Tukey's post hoc test (Fig. 1) between the control (174.8 ± 11.9) and rutin 30, 60 and 120 mg/kg, as well as Fluo 20 mg/kg (194.0 ± 4.8, 162.0 ± 22.9, 149.8 ± 19.5 and 180.6 ± 18.4, respectively) showed no significant difference at p = 0.925, 0.982, 0.828 and 0.999, respectively.

Assessment of Immobility Time in Mice Using Tail Suspension after Two Weeks of Chronic Mild Stressors without Treatment (TST 2)

The results of immobility time showed no significant difference among the control (240.6 ± 7.6), rutin 30, 60 and 120 mg/kg (220.4 ± 18.8, 228.8 ± 29.5, and 206.4 ± 27.9) and Fluo 20 mg/kg (210.2 ± 19.2) at p = 0.965, 0.995, 0.807 and 0.864 (control vs rutin 30, 60, 120 mg/kg and Fluo 20 mg/kg, respectively), (Fig. 2).

Meanwhile, paired t-test revealed significant differences between the mean immobility times of the control (p = 0.002), Rutin 120 mg/kg (p = 0.01), Fluo 20 mg/kg (p = 0.01) of TST 2 when compared with the respective groups in TST 1 (Table 3).

Assessment of Immobility Time in Mice Using Tail Suspension Test after Two Weeks of Treatment (TST 3)

The results of immobility time in the TST 3 (Fig. 3), showed significant decrease in the rutin 120 mg/kg (142. 4 ± 37.9) p = 0.048 compared with the control (246.0 ± 6.6). However, comparison between the means of the control and rutin 30, 60 mg/kg (225.0 ± 15.9 and 261.2 ± 22.6, respectively) and Fluo 20 mg/kg (174.4 ± 26.6) revealed no significant difference at p = 0.972, 0.991 and 0.265, respectively.

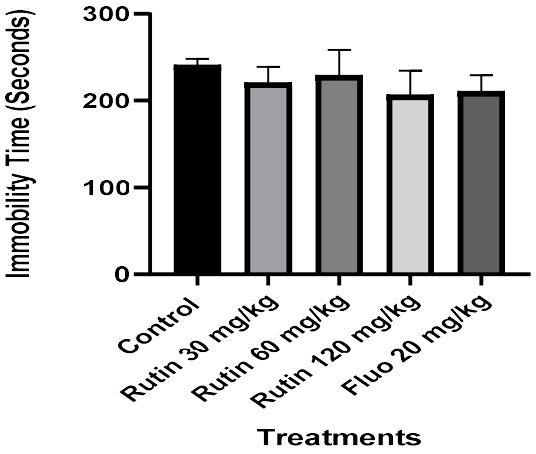

Fig. 1: Assessment of Immobility Time in Mice with the Tail Suspension Test before Applying Chronic Mild Stressors and Treatment (TST 1). The mean difference was not significantly different when compared to control group, p>0.05, (n = 5) GraphPad Prism version 8.0.2. Fluo: Fluoxetine

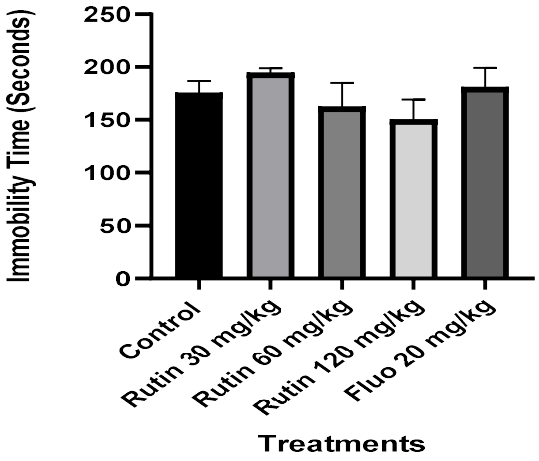

Fig. 2: Assessment of Immobility Time in Mice With the Tail Suspension after Two Weeks of Chronic Unpredictable Mild Stressors Without Treatment (TST 2). The mean difference was not significant compared to the control, p>0.05, (n = 5) GraphPad Prism version 8.0.2. Fluo: Fluoxetine

Effect of Rutin Supplement on Serum SOD Concentration in the Mice Subjected to Chronic Unpredictable Mild Stress

As shown in Figure 4, there was a significant decrease in SOD concentration in rutin 30 and 60 mg/kg (10.5 ± 1.4 and 8.8 ± 2.1) compared with the control (26.1 ± 2.3) at p = 0.0003 and 0.0001, respectively. Similarly, Fluo 20 mg/kg (10.9 ± 1.4) significantly decreased the SOD concentration when compared with the control group at p = 0.0004. However, rutin 120 mg/kg (17.7 ± 2.8) did not significantly p = 0.07 affect the SOD level in comparison with the control group.

Table 3: Comparison between the immobility time of TST1 and TST2 using paired t-test (two-tailed)

Groups	TST 1 (sec)	TST 2 (sec)	P-value	t-value
Control	174.8±11.9	240.6±7.6[a]	0.0028	6.556
Rutin 30 mg/kg	194.0 ±4.8	220.4±18.8	0.2551	1.327
Rutin 60 mg/kg	162.0± 22.9	228.8±29.5	0.0879	2.247
Rutin120mg/kg	149.0±19.5	206.4±27.9[a]	0.0166	3.964
Fluo 20 mg/kg	180.6±18.4	210.2±19.2[a]	0.0165	3.971

a- Significant compared to TST1 at p < 0.05, (n=5), using paired t-test (two-tailed), GraphPad Prism 8.0.2, Fluo: Fluoxetine, TST1: Tail Suspension Test before exposure to chronic unpredictable mild stressors, TST2: Tail Suspension Test after a two-week exposure to chronic unpredictable mild stressors.

Assessment of the Effect of Rutin Supplement on Serum Catalase Enzyme Activity in the Mice Subjected to Chronic Mild Stress

As shown in Figure 5, rutin groups 30, 60 and 120 mg/kg (15.4 ± 2.5, 19.9 ± 2.3 and 20.5 ± 3.2, respectively), as well as fluoxetine 20 mg/kg (19.9 ± 2.1) did not significantly (p > 0.05) affect the catalase concentration when compared with the control (14.7 ± 2.1) at p = 0.999, 0.575, 0.480 and 0.593 respectively.

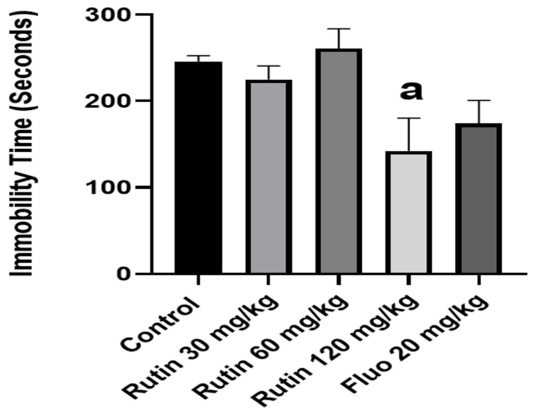

Fig. 3: Effect of rutin supplement on depression using tail suspension test after two weeks of treatment in mice subjected to chronic mild stressors (TST 3). a- Significant compared with the control, p <0.05, (n = 5) GraphPad Prism version 8.0.2. Fluo: Fluoxetine

Effect of Rutin Supplementation on MDA Level in Mice Subjected to Chronic Mild Stress

As shown in Figure 6, there was no significant (p > 0.05) difference on the MDA concentration when the control group (29.7 ± 3.2) was compared with the rutin 30, 60 & 120 mg/kg and Fluo 20 mg/kg (25.3 ± 4.6, 27.6 ± 5.6, 38.4 ± 7.0, and 38.0 ± 6.6) (at p = 0.980, 0.999, 0.801, and 0.826 respectively.

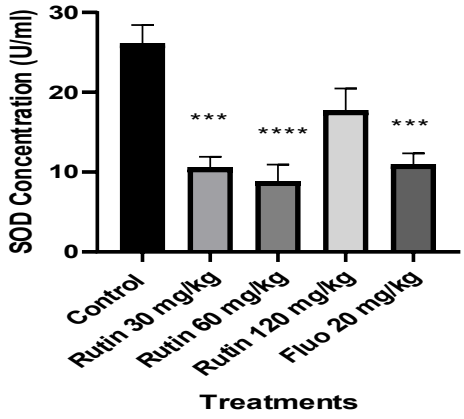

Fig. 4: Effect of rutin supplement on serum superoxide dismutase concentration in mice subjected to chronic unpredictable mild stress. ***- Significant compared to the control at p<0.0005, **** significant compared to the control at p<0.0001 (n = 5), GraphPad Prism 8.0.2. Fluo: Fluoxetine, SOD: Superoxide dismutase

DISCUSSION

Chronic mild stress is a model of depression that exposes mice chronically to constant stressors resulting in the development of behavioural changes which causes decreased response to reward: This can only be restored to normal level by chronic treatment with antidepressant (Willner 2017). Thus, this chronic exposure to stress leads to behavioural and pathological alterations in mice that are analogous to those observed in depressed patients (Luo et al. 2008). This study was conducted to evaluate the possible anti-depressant effect of rutin supplementation in chronic mild stress model of depression using the tail suspension test and the activities of anti-oxidants (SOD, MDA and catalase) as indices of oxidative stress.

Behavioural despair was found to be similar across the groups at the first TST because the first TST was conducted before the application of chronic mild stressors on the naïve animals. This is in accordance with the study of Yusha'u et al. (2019) who reported no difference in the immobility time (behavioural despair) between the normal saline group compared with the other groups in the first TST before exposure to chronic mild stress.

With the second TST, we reconfirmed similar behavioural despair across the groups as was also reported by Wang et al. (2008), Yadav et al. (2016) and Yusha'u et al. (2021) using forced swimming test and tail suspension test in mice and in rats, respectively. This might likely be due to lack of treatment at this stage. However, in TST2 we observed significant increase in behavioural despair in the control, rutin 120 and Flu 20 mg/kg groups when compared with their performances in TST 1. This might be due to the exposure of the mice to chronic mild stressors for two weeks without any treatment.

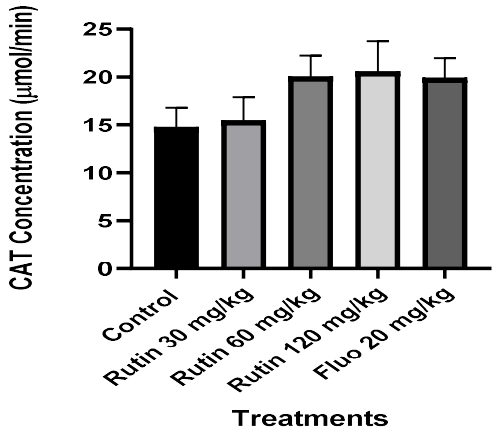

Fig. 5: Effect of rutin supplement on serum catalase concentration in mice subjected to chronic unpredictable mild stress. P > 0.05 (n = 5), GraphPad Prism 8.0.2. Fluo: Fluoxetine, CAT: Catalase

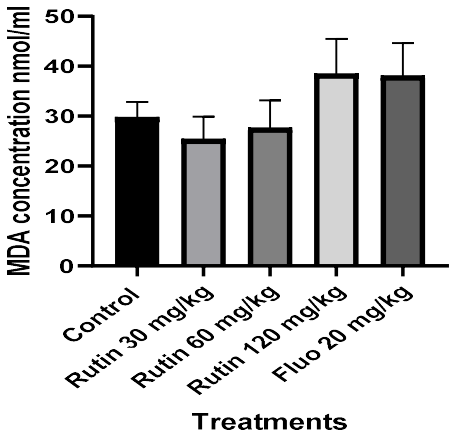

Fig. 6: Effect of rutin supplement on malondialdehyde concentration in mice subjected to chronic unpredictable mild stress. P > 0.05 (n = 5), GraphPad Prism 8.0.2. Fluo: Fluoxetine, MDA: Malondialdehyde

Rutin supplementation of 120 mg/kg showed an antidepressant-like effect by reversing the effect of chronic unpredictable mild stressors, hence, decreasing the immobility time (behavioural despair) at the last TST of the test. This reaffirms the antidepressant-like effect of chronic administration of rutin as demonstrated in studies by Sharma et al. (2009) and Yusha'u et al. (2017). Possibly, the mechanism via which rutin exerted this effect might be independent of its central antioxidant effect as observed in this study.

In contrast with the findings of Su et al. (2014), the lower doses of rutin supplement decreased the serum SOD concentration in our model of depression which differs from the model used by Su et al. (2014). In view of this, an increase or decrease in serum concentration might be model dependent. However, Kodydková et al. (2009) suggested that increased SOD activity is in response to the increased reactive oxygen species production. The observation made in SOD activity might be due to the time taken (time frame) for the research or may be dependent on the types of stressors used. Moreover, rutin supplementation of 120 mg/kg reversed the effect of chronic unpredictable mild stressors by increasing the serum SOD concentration.

Serum catalase concentration did not differ significantly in this study, although there was a biological response elicited by the elevated levels of catalase. However, a similar study by Su et al. (2014) showed an increased catalase activity in mice treated with rutin supplement at different doses. Similarly, a study by Merghem et al. (2019) revealed that catalase activity is increased in mice treated with a flavonoid (*Ruta montana*) extract at a dose of 300 mg/kg and 100 mg/kg. In contrast to our findings, a study by Rai et al. (2019), reported a decreased catalase activity in mice subjected to chronic unpredictable mild stress and treated with catechin 50 mg/kg. Rutin supplement therefore, have positive effects on the overall increase of serum catalase activity rather than decrease, which points at its possible effects on the reduction of oxidative damage.

It is paramount to note the controversy existing as regards the change in SOD and catalase activities in depressive patients. Disruptions in SOD activity usually exist in depressive patients, but the findings are still inconsistent (Paula et al. 2020). Declined SOD activity has been found in major depressive disorder (MDD) patients (Rybka et al. 2013). Increased SOD and catalase activities in depressive patients have also been reported (Kodydková et al. 2009). Furthermore, a study showed that serum SOD and catalase activities were significantly higher in the acute phase of MDD patients, showing a possibility that increased activities of both antioxidant enzymes might be indicators of acute depressive episodes in MDD (Tsai and Huang 2016).

The MDA serum concentration showed increased lipid peroxidation on chronic administration of 120 mg/kg rutin supplement, although not significantly as shown in our result. In line with this, changes in MDA (a lipid peroxidation marker) level in major depressive disorder have been reported in some clinical studies

(Bajpai et al. 2014). In animal models of chronic induced stress, enhanced oxidative stress is mainly expressed as increase in the product of lipid peroxidation (MDA level) (Duda et al., 2016). A study by Noldner and Schotz (2002) however, indicated that rutin is essential for the antidepressant activity of *Hypericum perforatum* extract, a plant used in many countries for the treatment of mild to moderate forms of depression (Linde and Knüppel 2005).

Conclusion

From the results obtained, it can be concluded that rutin supplement have the potentials of positively reversing behavioural despair in mice. Nonetheless, there is need for further studies on the changes of the antioxidant enzymes in depressed animal models for better understanding of the rutin possible antidepressant mechanism of action via the oxidative stress theory of depression. It is also important to note the limitation of this study for only determining depressive phenotypes. Hence, the need for further studies on the molecular biomarkers of depression including serotonin, dopamine and norepinephrine.

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Conflict of Interest

None declared.

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Authors' Contribution

YY and UMA conceptualized the design of the study. AMK, SI and MDM carried out the laboratory work. YY and UMA supervised the study. AMK prepared the first draft of the manuscript. YY and UMA improved the drafted manuscript, and helped in the statistical analyses. All authors contributed and agreed to the final version of the manuscript.

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