

Effects of *Withania Somnifera* on Model of Chronic Stress and Brain Toll-Like Receptors 2 and 4 Gene Expression in Male Rats

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Abstract

Background: Depression is one of the prevalent psychiatric disease, in which social function impairment and large suicide rates are very common. Toll-Like receptors have a essential role in the pathophysiology of depression. One of the major concern of current depression treatment are side effects, toxicity, and delayed onset of action. Recently, herbal drugs, e.g. *Withania Somnifera*, are gaining a great deal of attention in depression treatment because of their safety, efficacy, and cost-effectiveness.

Objective: To establish a chronic unpredictable stress(CUS) model in male rats, to investigate the antidepressant effects of the *W. Somnifera* roots extract and to define the relationship between the stress and gene expression of TLR 2/4 in the brain of the male rats and the effects of an extract of the roots of *W. Somnifera* on that gene expression.

Material and methods: Sixty rats have been involved in this experiment. The animals were randomly distributed into 6 groups, each group with 10 rates. Group 1 (control group, untreated and unexposed to CUS), group 2 (untreated and exposed to CUS), group 3 (treated with 10mg/kg fluoxetine for 14 days), group 4 (treated with 50mg/kg *W.Somnifera* extract for 14 days), group 5 (treated with 100mg/kg *W.Somnifera* extract for 14 days), group 6 (treated with 150mg/kg *W.Somnifera* extract for 14 days), no.of rats=10 for each group.

The antidepressant effect of fluoxetine and *w. somnifera* was evaluated by using forced swimming test (FST) and the gene expression of toll like receptor 2 and 4 was determined by RT_PCR.. FST was done for each animal on day 0, 10 and 25. Each animal of groups 2, 3, 4, 5, and 6 exposed to CUS for 24 days.

Keywords: Chronic unpredictable stress; Depression; withania somnifera; Toll-like receptor 2; Toll-like receptor 4; gene expression

Results: All animals which were exposed to CUS protocol showed a significant ($P < 0.05$) increase in duration of immobility when compared with the baseline (at day 0), at the end of day 10. In CUS group the immobility time was statistically significantly larger than the baseline ($P < 0.05$). Stressed animals have shown a statistically significant decrease in the immobility time when compared with CUS group (group2) (P value < 0.05). Fluoxetine significantly reduced the duration of immobility ($P < 0.05$) when compared to CUS group.

In terms of gene expression and in group 2, the mean of fold changes of TLR2/4 mRNA level was significantly increased (P -value < 0.05) as compared with group 1. While in groups 3, 4, 5 and 6 the means of the fold changes of TLR2/4 mRNA level were significantly decreased (P -value < 0.05) as compared with group 2.

Conclusions: The CUS-induced increases in the TLR2/4 mRNA levels. According to the present study *w.somnifera* may have the antidepressant-like activity and reverse the effect of stress-induced TLR-2/4 upregulation.

INTRODUCTION

Depression is one of the prevalent psychiatric disease, in which social function impairment and high suicide rates are very common. Major depressive episode is defined as a period of 2 weeks or longer during which there is either a depressed mood or loss of interest or pleasure (i.e., anhedonia) and at least 4 other symptoms that reflect a change in a person's baseline activity, e.g. fatigue, suicide, change in sleep, or change in activity (e.g., psychomotor agitation or retardation) (1). In the United States, the lifetime incidence of depression is approximately 16.2% and it is predicted to be one of the most serious global health concerns during the 21st century.

In most countries, the incidence rate of major depressive disorder ranges from 8% to 12%. Globally, more than 264 million people of all population ages have depression, and it appears that women are affected more than men. MDD, further have a substantial burden on disability among other mental and behavioral disorders. depression can lead to suicide and it is estimated that a huge number of people (800,000) die due to committing suicide every year. Among 15-29-year-olds population, suicide is the second leading cause of death.

Despite the high incidence rate of depression, the mechanism of depression remains unclear. One of the mechanisms which could explain the pathophysiology of depression is the low levels of monoamine neurotransmitters, especially dopamine (DA), serotonin (5-HT), and norepinephrine (NE) (2). Another important mechanism, which could explain the mechanism of depression is the abnormalities of Toll-like receptors (TLRs) (3). Toll-like receptors (TLRs) are pattern-recognition receptors family of innate immunity, which have a unique molecular signature of microbes, called pathogen-associated molecular patterns (PAMPs). The first line of host innate is the activation of TLRs. Activation of these receptors by their respective PAMPs

can lead to proinflammatory cytokine cascades and innate and adaptive immune responses induction (4).

Treatment of depression includes several medication classes. These are tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibition (SNRI), monoamine oxidase inhibitors MAOIs, Norepinephrine reuptake inhibitor with serotonin receptors antagonism (NRISA) such as maprotiline, Noradrenergic α 2-receptor antagonist with specific serotonergic receptors-2 and-3 antagonism (NASSA) (5). One of the major concern of current depression treatment are side effects, toxicity, and delayed onset of action. Recently, herbal drugs are gaining a great deal of attention in depression treatment because of their safety, efficacy, and cost-effectiveness.

W. Somnifera (WS) commonly known as 'Ashwagandha' is a further example of herbal medicine, which has a wide ray of uses, e.g. depression. Additionally, WS is used in the management of neurological conditions such as anxiety, cognitive disorders, senile dementia, and neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. It was shown that the neuroprotective activity of *W.somnifera* root extract may be related to the of one of its constituents called glycowithanolides and their lipid peroxidation inhibitory activity (6). Extracts from WS extract has shown to improve motor function and decrease mortality and DNA fragmentation in the brain of a stroke model (7)

Hence, the aim of this study was to evaluate the effects of *W. Somnifera* on the model of chronic unpredictable stress (CUS) and brain TLR 2/4 gene expression in male rats.

MATERIALS AND METHODS:

Animals

Sixty male, adult albino rats were used in this experiment. Their weights were 150-250 g. The rats were kept in the

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animal house of the College of Medicine/ University of Babylon and kept on 25 °C and 14 h light and 10 h dark cycle with water and food and libitum. After two weeks of adaptation, the animals were randomly divided into groups according to the experiment protocol.

Plant preparation

The dried root of *W. somnifera* was purchased from Lamar Natural Pvt. Ltd., Mumbai, India in July 2019. The plant was approved to be *W. Somnifera* with the help of the college of agriculture /Medicinal Plant Department, the University of Babylon according to document no.3737 on 16/9/2019. The dried root of *W. Somnifera* sample (1 kg) was minced and ground with a mechanical grinder into powder and stored at 4 °C. Powdered *W. somnifera* sample (50 g) was extracted with 500 ml of 80% ethanol for 5–10 h using soxhlet extraction apparatus, at 60 °C (8). The obtained extract was filtered and concentrated to dryness with subsequent evaporation of alcoholic content by using a microwave oven. The dried extract (1g) was dissolved in 20 ml of distilled water and the final product stored in a concentration of 50 mg in each ml.

General Experimental Procedure

1. FST was conducted to each animal on day 0, 10 and 25
2. The animals of group 1 (control) received no treatment and did not exposed to stress.
3. Each animal of groups 2, 3, 4, 5, and 6 exposed to CUS (as discussed below) for 24 days.
4. Each animal of group 2 received 0.2 ml of distilled water without treatment.
5. Each animal of group 3 received fluoxetine 10mg/kg P.O. for 14 days. (9)
6. Each animal of groups 4, 5 and 6 received daily treatment with *W. Somnifera* extract (50mg/kg, 100mg/kg, and 150mg/kg respectively) for 14 days. (10)

Chronic unpredictable stress (CUS)

For the induction of chronic stress, the Katz method was used with some modifications. The animals in stress groups were exposed to the CUS protocol as follows: Day 1, 15 min forced swim (20 °C), tail pinch; day 2, 12 h cage tilting (45 °C), 1 h cage rotation; day 3, reversal of the light/dark cycle, day 4, 12 h wet bedding, crowded cage; day 5, 24 h food deprivation, 1 h restraint; day 6, 12 h cage tilting (45 °C), crowded cage; day 7, 24 h water deprivation, 1 h cold room isolation; day 8, reversal of the light/dark cycle, tail pinch; day 9, 1h cage rotation; day 10, 24 h water and food deprivation, 12 h cage tilting (45°C); day 11, 15 min forced swim (20 °C), 1 h restraint; day 12, reversal of the light/dark cycle, 24 h food deprivation; day 13, tail pinch; day 14, 24 h water deprivation, 1 h restraint; day 15, 12 h wet bedding, 12 h cage tilting (45 °C); day 16, 1 h cage rotation, reversal of the light/dark cycle; day 17, 1 h restraint, crowded cage; day 18, 12 h wet bedding, tail pinch; day 19, reversal of the light/dark cycle, 12 h cage tilting (45 °C); day 20, 15 min forced swim (20 °C), 24 h water deprivation; day 21, 1 h cage rotation, crowded cage; day 22, 24 h food deprivation, tail pinch; day 23, 1 h restraint, 12 h wet bedding; day 24, 24 h water and food deprivation, crowded cage.

Forced swimming test

A cylindrical glass box (30cm*30cm*70cm) was made as

Table 1. Comparison in immobility time ± SEM between groups on days 0,10,25

FST	Group 1 (Sec)	Group 2 (Sec)	Group 3 (Sec)	Group 4 (Sec)	Group 5 (Sec)	Group 6 (Sec)
Day0	47.10± 5.01210	33.0000±4.5117	34.5000±4.6025	43.3000±5.6036	43.1000±3.0639	37.2000±3.8493

described by (11). In a cylindrical glass container in which tap water (25 ± 1 °C) to a depth of 30 cm was contained, rats were enforced to swim individually. The animals were individually allowed to swim for 5 min.

Sessions were recorded and scored by an observer, who was blind to the animal's groups. The immobility time of the rats during the first 5 min of swimming sessions was recorded at day 0, 10, and 25.

Tissues samples preparations

On the 25th day, the animals were sacrificed by a decapitation 24 h after the last treatment. The brains were removed after dissection of a skull from foramen magnum posteriorly. Olfactory pulps and cerebellum were cut and the brain was removed gently from the skull. The tissues covered the medial surface of the hippocampus was removed, and the striatum and thalamus was eliminated gently. The hippocampus was clearly visible in a banana-like shape. Using two spatulas, the cortex was held and the hippocampus was roll up. The isolated hippocampus flashed in a liquid nitrogen and kept in an eppendorf contained 500 ml DEPC water and was frozen on dry ice.

Quantitative polymerase chain reaction

Relative RNA expression was quantified using established methods using real time quantitative polymerase chain reaction (qPCR) with the SYBR green reporter dye. Template cDNAs in each sample tested for quantitative expression levels of TLR-2 and TLR-4 genes and housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) and Bio-Rad iQ5 detection system (Bio-Rad, Richmond, USA) were used in RT-PCR.

Statistics analysis

Statistical analysis was carried out by using the 24th edition of Statistical Package of Social Sciences (SPSS v24) statistics for Windows[®] 7. The results were shown as mean ± standard error of the mean (SEM). Statistical analysis was carried out by using a repeated measure ANOVA, one way ANOVA, and test of proportion. Differences were considered statistically significant if the P-value is ≤ 0.05.

RESULTS

Forced swimming test

In group 1 (control group, untreated and unexposed to CUS), there were no significant differences (p value > 0.05) in the immobility time on days 10 and 25 as compared with day 0, while in group 2, (untreated and exposed to CUS) the mean of the immobility time on day 25 significantly increased (p value < 0.05) as compared with days 0 and 10. (Table 1).

Furthermore, in groups 3 (treated with 10mg/kg fluoxetine for 14 days), 4 (treated with 50mg/kg *W. Somnifera* extract for 14 days), 5 (treated with 100mg/kg *W. Somnifera* extract for 14 days), and 6 (treated with 150mg/kg *W. Somnifera* extract for 14 days) the means of the immobility time on day 10 significantly increases (P-value < 0.05) as compared with day 0 (Table 3.1 and Figure 3.1). In groups 3, 4, 5, and 6, the means of the immobility time on day 25 significantly decreased (P-value < 0.05) as compared with day 10 (Table 1)

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		1	4	7	5	9
Day10	43.2000±3.3691 4	39.8000±4.5898 4*	59.6000±5.8522 6*	51.9000±9.9548 4*	53.8000±4.1467 5*	49.3000±3.9918 0*
Day25	44.3000±4.0553 1	47.2000±4.6638 1 [∞]	40.0000±4.3563 5 ^α	31.7000±3.4223 1 ^α	36.7000±3.3699 7 ^α	32.4000±4.0666 7 ^α

* = significantly increased (*P* value <0.05) as compared with day 0.
[∞] = significantly increased (*P* value <0.05) as compared with day 0.
^α = significantly decreased (*P* value <0.05) as compared with day 10.

Group 1 (control group, untreated and unexposed to CUS), group 2 (untreated and exposed to CUS), group 3 (treated with 10mg/kg fluoxetine for 14 days), group 4 (treated with 50mg/kg *W.Somnifera* extract for 14 days), group 5 (treated with 100mg/kg *W.Somnifera* extract for 14 days), group 6 (treated with 150mg/kg *W.Somnifera* extract for 14 days), no.of rats=10 for each group.

While on the day 25, the mean of the immobility time of group 2 significantly increased (*P*-value <0.05) as compared with group1, while in groups 3, 4,5, and 6 the means of the immobility time significantly decreased (*P*-value <0.05) as compared with group 2. In group 6 the mean of the immobility time significantly decreased (*P*-

value <0.05) as compared with group 3 (Figure 1).

Gene expression results
Toll-Like Receptor 2 (TLR2)

In group 2, the mean of the fold changes of TLR2 significantly increased (*P*-value <0.05) as compared with group 1, while in groups 3, 4, 5 and 6 the means of the fold changes of TLR2 significantly decreased (*P*-value <0.05) as compared with group 2. In groups 3 and 4, the means of the fold changes of TLR2 significantly increased (*P*-value <0.05) as compared with group 5. In group 6 the mean of the fold changes of TLR2 significantly decreased (*P*-value <0.05) as compared with group 3 (Figure 2).

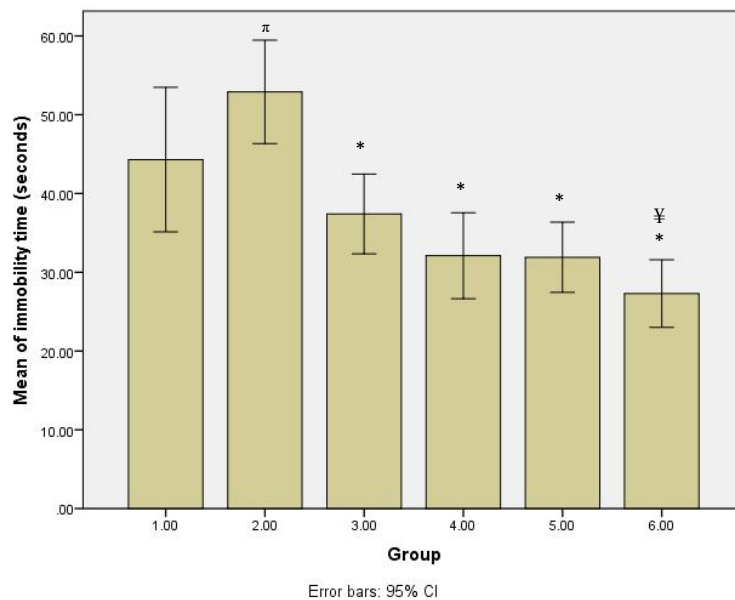
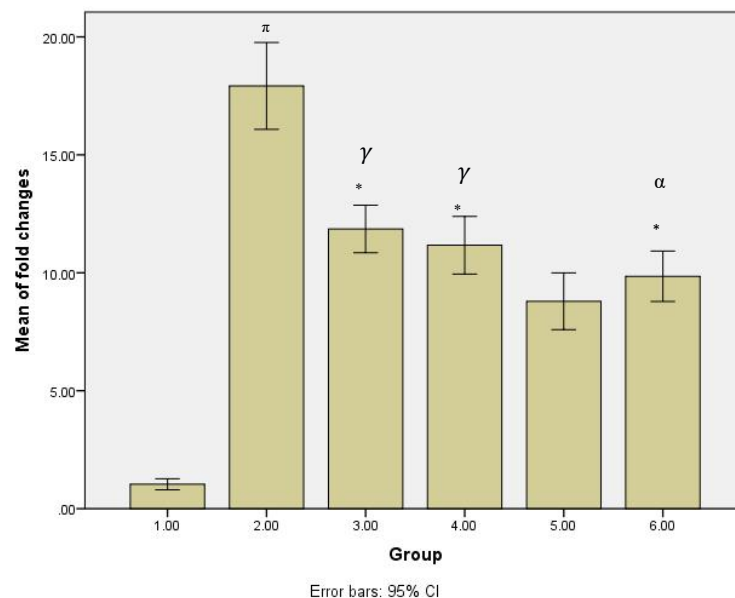


Figure 1. Means ± SEM of the immobility time of forced swimming test on day 25 for all groups.

π = significantly increased (*P* value <0.05) as compared with group 1,
* = significantly decreased (*P* value <0.05) as compared with group 2,
¥ = significantly decreased (*P* <0.05) as compared with group 3,



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Figure 2. means \pm SEM of fold changes of TLR2.

π = significantly increased ($P < 0.05$) as compared with group 1,
 *= significantly decreased ($P < 0.05$) as compared with group 2,
 α = significantly decreased ($P < 0.05$) as compared with group 3,
 γ = significantly increase ($P < 0.05$) as compared with group 5.

Toll-Like Receptor 4

In group 2, the mean of the fold changes of TLR4 significantly increased (P -value < 0.05) as compared with group 1. In groups 3,4, 5, and 6 the means of the fold changes of TLR4 significantly decreased (P -value < 0.05) as compared with group 2.

In group 3, the mean of the fold changes of TLR4

significantly increased (P -value < 0.05) as compared with group 5. In groups 3 and 4 the means of the fold changes of TLR4 significantly increased (P -value < 0.05) as compared with group 5. In group 6 the mean of the fold changes of TLR4 significantly decreased (P -value < 0.05) as compared with group 4 (Figure3).

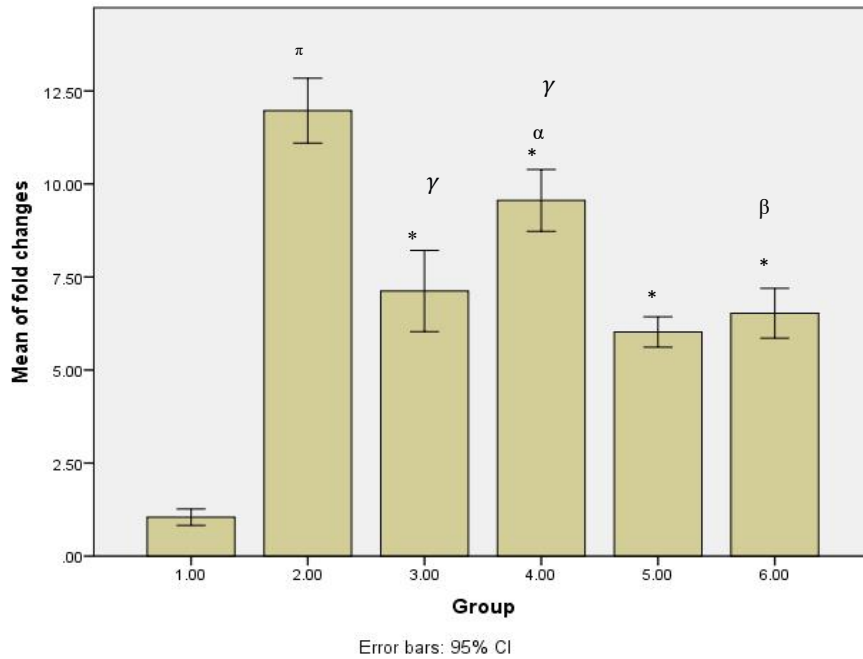
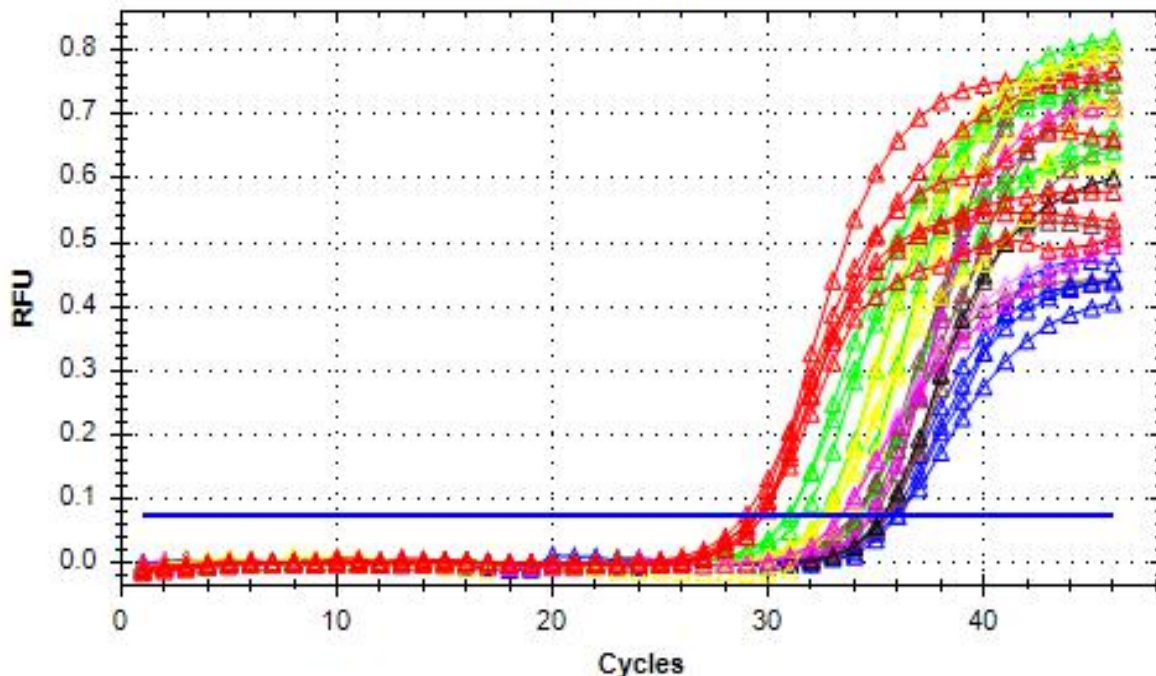


Figure 3. means \pm SEM of fold changes of TLR4.

π = significantly increased ($P < 0.05$) as compared with group 1,
 *= significantly decreased ($P < 0.05$) as compared with group 2,
 α = significantly decreased ($P < 0.05$) as compared with group 3,
 β = significantly decrease ($P < 0.05$) as compared with group 4,
 γ = significantly increase ($P < 0.05$) as compared with group 5.

Amplification



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Figure 4. Real-time PCR amplification plot for TLR4 gene tissue samples that showed a clear difference in threshold cycle numbers (Ct value) between treatment and control groups. Where: Blue plot: Group 1 (control group, untreated and unexposed to CUS), Red plot: group 2 (untreated and exposed to CUS), Green plot: group 3 (treated with 10mg/kg fluoxetine for 14 days), Yellow plot: group 4 (treated with 50mg/kg W.Somnifera extract for 14 days), Violet plot: group 5 (treated with 100mg/kg W.Somnifera extract for 14 days), Black plot: group 6 (treated with 150mg/kg W.Somnifera extract for 14 days), no.of rats=10 for each group.

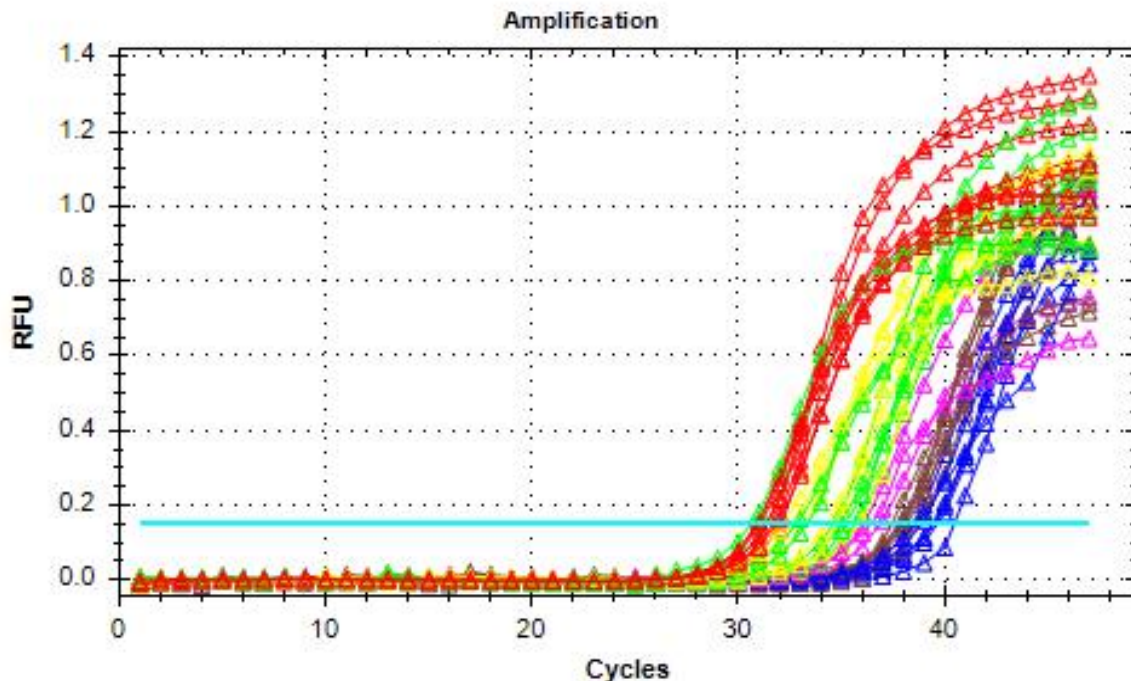


Figure 5. Real-time PCR amplification plot for TLR2 gene tissue samples that showed a clear difference in threshold cycle numbers (Ct value) between treatment and control groups. Where: Blue plot: Group 1 (control group, untreated and unexposed to CUS), Red plot: group 2 (untreated and exposed to CUS), Green plot: group 3 (treated with 10mg/kg fluoxetine for 14 days), Yellow plot: group 4 (treated with 50mg/kg W.Somnifera extract for 14 days), Violet plot: group 5 (treated with 100mg/kg W.Somnifera extract for 14 days), Black plot: group 6 (treated with 150mg/kg W.Somnifera extract for 14 days), no.of rats=10 for each group.

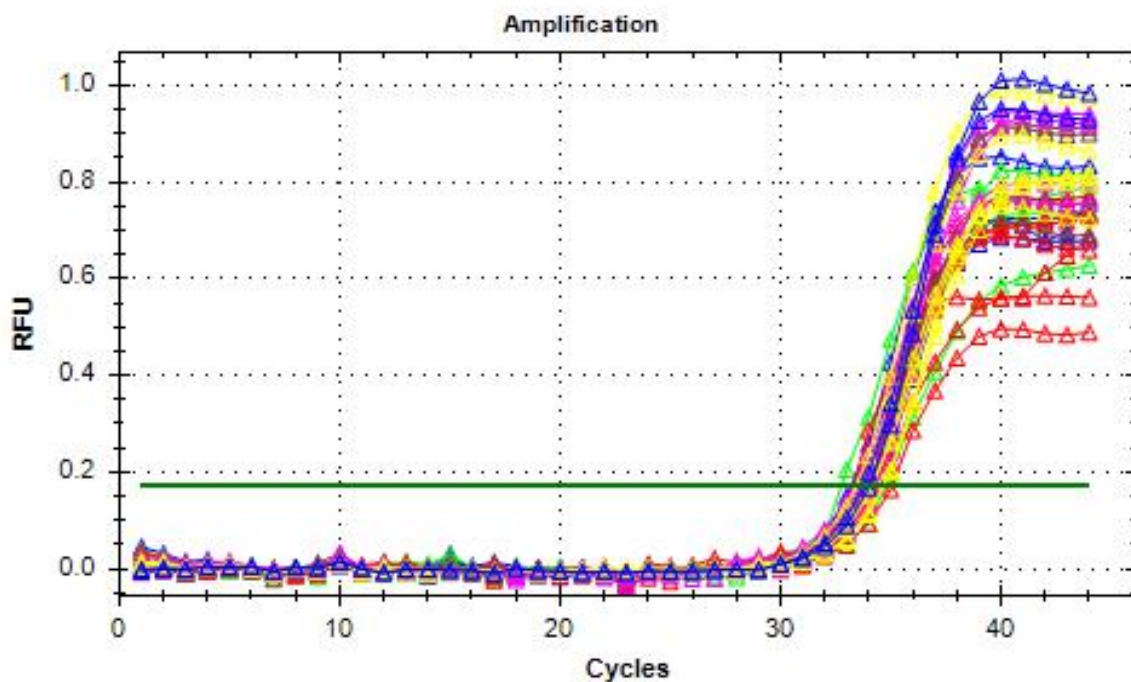


Figure 6. Real-time PCR amplification plot for GAPDH housekeeping gene tissue samples that showed less difference in

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threshold cycle numbers (Ct value) between treatment and control groups. Where: Red plot: group 1 (control group, untreated and unexposed to CUS), Blue plot: group 2 (untreated and exposed to CUS), Yellow plot: group 3 (treated with 10mg/kg fluoxetine for 14 days), Violet plot: group 4 (treated with 50mg/kg *W.Somnifera* extract for 14 days), Green plot: group 5 (treated with 100mg/kg *W.Somnifera* extract for 14 days), Black plot: group 6 (treated with 150mg/kg *W.Somnifera* extract for 14 days), no.of rats=10 for each group.

DISCUSSION

Depression is one of the debilitating diseases affecting a decent percentage of the general population. Hence, this study was designed to investigate the anti-depressant effect of *W. Somnifera* against CUS-induced depression by using FST and its effect on the gene expression of TLR 2 and 4. To the best of our knowledge, this is the first study to correlate the effects of *W. Somnifera* extract on the chronic stress-induced TLR2/4 gene expression in the hippocampus of the male rat.

In this study, it was noted that on day 0 (baseline), there were no statistically significant differences between animals from all groups in the immobility time. While after exposure to 10 days of unpredictable stress, there was a significant increase in the immobility time during FST. This means that these animals have developed a model of depression. Previous studies had demonstrated similar results, which were after exposing rats to unpredictable stress procedures using different stressors, they have developed a behavioral model of depression (12,13)

On day 25, group 2 (untreated and exposed to CUS) had a depressive-like behavior manifested by a significant increase in the immobility time during FST as compared with group 1 (untreated and unexposed to CUS). These results were compatible with a previous study (14). While in groups 3,4,5, and 6, there were a significant decrease in the immobility times during FST as compared with group 2. These results explained that fluoxetine and *W. Somnifera* extracts have an antidepressant action, and this has been explained by another study (15).

Moreover, this study has revealed that after the treatment of rats with either fluoxetine or three concentrations of *W. Somnifera*; antidepressant effect has been noted. For instance, the immobility time was significantly decreased on day 25 as compared with day 10 for group 3 rats (treated with 10 mg/kg fluoxetine for 14 days from CUS). This result has been demonstrated in the previous study (2). Shen et al. have reported that fluoxetine increased serotonin levels in the brain, where neurobiological mechanism of depression are attributed to the low level of neurotransmitter, particularly dopamine (DA), 5-HT, and NE.

In groups 4,5 and 6 (treated with *W.Somnifera* extract 50 mg/kg,100 mg/kg and 150 mg/kg respectively), there was a significant increase in the immobility time after 10 days of CUS exposure as compared to day 0 (baseline). This demonstrates a depressive state and a model of depression. While after treatment with different concentrations of *W.Somnifera* for 14 days from CUS, there was a significant decline in the immobility time on day 25 as compared with day 10. These results indicate that *W. Somnifera* extract has an antidepressant action via reversing the behavioral alterations observed in the CUS model.

In terms of TLR2 and TLR4 gene expression, this study has found that after 24 of days chronic unpredictable stress exposure, TLR-2, and TLR-4 mRNA expression levels have been unregulated at the hippocampus of the male rats. In group 2 (untreated and exposed to CUS), there were a significant increase (p-value <0,05) in fold changes of TLR2 and TLR4 mRNA expression levels as

compared with the control group (group 1). This increment in gene expression was consistent with a previous study (16). Hung *et al.* found that exposure of animals to different stressors causes an increase in fold changes of TLRs mRNA expression level. In group 3 (treated with fluoxetine for 14 days), a significant reduction in the fold changes of TLR-2 and TLR-4 mRNA expression levels was found as compared with group 2. This result is consistent with (17)

Interestingly and in groups 4,5 and 6, which were treated with *W.Somnifera* extract, there was a significant downregulation in fold changes of TLR-2 and TLR-4 mRNA expression levels as compared with group 2 (p-value < 0.05). This could explain the correlation between MDD and TLRs. There is increasing attention to TLRs due to their potential role in neuropsychiatric disease.

In summary, this study has demonstrated that the exposure of rats to chronic unpredictable stress results in the development of depressive-like behavior and high expression of TLR2/4 mRNA levels in the hippocampus. The extract of *W.somnifera* has anti-depressant like activity evaluated by FST and stress-induced TLR- 2/4 upregulation of mRNA expression levels is prevented by *w.somnifera* treatments.

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