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Original Article

Study of the Effects of *Cordia myxa* Fruit Extract on Induced Animal Model of Depression in Male Rats

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Abstract

Depression is one of the most common mental illnesses. Herbal medications such as ginseng and peony have recently gained popularity in treating depression due to their safety, efficacy, and cost-effectiveness. Therefore, the present study aimed to evaluate the actions of Cordia myxa **(***C. myxa*) fruit extract on the model of chronic unpredictable mild stress (CUMS) and antioxidant enzymes system in male rats' brains. Sixty male rats were divided into six groups (n=10). Group 1 (control) was neither exposed to CUMS nor received any treatment, while group 2 was exposed to CUMS for 24 days with normal saline treatment for 14 days, group 3 was exposed to CUMS for 24 days and received 10 mg/kg fluoxetine daily on day 10 for 14 days, and group 4, 5, and 6 were exposed to CUMS for 24 days and received *C. myxa* extract (125, 250, and 500 mg/kg, respectively) on day 10 for 14 days. The antidepressant effect of fluoxetine and *C. myxa* extract was evaluated using a forced swim test (FST). At the end of the experiments, animals were sacrificed by decapitation; and antioxidant enzyme levels, catalase (CAT), and superoxide dismutase (SOD) were determined by enzyme-linked immunosorbent assays kits (ELISA) on rats' brain tissues. All groups subjected to CUMS showed a significant rise in duration of immobility on the tenth day compared to day zero. The CUMS showed a decrease in antioxidant enzyme levels, and groups treated with extract showed significant rise in enzyme levels (SOD and CAT) compared to group 2. According to this recent study, *C. myxa* may have an antidepressant-like action.

Keywords: Chronic unpredictable mild stress, Depression, *Cordia myxa*, Superoxide dismutase, Catalase, Antioxidant enzyme system

1. Introduction

Depression is a serious and prevalent mental illness that affects around 350 million individuals of all ages worldwide. It is the fourth major cause of disability (1). Approximately 1 in every 5 women and 1 in every 8 men will have a significant depressive episode at some point in their lives (2). Sadness, loss of interest or pleasure, feelings of guilt or low self-worth, interrupted sleep or food consumption, weariness, and impaired concentration are all symptoms of depressive disorder. Depression, in its most extreme form, can lead to suicide and an increased risk of death. It is frequently a long-term condition (3). Even in its mildest form, it has a negative impact on people's quality of life and performance (4).

Because depression is a diverse illness with complicated phenomena and numerous probable aetiologies, the specific mechanism through which it develops is yet unknown. Significant variations in the hypothalamic– hypophyseal–adrenal axis and inadequate levels of monoamine neurotransmitters appear to be caused by genetic, epigenetic, and environmental factors. Monoamine levels are essential in the pathophysiology of depression, as seen by lower dopamine levels in

patients with significant depression and decreased cerebrospinal fluid monoamine metabolite levels in individuals who tried to commit suicide and displayed depression. Furthermore, in people with depression, activation of the serotonergic system can boost dopamine release. As a result, the serotonergic and dopaminergic systems may interact (5).

Antidepressant drugs are divided into several categories, including TCAs, MAOIs, SSRIs, SNRIs, NDRI. Selective NRIs, atypical antidepressants, and others.

For children and adolescents with depression, selective serotonin reuptake inhibitors are the first-line antidepressants. The Food and Drug Administration has authorized fluoxetine for children aged 8 years and older. Escitalopram was authorized for those aged 12 years and older. Fluoxetine is the drug with the most evidence for usage in children with depression. While sertraline and escitalopram are considered as first-line antidepressants for the initial treatment of major depression in adults (6).

Herbal medications have received much attention because of their potential to treat mental illnesses. There has been an increase in their usage in depression therapies because of their high effectiveness, safety, and low cost (5). Therefore, the current study aimed to evaluate the antidepressant effects of the ethanolic extract of the Cordia myxa fruit (*C. myxa*) in male rats and to define the relationship between the depression and antioxidant enzyme system in male rats, especially in brain tissue and the effects of fruit extract of *C. myxa* on these mediators.

2. Materials and Methods

2.1. Animals

This study included 60 male adult albino rats. Their weights ranged from 185 to 245 g. The rats were maintained at the Animal House of medical college, University of Babylon at a temperature of approximately 25 °C with a 14/10 h light-dark cycle and free access to water and food. They were kept in 12 cages, and each cage contained five rats. The animals have been randomly separated into six groups following three weeks of adaptation period as the trial planning.

2.2. Plant preparation

The dried fruit of *C. myxa* was purchased from Diyala, Iraq, in July 2021. According to document no. 3,115 on 18 November 2021, the plant was authorized as *C. myxa* with the aid of the University of Al-Qasim green/ faculty of agriculture/medicinal plant department. The dried fruits of *C. myxa* were crushed into powder with a mechanical grinder, then kept at 4 °C.

Powder of fruits was extracted using maceration with ethanol. The powdered fruits were macerated in 70% (v/v) ethanol (5:10 w/v) at room temperature for 72 h. Then, it was shaken for 4 h. The mixture was filtered with Buchner funnel and Whatman filter paper No.1. The resulting extract is concentrated under pressure using a rotary evaporator in 40 °C. The extract was stored in the refrigerator. For tests, the extract was suspended with normal saline. The solution was prepared at a concentration of 200 mg/1ml, and normal saline was taken as a control (7).

2.3. General Experimental Procedure

1. On days 0, 10, and 25, each animal underwent a behavioral forced swim test (FST).

2. The animals in group 1 (control) did not receive any medication and were not stressed.

3. For 24 days, all the animals in groups 2, 3, 4, 5, and 6 were exposed to chronic unpredictable mild stress (CUMS).

4. Each rat in group two was given 0.2 milliliters of normal saline by oral gavage for 14 days starting from the tenth day of CUMS without any treatment.

5. For 14 days, each animal in group 3 was given fluoxetine treatment 10 mg/kg through oral gavage.

6. For 14 days, each rat in groups 4, 5, and 6 was given a daily dose of *C. myxa* fruit extract of 125 mg/kg, 250 mg/kg, and 500 mg/kg, respectively, by oral gavage (8).

2.4. Chronic Unpredictable Mild Stress (CUMS)

The Katz method, with minor modifications, was used to induce chronic stress. This protocol was chosen since it has previously been used to generate anxiety in

animals. The animals under stress were subject to CUMS, as shown in table 1 (9).

Table 1. Chronic unpredictable mild stress (CUMS) protocol

Day	CUMS protocol
1	15 min forced swim (20 ℃), crowded cage
2	12 h cage tilting (45 ℃), 1 h restraint
3	reversal of the light/dark cycle
4	12 h wet bedding, crowded cage
5	24 h food deprivation, 1 h cage rotation
6	12 h cage tilting (45 ℃), tail pinch
7	1 h cage rotation, 1 h cold room isolation
8	reversal of the light/dark cycle, tail pinch
9	24 h water and food deprivation
10	12 h cage tilting (45 ℃), 15 min forced swim (20 ℃)
11	1 h restraint , 24 h water deprivation
12	reversal of the light/dark cycle, 24 h food deprivation
13	12 h cage tilting (45 ℃)
14	24 h water deprivation, 1 h restraint
15	12 h wet bedding, 12 h cage tilting (45 ℃)
16	1 h cage rotation, reversal of the light/dark cycle
17	1 h restraint, crowded cage
18	12 h wet bedding, 12 h cage tilting (45 ℃)
19	reversal of the light/dark cycle, tail pinch
20	15 min forced swim (20 ℃), 1 h cold room isolation
21	1 h cage rotation, crowded cage
22	reversal of the light/dark cycle , tail pinch
23	24 h food and water deprivation
24	12 h cage tilting (45 ℃), crowded cage

2.5. Forced Swimming Test (FST)

A glass container was used in this experiment as designed by Cryan, Valentino (10) with dimensions of 30×30×70 centimeters.

Individual rats were forced to swim in a cylindrical glass container filled to a depth of 0.3 meters with tap water (25 ℃). Individually, the laboratory animals were permitted to swim for 5 min. An observer who was blind to the animal groups recorded and evaluated the test sessions. At 0, 10, and 25 days, the duration of immobility during the first 5 min of the swimming exercise was recorded. After each rat testing, the water in the container was replaced. A depressed phenotype is defined as spending more time stationary or immobile and less time actively swimming or struggling in response to a threat or challenge.

2.6. Tissue Samples Preparations

The animals were decapitated one day after the final treatment on the twenty-fifth day. The brains were extracted after dissecting a skull from the foramen magnum posteriorly. The brain was carefully removed from the skull after the olfactory pulps and cerebellum were severed. The mid and forebrain were dissected and weighted after being washed in phosphate buffer saline solution and were maintained in a sanitized eppendorf tube, then deep-frozen at -20 ℃ on dry ice.

2.7. Rat Superoxide Dismutase (SOD) ELISA Kit

The principle of this kit was that Rat SOD antibody had been pre-coated on the plate. The SOD from the sample was introduced to the wells, where it was bound to antibodies. The biotinylated Rat SOD antibody was then added to the sample and bound to SOD. Streptavidin-HRP then bound the biotinylated SOD antibody. All unbound Streptavidin-HRP was washed away during the washing process following the incubation. The substrate solution was then added, and the color developed in direct proportion to the quantity of Rat SOD present. The process may be stopped by adding an acidic stop solution and monitoring the absorbance at 450 nm.

2.8. Rat Catalase (CAT) ELISA Kit

The principle of this kit was that Rat CAT antibody has been pre-coated on the plate. The CAT from the sample was introduced to the wells, where it bound to antibodies. The biotinylated Rat CAT antibody was then added to the sample and bound to CAT. The biotinylated CAT antibody was then bound by Streptavidin-HRP. All unbound Streptavidin-HRP has washed away during the washing process following the incubation. The substrate solution was then added, and the color developed in direct proportion to the quantity of Rat CAT present. The process may be stopped by adding an acidic stop solution and monitoring the absorbance at 450 nm.

2.9. Analysis of Statistics

The data were presented as a mean with a standard error of the mean (SEM). The post-hoc test and one-way analysis of variance (ANOVA) were used in the statistical analysis (LSD and Bonferoni). A *P*-value of less than 0.05 was considered statistically significant. The SPSS software (version 23) for Windows ® 10 was used for statistical analysis.

3. Results

3.1. Forced Swimming Test

No significant differences were seen in immobility duration on the days 10th and 25th in comparison to day zero in group 1 (control, not given any drug and unexposed to CUMS), whereas in group 2 (not given any treatment and subject CUMS-protocol), the mean of immobility duration on day 25th showed significant increases (P<0.05) as compared to days zero and 10th. Furthermore, the mean of immobility duration on day 10th significantly decreased (P<0.05) in comparison to day zero and in group 3 (given doses of 10 mg/kg fluoxetine for 14 days), 4 (given plant 125mg/kg *C. myxa* fruit extract for 14 days), 5 (given dose 250 mg/kg *C. myxa* fruit extract 14 days), and 6 (given dose 500mg/kg *C. myxa* fruit extract for 14 days). On the 25th day, the immobility duration in groups 3, 4, 5, and 6 dropped significantly (P<0.05) in comparison to day 10th (Table 2).

On the 25th day, Group 2's mean immobility time rose substantially (P-value less than 0.05) when compared to group 1. However, groups 3, 4, 5, and 6's mean immobility time dropped significantly (P-value less than 0.05) compared to group 2 (Figure 1).

3.2. Brain Antioxidant Enzyme System Results

3.2.1. Superoxide Dismutase Enzyme Concentration (SOD)

The means of SOD concentrations in brain tissue in group 2 considerably dropped (P<0.05) compared to group 1; however, the means of SOD concentrations in brain tissue in groups 4, 5, and 6 significantly rose (P<0.05) compared to group 2. When comparing group 3 to group 2, there was no significant rise in the means of SOD concentrations in brain tissue (P<0.05; Table 3 and Figure 2).

3.2.2. Catalase enzyme concentration (CAT)

The means of CAT concentrations in brain tissue in group 2 considerably reduced (P<0.05) compared to group 1, however, the means of CAT concentrations in brain tissue in groups 4, 5, and 6 significantly rose (P<0.05) compared to group 2. When comparing group 3 to group 2, there was no significant rise in the means of CAT concentrations in brain tissue (P>0.05; see Table 3 and Figure 3).

Table 2. Comparison in immobility time ± SEM between groups on days 0, 10, and 25

FST	Group 1 (secs)	Group 2 (secs)	Group 3 (secs)	Group 4 (secs)	Group 5 (secs)	Group 6 (secs)
Day 0	47.9000±1.52352	47.5000±1.43952	46.4000±4.48504	48.4000±3.23934	44.4000±1.65462	42.5000±2.65937
Day 10	48.6000±1.82087	64.8000±4.07104*	66.0000±4.17665*	67.7000±4.86952*	63.5000±3.14201*	64.0000±4.98219*
Day 25	47.7000±1.26535	67.0000±5.55578∞	38.9000±2.19823α	49.2000±2.25979α	38.1000±1.87646α	48.9000±1.13969α

* = Significant increases (P-*value* less than 0.05) in comparison to day zero
∞ = Significant increases (P-*value* less than 0.05) in comparison to day zero
α = significant decreases (P-*value* less than 0.05) in comparison to day zero
Group 1 (control group, not given any treatments and not subjected to CUMS-protocol), group 2 (not given any treatments and subjected to CUMS-protocol), group 3 (was given doses of 10mg/kg fluoxetine for 14 days), group 4 (was given 125 mg/kg *C. myxa* fruit extract for 14 days), group 5 (was given 250 mg/kg *C. myxa* fruit extract for 14 days), group 6 (was given 500 mg/kg *C. myxa* fruit extract for 14 days), the number of laboratory rats was ten per one group.

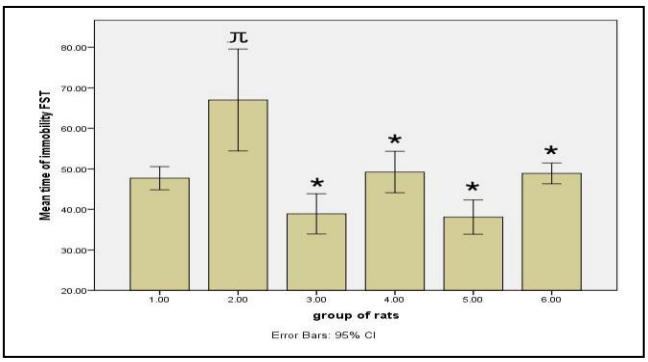

Figure 1. Immobility duration means by forced swim test by day twenty-five
*= significant drop (P-*value* less than 0.05) compared to group 2. π = significant rise (P-*value* less than 0.05) compared to group 1

Table 3. ELISA results of IL-6, SOD, and CAT concentrations in brain tissue

Concentrations	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
SOD (ng/ml)	5.0756±0.31169	3.7913±0.54339$^{£}$	4.4937±0.51124	5.3509±0.24535^{β}	5.8615±0.35779^{β}	5.7067±0.33619^{β}
CAT (ng/ml)	34.7461±1.90833	27.2349±1.72104$^{£}$	33.8669±2.01764	36.8036±2.25583^{β}	40.3442±2.66987^{β}	37.2042±1.83820^{β}

π = Significant rise (*P-value* less than 0.05) compared to group 1
β = Significant rise (*P-value* less than 0.05) compared to group 2
£ = Significant drop (*P-value* less than 0.05) compared to group 1
Group 1 (control group, not given any treatments and not subjected to CUMS-protocol), group 2 (not given any treatments and subjected to CUMS-protocol), group 3 (was given doses of 10 mg/kg fluoxetine for 14 days), group 4 (was given 125 mg/kg *C. myxa* fruit extract for 14 days), group 5 (was given 250 mg/kg *C. myxa* fruit extract for 14 days), group 6 (was given 500 mg/kg *C. myxa* fruit extract for 14 days), the number of laboratory rats was ten per one group.

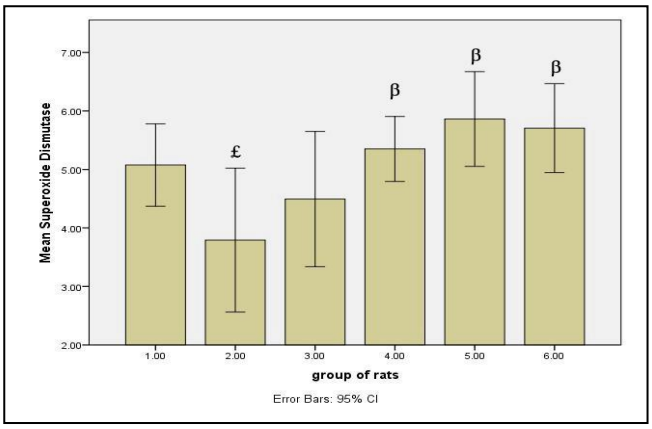

Figure 2. Means ± SEM of the Superoxide dismutase enzyme concentrations (ng/ml) in brain tissue
β = Significant rise (*P-value* less than 0.05) compared to group 2
£ = Significant drop (*P-value* less than 0.05) compared to group 1
Group 1 (control group, not given any treatments and not subjected to CUMS-protocol), group 2 (not given any treatments and subject to CUMS-protocol), group 3 (was given doses of 10 mg/kg fluoxetine for 14 days), group 4 (was given 125 mg/kg *C. myxa* fruit extract for 14 days), group 5 (was given 250 mg/kg *C. myxa* fruit extract for 14 days), group 6 (was given 500 mg/kg *C. myxa* fruit extract for 14 days), the number of laboratory rats was ten per one group.

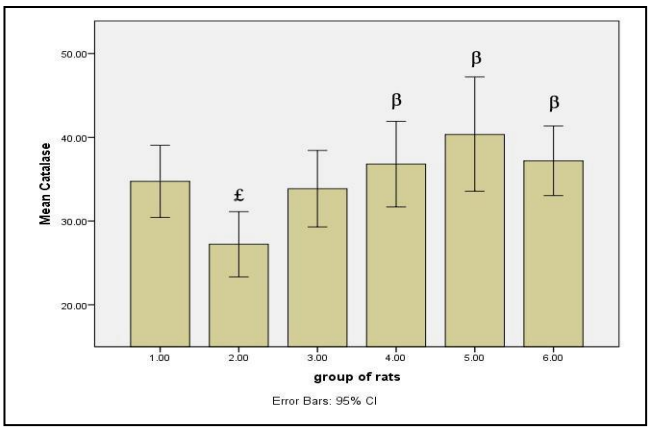

Figure 3. Means ± SEM of the Catalase enzyme concentrations (ng/ml) in brain tissue
β = Significant rise (*P-value* less than 0.05) compared to group 2
£ = Significant drop (*P-value* less than 0.05) compared to group 1
Group 1 (control group, not given any treatments and not subjected to CUMS-protocol), group 2 (not given any treatments and subjected to CUMS-protocol), group 3 (was given doses of 10 mg/kg fluoxetine for 14 days), group 4 (was given 125 mg/kg *C. myxa* fruit extract for 14 days), group 5 (was given 250 mg/kg *C. myxa* fruit extract for 14 days), group 6 (was given 500 mg/kg *C. myxa* fruit extract for 14 days), the number of laboratory rats was ten per one group.

4. Discussion

Depression is a debilitating disorder that affects a significant portion of the population. This illness is highly heterogeneous and can be caused by a variety of reasons. This necessitates the use of animal models to verify the relationship between stress and depression, which should exclude animals that are tolerant to stress in order to simulate the real-life scenario in humans. Animal models created with maternal deprivation and chronic unpredictable stress exposure, are commonly used models that simulate stress experienced by humans during childhood and maturity (11). Several studies have found that CUMS can cause long-term behavioral changes that are similar to clinical depressive symptoms. This study looked into the antidepressant effect of *C. myxa* fruit extract against CUMS-induced depression using the behavioral test the FST, as well as the impact on antioxidant enzyme systems (e.g., SOD and CAT). To the best of our knowledge, this is the first study correlating the action of C. myxa fruit extract on stressed male rats.

The immobility duration was not significantly different between animals from all groups on day 0 (baseline) in this study. Compared to baseline (day 0), there was a substantial rise in immobility duration on FST for all groups of rats exposed to CUMS after 10 days of unpredictable stress. This indicates that these animals have evolved a depression model. These results are in line with previous studies, which found that rats acquired a behavioral model of depression after being exposed to unpredictable stress procedures utilizing various stressors, as seen by increased immobility periods in behavioral despair tests, which indicates "behavioral despair" and hence may reflect "recurrent thoughts of death," a common hallmark of severe depression in humans (12).

Clinical and laboratory studies show that chronic stress causes oxidative stress, which causes an imbalance in the body's free radical generation and antioxidative system. Reactive oxygen species (ROS)-based oxidative stress may damage proteins, lipids, and DNA and cause chromatin remodeling by interfering with histone acetylation and deacetylation (13).

Compared to group 1, group 2 (untreated and subjected to CUMS) had depressive behavior on day 25, as evidenced by a significant rise in immobility duration on FST and these findings matched those of prior research (14). Compared to group 2, there was a significant decrease in immobility durations during FST in groups 3, 4, 5, and 6. These findings showed the antidepressant properties of fluoxetine and *C. myxa* fruit extracts.

Stress is a pathogenic component that promotes neuroinflammation and neuronal degeneration (15). At the end of the experiment, the animals were decapitated, their brains were removed from the skull, and homogenized to evaluate the antioxidant enzyme system concentrations (e.g., SOD and CAT).

One of the primary causes of neurodegeneration and the progression of depression and its symptoms is oxidative stress. Chronic stress causes the activation of various proapoptotic proteins, the production of excessive ROS, and necrosis, resulting in neurodegeneration, which plays an integral part in the pathophysiology of depression. The brain homogenate's antioxidant capacity is a sign of oxidative stress (16).

In group 2 (exposed to CUMS and received normal saline for 14 days), there was a significant decrease in SOD and CAT levels as compared to group 1 (control; untreated and unexposed to CUS), which means the development of oxidative stress status and elevated levels of free radicals which are superoxide radicals and hydrogen peroxide radicals due to consumption of enzymes.

Lower antioxidant levels define CUS-induced depression in rats and also reported in previous study that CUS has been shown to elicit depressive and anxiety-like symptoms and lower SOD and GPX levels (17). Overproduction of ROS results from the activation of immune cells by pro-inflammatory cytokines, which increases lipid peroxidation levels. Overproduction of ROS interferes with the structure

and ratio of polyunsaturated fatty acids, causing a loss of fluidity in the biological membrane. Consequently, the biological membranes trigger cytokine production due to these changes. On the other hand, increased lipid peroxidation activates phospholipase A2, which alters receptor activities in cell membranes, activates immune cells, and causes T cells to secrete ILs, potentially increasing lipid peroxidation (18). Previous studies show that superoxide generation was enhanced in all brain areas studied in a CUS rat model, which mimics some of the symptoms of a severe depressive episode in humans (19).

Increased monoamine metabolism could be a factor in increased ROS production in depression. Sanacora, Treccani (20), noted that depression is associated with increased glutamatergic transmission. In the presence of high glutamate levels, can cause excitotoxicity by allowing pathologically high levels of calcium to enter the cell, which can stimulate the production of ROS.

Excessive oxidative stress lowers catalase levels, resulting in a buildup of harmful hydrogen peroxide and ROS in the body. Reduced CAT concentrations mean that significant amount of H_2O_2 is accessible to react with transition metals and release the radical hydroxyl (the most dangerous radical), resulting in increased lipid peroxidation and, as a consequence, neuronal damage (21).

Reactive oxygen species can be antibacterial, killing antigen-bearing cells by damaging lipid membranes and protein structures. However, oxidative damage is not confined to microbial targets, and severe damage to host tissue can occur. Reactive oxygen species produced by active phagocytes can damage DNA bases and cause strand breaks in surrounding cells, leading some to believe that hydroxyl radicals and peroxynitrite produced during inflammation are the main contributors to DNA oxidation (22).

In our study, fluoxetine improved the behavioral deficits caused by CUS but had no protective benefits against oxidative stress antioxidant enzymes concentration showed no significant differences between group 3 (exposed to CUS and received fluoxetine treatment for 14 days) and group 2 (exposed to CUS and received normal saline treatment for 14 days). This finding support Zhao et al., results because fluoxetine primarily exerts its antidepressant effect by selectively blocking serotonin reuptake (23).

There is limited information on fluoxetine's effect on antioxidant enzymes. While some studies suggest that this antidepressant improves antioxidant capacity in the brain, others claim that it affects the liver antioxidant system in rats (24).

In groups 4, 5, and 6 (exposed to CUS and treated with 125mg/kg, 250mg/kg, and 500mg/kg of *C. myxa* extract, respectively), there was a significant increase in SOD and CAT concentrations as compared to group 1 (control; untreated and unexposed to CUS) that associated to antioxidant properties of the plant constituents. According to these findings *C. myxa* extract have a different mechanism of antidepressant-like activity than fluoxetine.

Increased reactive oxygen generation during depression may result in depletion of endogenous scavenging chemicals, as mentioned before. Our results demonstrate that the plant has antioxidant action, as noted by previous studies. Aljeboury report that flavonoids, one of the most important constituents of *C. myxa* fruits, may have an additive effect on endogenous scavenging compounds. Endogenous antioxidants may benefit from flavonoids as well. When flavonoids are combined with radicals, the flavonoid hydroxyl groups render the radicals inactive (25).

As a result, this study found that *C. myxa* extract reduces stress-induced depletion of antioxidant enzymes and increases antioxidant enzyme levels. This could be linked to the plant's antioxidant, anti-inflammatory, and ability to scavenge free radicals produced during oxidative stress. Despite the small size of the groups in this study, the findings support the oxidative stress hypothesis that plays a role in MDD pathogenesis. Increased antioxidant enzyme levels and their relationship with treatment outcome in depressed rats suggest a link between oxidative stress, depression,

and treatment. The current study did not look at monoamine levels or changes in protein expression in the brain; however, future studies may investigate these features. Future research may look at more fundamental processes in addition to the antioxidant axis.

5. Conclusion

Based on the findings of the current study, prolonged unpredictable stress causes depressive-like behavior in rats, as well as lower levels of brain antioxidant enzymes, including CAT and SOD, which are reversed by *C. myxa* treatments in treated groups, in addition to its antidepressant-like action as shown by behavioral tests. In addition, it was revealed that CAT and SOD measured were not significantly increased during antidepressant treatment (fluoxetine) in animals with major depressive disorder. Lastly, the development of oxidative stress plays a vital role in the pathogenesis of depression.

Authors' Contribution

Study concept and design: S. M. S.
Acquisition of data: Y. J. H.
Analysis and interpretation of data: Y. J. H.
Drafting of the manuscript: A. J. M.
Critical revision of the manuscript for important intellectual content: S. M. S.
Statistical analysis: A. J. M.
Administrative, technical, and material support: Y. J. H.

Ethics

The study was conducted in accordance with University of Babylon, Babel, Iraq ethics committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

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