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*Original Article*

# Effects of oregano essential oil on brain TLR4 and TLR2 gene expression and depressive-like behavior in a rat model

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## *Abstract*

The aim of the present study was to evaluate the effects of oregano essential oil (OEO) on the hippocampus and prefrontal cortex TLR 2/4 gene expression and depressive like behavior induced by chronic unpredictable stress (CUS). Sucrose preference and forced swim tests were adopted to examine the antidepressant effect. Control (CON), OEO, CUS, and CUS + OEO groups were used. The OEO and CUS + OEO groups received OEO (0.2 mL/kg, i.p.), CON and CUS received saline (0.2 mL/kg, i.p.), and the positive drug groups of CUS rats received fluoxetine (10 mg/kg) and diazepam (3 mg/kg) once daily for 14 days. The expression of TLR 2/4 was determined using real time quantitative polymerase chain reaction with the SYBR green reporter dye. The compositions of the OEO were determined by gas chromatography-mass spectroscopy. The main constituents were thymol (20.72%), gamma-terpinene (8.83%), borneol (8.72%), cymene (6.83%), carvacrol (6.274%), alfa-terpinene (5.26%), and sabinene (4.92%). Administration of OEO significantly alleviated the depressive symptoms of CUS. A higher level of TLR2/4 mRNA was seen in the brain of CUS group ($P < 0.05$). The CUS-induced increases in the TLR2/4 levels were not reversed by OEO. According to the present study OEO may have the antidepressant-like activity but have no effect on the stress-induced TLR-2/4 upregulation.

***Keywords:*** Chronic unpredictable stress; Depression; *Origanum vulgare L.*; Toll-like receptor 2; Toll-like receptor 4.

## INTRODUCTION

There are a great number of unexpected environmental, social, or pathological stimuli that happen during life and that can trigger stress. Being exposed to external stressors is recognized to increase the risk of psychiatric disorders such as depression, high susceptibility to infections, cancer development, and immune dysfunctions especially due to the higher levels of glucocorticoids (1). There is growing evidence in recent years indicating that exposure to certain psychological experiences, including stress-induced diseases, is closely connected with variations in immune parameters. In some cases, it has been noticed that both depression and chronic stressors have had significant connections with reduced adaptive/acquired immunity and inflammation; however, only recent studies have pointed out that after being exposed to stress or during certain episodes of depression, an innate inflammatory/immune response is strongly activated (2,3). The study by Munhoz, *et al.*, for instance, reveals that chronic unpredictable stress potentiated lipopolysaccharide (LPS)-induced nuclear factor kappa B (NFκB) activation and mRNA expression of pro-inflammatory genes in the frontal cortex and hippocampus (4). Among the essential signaling components of the mammalian host defense system are Toll-like receptors (TLRs) (5). Recently, a number of research works have shown interest in TLRs and their potential roles in neuropathology (6). The finding that neurons, astrocytes and resident microglia express TLRs, just like immune cells (7), has challenged the way neuroscience explains the role of the brain's immune system (8). In an experimental model of depression in rodents, the expression of mRNA levels for Toll-like receptor 4 (TLR-4) in the brain cortex overweighed those in a normal brain (9).

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Currently, there is a growing interest in the use of medicinal plants as a group of natural substances that could be a good source for managing of disorders. Considerable attention has been invested in the positive effects of natural medication on alleviating stress-related neuropsychiatric disorders, such as depressive disorders (10). The Lamiaceae family is among the largest and most distinctive families of flowering plants. It has about 220 genera and almost 4000 species worldwide (11). Labiatae are best known for the essential oils of many family members. *Origanum vulgare L.* "Oregano" as an aromatic plant is rich in phenolic compounds with therapeutic actions widely distributed throughout Asia, including Iran. Oregano is the herb widely used in cooking and natural medicine. It has applied for the treatment of respiratory and hypoglycemic diseases and microorganisms inhibition (12-16). Previous studies have shown that oregano oil contains thymol, γ-terpinene, carvacrol, cis-terpinene, p-cymene, linalool, terpinen-4-ol, and sabinene hydrate as the main components (17,18). It has been reported that thymol exhibit significant anti-anxiety activity in rat (19). Moreover, in the plus maze test, anxiolytic effects are presented by carvacrol (5-isopropyl-2-methylphenol) (20), which is one of the major components of the essential oil of oregano (18).

The identification of active components such as thymol and carvacrol from oregano that have been shown to induce anti-anxiety effects and other bioactive polyphenols, prompted us to assess the antidepressant potential of oregano essential oil. To the best of our knowledge, the antidepressant effects of oregano essential oil (OEO) have not been fully investigated. In this present study, the chronic unpredictable stress (CUS) model was used to further elucidate the antidepressant-like effects of oregano. In the (CUS) model (21), animals are subjected to a variety of unpredictable stressors continuously leading to neurochemical and behavioral changes that are similar to those found in people with depression. Furthermore, since the hippocampus and prefrontal cortex are critical for stress (22-24), the corresponding changes in TLR2/4 gene expression in these important brain areas were examined along with the behavioral evaluation.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats with an average weight of 190-240 g were obtained from the animal house of the University of Medical Sciences, Kerman, Iran and animals were housed in a temperature-controlled room with a 12:12 h light/dark cycle condition with free access to food and water. The rats were acclimated to the laboratory for 1 week prior to the start of the experiment. All procedures were in accordance with guidelines for caring and using of laboratory animals in Neuroscience Research Center of Kerman University of Medical Sciences and the Neuroscience Ethic Committee (EC/KNRC/89-5A).

### *Plant material and essential oil extraction*

The aerial parts of oregano were collected in early summer 2014, near the town of Yazd (Yazd Province, Iran). The plant materials were identified by Dr Seyed Mansour Mirtadzadini, a botanist at Shahid Bahonar University of Kerman, Kerman, Iran. The aerial parts of the plant were dried at room temperature and the essential oil (EO) was extracted by hydrodistillation for approximately 3 h using a Clevenger-type apparatus.

### *Gas chromatography/mass spectroscopy analysis*

Gas chromatography/mass spectroscopy (GC/MS) analysis was carried out on a Hewlett-Packard 5973 connected with a mass detector HP 6890 fitted with a HP-1 column (60 m × 0.25 mm, film thickness 0.25 μm). Helium (at a flow rate 1 mL/min) was used as a carrier gas. The initial oven temperature was programmed 40 °C and then raised at a rate of 3 °C/min to 250 °C; injector and detector temperatures were 250 ºC and 230 ºC, respectively. The mass spectra were obtained by electron ionization at 70 eV. The identification of the components of the essential oil was accomplished by comparing

their retention indices and mass spectra with those of authentic samples also with NIST mass spectral library or those reported in the literature data (25).

***General experimental procedure***

Rats were assigned randomly into seven groups (n = 7 per group): intact animal (lack of exposure to stress or treatment with drugs), control (daily treatment with saline (0.2 mL/kg, i.p, 14 days), OEO daily treatment with essential oil of oregano (0.2 mL/kg, i.p, 14 days). For another four groups, the animals were treated with CUS procedures for 24 days. The details of CUS are explained in fallowing section. Afterwards, these CUS treated rats were given different drugs accompanied with CUS procedures. Administration of drugs was done daily in the evening (3-4 p.m.) for 14 consecutive days, starting on the tenth day of the CUS procedure.

The rats in the CUS + OEO received essential oil of oregano (0.2 mL /kg, i.p.), CUS + fluoxetine (FLX) group was given fluoxetine (10 mg/kg, i.p. Aria Pharmaceutical Co. Iran), CUS + diazepam (DZ) rats treated with diazepam (3 mg/kg, i.p. Sobhan Darou Co. Iran) and the CUS group only supplied with saline (0.2 mL/kg, i.p.).

Chronic unpredictable stress has influence on animal behavior. Here, we examined the depressive like behavior using the sucrose preference test (SPT) and forced swimming test (FST) in rat CUS model. Before the start of the CUS protocol the animals were tested with the FST and SPT to determine the animals' depressive state (baseline, 1 day). In the period of CUS exposure (10 days) depressive like behavior was evaluated using SPT and FST as well. At the end of treatments (25 days), anti-depressive effects of different drugs were evaluated using SPT and FST. The weights of rats were determined in parallel with behavioral tests at 1, 10, and 25 days.

In the previous pilot study, our results indicated that acute administration of the OEO (50, 100, and 200 µL/kg, i.p.) produced a dose response antidepressant-like effect in the FST (unpublished data). According to results of the pilot study, the most effective dose (200 µL/kg) has been chosen for repeated administration in chronic stressed animals.

***Chronic unpredictable stress***

For induction of chronic stress, the Katz method was used with some modifications (21). We used this protocol because it was used as an animal model to induce anxiety. This animal model of stress consists of chronic exposure to variable unpredictable stressors, none of which is sufficient alone to induce long-lasting effects. Vary stressors were used and applied in a different sequence each week to avoid any habituation. Each animal received two stresses per day individually for a period of 24 days. After each stressor, animals were kept in a recovery room for 1 h, following which they were placed in clean cages with fresh bedding and returned to the housing facility. Control rats were individually housed for the same period of time, and were handled daily for 1 min in the housing room, but were not stressed.

The animals in stress groups were exposed to the CUS protocol as follows: Day 1, 15 min forced swim (20 ºC), tail pinch; day 2, 12 h cage tilting (45 ºC), 1 h cage rotation; day 3, reversal of the light/dark cycle, 1 h cold room (4 ºC); day 4, 12 h wet bedding, crowded cage; day 5, 24 h food deprivation, 1 h restraint; day 6, 12 h cage tilting (45 ºC), 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, cold room (4 ºC), 1h cage rotation; day 10, 24 h water and food deprivation, 12 h cage tilting (45ºC); day 11, 15 min forced swim (20 ºC), 1 h restraint; day 12, reversal of the light/dark cycle, 24 h food deprivation; day 13, tail pinch, cold room (4 ºC); day 14, 24 h water deprivation, 1 h restraint; day 15, 12 h wet bedding, 12 h cage tilting (45 ºC); 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 ºC); day 20, 15 min forced swim (20 ºC), 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. Definitions: restraint (rats were placed in a restraining device made of plexiglass and flexible nylon, which restricting movement but allowing free respiration and air circulation), tail pinch (tail pinch involved placing the rat in the previously described restraining device, and applying a clothespin 1 cm from the base of the tail), busy cage (high-density housing was 8 rats per cage).

***Forced swimming test***

The method described by Porsolt, *et al*. was used in our study (26). Briefly, rats were forced to swim individually in a cylindrical glass container (70 cm height, 30 cm diameter), which contained tap water (25 ± 1 °C) to a depth 30 cm. The animals were individually allowed to swim for 15 min in the swim tank and after the test; rats were dried with a towel and returned to their home cages. After 24 h, same procedure was followed to conduct the test swim session for 5 min. The test sessions were recorded and scored by an observer who was blind to the groups of animals. The total duration of immobility during the first 5 min of the swimming session was recorded at 1, 10, and 25 days. The rat was judged to be immobile when it made only the necessary movements to keep its head above water level.

***Sucrose preference test***

The rats were individually housed in a cage and given two bottles of 1% w/v sucrose solution 72 h before the test. After 24 h, 1% sucrose in one bottle was replaced with tap water for next 24 h. After adaptation period, the animals were deprived from food and water for 24 h and sucrose preference was determined by 1 h exposure to two identical bottles filled with either sucrose solution or water. Sucrose preference was defined as the ratio of the volume of sucrose *vs.* total liquid (water + sucrose) consumed during the 1-h test.

***Tissue isolation and RNA extraction***

The animals were decapitated 24 h after the last treatment. Their hippocampus and prefrontal were quickly removed and were frozen on dry ice. They were kept at -80 °C until the use. Total RNA was extracted from prefrontal cortex and hippocampus tissues (6 brain tissue samples from each group, n = 6) by the Trizol method total RNA was treated with DNase (RNase-free DNase, Roche, Manheim, Germany). The purification of RNA (A260⁄A280 ratio was ≥ 1.9) was determined according to OD 260/280 ratio by a spectrophotometer (Sigma, Germany). The integrity of RNA was analyzed on a 1.5% agarose gel electrophoresis (Sigma, Germany). Total RNA was stored at -80 °C until further analysis.

***Reverse transcription***

Briefly, the reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase (Fermentas, Germany) based on the manufacture's protocol. The reproducibility of single results was determined with two strategies: two time measurement of complementary DNA (cDNA) aliquots, and analysis of two different cDNA prepared from the same RNA extract.

***Quantitative polymerase chain reaction***

Quantification of relative RNA expression followed 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)), using a Bio-Rad iQ5 detection system (Bio-Rad, Richmond, USA).

The 2× universal master mix (Takara, Japane) was used in the polymerase chain reaction (PCR) reactions. The amplification program was as follows: a denaturation step of 30 s at 95 ºC and 35 cycles for real-time PCR including 5 s at 95 ºC (denaturation), 30 s at 62 ºC (annealing), and 1 min at 72 ºC (polymerase elongation). A final melting curve of fluorescence *vs* temperature was generated to screen for primer dimers and to document single product formation. To confirm the amplification specificity of the PCR, products from each primer pairs, in addition to melting curve analysis, were subjected to subsequent agarose gel (1.5%) electrophoresis.

**Table 1.** Primer sequences

| Primer name | Primer sequence | Size of PCR product | NCBI accession number |
|---|---|---|---|
| GAPDH | R:ATGCCAGTGAGCTTCCCGTTCAGC<br>F: GTCTTCACCACCACGGAGAAGGC | 392 | NM-017008/4 |
| TLR2 | F:GGGTTCTGACATTGGAGTCC<br>R:CAGTGTCCTGTAAGGATTTCC | 182 | NM-008761102/1 |
| TLR4 | F:CGGAAAGTTATTGTGGTGGTGT<br>R:GGACAATGAAGATGATGCCAGA | 419 | NM-019178/1 |

Primer sequences, PCR fragment length and National Center for Biotechnology Information (NCBI) accession number are reported in Table 1. The amount of PCR products were normalized with HKG primers in separate reactions. All samples were assayed in duplicate. Linearity and efficiency of PCR amplification were assessed using standard curves generated by decreasing amount of cDNA, using five points, diluted over a twofold range. In qPCR, the relative mRNA levels were calculated by the expression 2-ΔΔCT (27).

### *Statistical analysis*

All the results were expressed as mean ± SEM. Statistical comparison were performed using Independent t-tests. One-way ANOVA followed by Tukey's post hoc test was used for comparison of more than two independent groups. SPSS 15.0 was used for statistical analysis. *P*-values less than 0.05 ($P < 0.05$) were used as the significant level.

## RESULTS

### *Gas chromatography/mass spectroscopy analysis*

The EO was subjected to GC/MS. The chemical composition of the oregano essential oil is given in Table 2. Seventy-seven components of the oil were identified. The major components identified using GC/MS analysis were thymol (20.72%), gamma-terpinene (8.83%), borneol (8.72%), cymene (6.83%), carvacrol (6.274%), alfa-terpinene (5.26%), and sabinene (4.92%).

### *Body weight*

Prior to the start of the experiment at day 1, there were no significant weights differences between the rats among all of the groups. In the period of CUS exposure (10 days) the weight of rats markedly decreased compared to control group ($P < 0.001$, Table 3). Moreover, at the end of treatment (25 days), body weight also decreased in the CUS + FLX compared with the baseline (($P < 0.01$) and CUS + saline group ($P < 0.05$; Table. 3).

### *Effects of oregano essential oil on behavioral tests in the chronic unpredictable stress-treated rats*

*Force swimming test*

At the end of day 10, all animals which had undergone CUS protocol showed a significant ($P < 0.001$) increase in duration of immobility in FST when compared with the baseline (Fig. 1A). The OEO administered to the stressed animals produced significant ($P < 0.05$) decrease in the duration of immobility when compared with CUS group. Fluoxetine significantly reduced the duration of immobility ($P < 0.01$) when compared to CUS group. In CUS + saline group the immobility time was significantly higher than the baseline ($P < 0.05$) (Fig. 1A).

*Sucrose preference test*

As shown in Fig. 1B, the mean of baseline sucrose preference index was 76.2 %. Chronic unpredictable stress procedure caused a significant drop of sucrose preference. During the CUS procedure, significant decrease was observed on day 10 compared to the baseline ($P < 0.001$). After the administration of OEO for two weeks, the sucrose preferences of the CUS + OEO group was significantly higher than those of the CUS group ($P < 0.05$). Meanwhile, treatment with fluoxetine and diazepam also significantly increased the percentage of sucrose consumption as compared to the CUS group ($P < 0.05$). Treatment with saline for two weeks did not affect sucrose preference index. In CUS + saline group the sucrose preference was significantly lower than the baseline ($P < 0.01$) (Fig. 1B).

**Table 2**. GC-MS analyses of *Origanum vulgare L.* essential oil.

| No. | Retention time | Compound | Chemical composition (%) | RI |
|---|---|---|---|---|
| 1 | 3.977 | Butanoic acid, 2-methyl-, methyl ester | 0.086 | |
| 2 | 4.402 | Octane | t | |
| 3 | 5.815 | 2-Hexenal | 0.066 | |
| 4 | 8.076 | Tricyclene | t | |
| 5 | 8.489 | Alpha-Thujene | 2.782 | |
| 6 | 8.666 | Alpha-Pinene | 0.795 | |
| 7 | 8.988 | 2,4(10)-Thujadiene | t | 605 |
| 8 | 9.146 | Camphene | 0.523 | 611 |
| 9 | 9.588 | Benzaldehyde | 0.077 | 629 |
| 10 | 10.249 | Sabinene | 2.179 | 655 |
| 11 | 10.718 | 3-Octanone | 0.225 | 673 |
| 12 | 11.008 | Beta-Myrcene | 2.025 | 684 |
| 13 | 11.449 | l-Phellandrene | 0.592 | 701 |
| 14 | 12.387 | Alpha-Terpinene | 5.521 | 725 |
| 15 | 12.762 | O-Cymol | 4.029 | 734 |
| 16 | 12.877 | Beta-Phellandrene | 1.316 | 737 |
| 17 | 13.473 | Beta-Ocimene Y | t | 752 |
| 18 | 14.329 | Gamma-Terpinene | 8.240 | 773 |
| 19 | 14.81 | Trans-Sabinene hydrate | 2.319 | 785 |
| 20 | 15.547 | Alpha-Terpinolene | 2.066 | 803 |
| 21 | 16.285 | cis-Sabinene hydrate | 9.908 | 818 |
| 22 | 17.713 | Cis-p-2-Menthen-1-ol | 0.527 | 847 |
| 23 | 18.909 | Borneol | 0.455 | 871 |
| 24 | 19.914 | 4-Terpineol | 12.202 | 891 |
| 25 | 21.968 | Alpha-Terpineol | 0.822 | 930 |
| 26 | 22.542 | Carvacrol methyl ether | 1.712 | 941 |
| 27 | 23.059 | 2-Cyclohexen-1-ol, 3-methyl-6-(1- | 0.205 | 951 |
| 28 | 24.267 | Borneol acetate | 0.327 | 973 |
| 29 | 25.379 | Thymol | 19.027 | 994 |
| 30 | 26.594 | Carvacrol | 6.274 | 1017 |
| 31 | 26.653 | P-Cymen-alpha-ol | 0.071 | 1019 |
| 32 | 27.408 | Chrysanthenone | t | 1033 |
| 33 | 27.596 | Alpha-Terpinenyl acetate | 0.072 | 1037 |
| 34 | 28.498 | Benzoic acid, 4-methoxy-, methyl ester | t | 1054 |
| 35 | 28.754 | Beta-Bourbonene | 0.065 | 1059 |
| 36 | 28.876 | Beta-Damascenone | t | 1061 |
| 37 | 30.318 | Trans-Caryophyllene | 3.035 | 1088 |
| 38 | 30.903 | Alpha-Cubebene | 0.303 | 1100 |
| 39 | 31.469 | Alpha-Humulene | 0.432 | 1111 |
| 40 | 31.548 | Trans-beta-Farnesene | 0.166 | 1112 |
| 41 | 31.986 | Isothiocyanic acid, phenethyl ester | 0.089 | 1121 |
| 42 | 32.37 | Gamma-Muurolene | 0.185 | 1129 |
| 43 | 32.558 | Germacrene D | 0.246 | 1133 |
| 44 | 32.739 | Beta-Selinene | t | 1136 |
| 45 | 33.194 | Bicyclogermacrene | 0.667 | 1145 |
| 46 | 33.574 | Beta-Bisabolene | 0.181 | 1153 |
| 47 | 33.805 | Alpha-Amorphene | 0.107 | 1157 |
| 48 | 34.175 | Delta-Cadinene | 0.301 | 1165 |
| 49 | 34.473 | Cadina-1,4-diene | t | 1171 |
| 50 | 34.67 | Alpha-Cadinene | t | 1175 |
| 51 | 34.876 | Alpha-Calacorene | t | 1179 |
| 52 | 35.263 | Elemol | 0.627 | 1186 |
| 53 | 35.803 | Nerolidol | 0.072 | 1197 |
| 54 | 36.512 | Spathulenol | 1.187 | 1211 |
| 55 | 36.644 | Caryophyllene oxide | 0.825 | 1214 |
| 56 | 36.809 | Veridiflorol | t | 1217 |

**Table 2. Continued**

| No. | Retention time | Compound | Chemical composition (%) | RI |
|---|---|---|---|---|
| 57 | 36.884 | Salvial-4(14)-en-1-one | t | 1219 |
| 58 | 38.326 | Gamma-Eudesmol | 0.667 | 1248 |
| 59 | 38.569 | Valencene | 0.243 | 1253 |
| 60 | 39.402 | Alpha-Eudesmol | 3.262 | 1270 |
| 61 | 39.507 | Beta-Patchoulene | 0.469 | 1272 |
| 62 | 39.793 | Cis-Z-.alpha.-Bisabolene epoxide | 0.209 | 1278 |
| 63 | 40.446 | Eremophilene | t | 1291 |
| 64 | 42.961 | Benzyl benzoate | t | 1348 |
| 65 | 44.164 | Isoledene | t | 1377 |
| 66 | 45.573 | Unknown | 0.152 | 1410 |
| 67 | 45.672 | Hexahydrofarnesyl acetone | 0.133 | 1412 |
| 68 | 47.078 | Unknown | 0.417 | 1445 |
| 69 | 47.659 | 2-Methoxymesitylene | 0.251 | 1458 |
| 70 | 52.382 | Dehydroabietane | 0.061 | 1575 |
| 71 | 53.242 | 1-Hexadecene | 0.264 | 1597 |
| 72 | 54.083 | Phytol | 0.133 | 1618 |
| 73 | 56.982 | 12,13-dimethoxypodocarpa-8,11,13- | 0.076 | 1692 |
| 74 | 58.293 | (4bS,8aS,9R)-4b,5,6,7,8,8a,9,10- | 0.193 | 1729 |
| 75 | 59.007 | 1-Octadecanol | t | 1750 |
| 76 | 59.811 | (+)-13-Methoxypodocarpa-8,11,13- | t | 1773 |
| 77 | 60.988 | 4-Epidehydroabietol | 0.111 | 1807 |
| Total | | | 100 | |

(RI), retention indices in elution from HP-1 column; (t), less than 0.05%.

**Table 3**. Changes in the mean body weight (g).

| groups/treatments | | Number | Weight (mean/gr) | S.E.M | *P* value[1] | *P* value[2] |
|---|---|---|---|---|---|---|
| Baseline (1 day) | | 28 | 219 | 2.5 | | |
| CUS (10 days) | | 28 | 207 | 3.4 | $P < 0.01$ | |
| CUS (25 days) | SAL | 7 | 218 | 4.7 | | |
| | OEO | 7 | 212 | 4.9 | | |
| | FLX | 7 | 202 | 4.5 | $P < 0.01$ | $P < 0.05$ |
| | DZ | 7 | 214 | 5.8 | | |

(Baseline), initial body weight; (CUS), chronic unpredictable stress; (SAL), saline; (OEO), oregano essential oil; (FLX), fluoxetine; (DZ), diazepam; (*P* value[1])**,** comparison between all groups with baseline (1 day); (*P* value[2])**,** comparison between all groups with CUS (25 days) + SAL group.

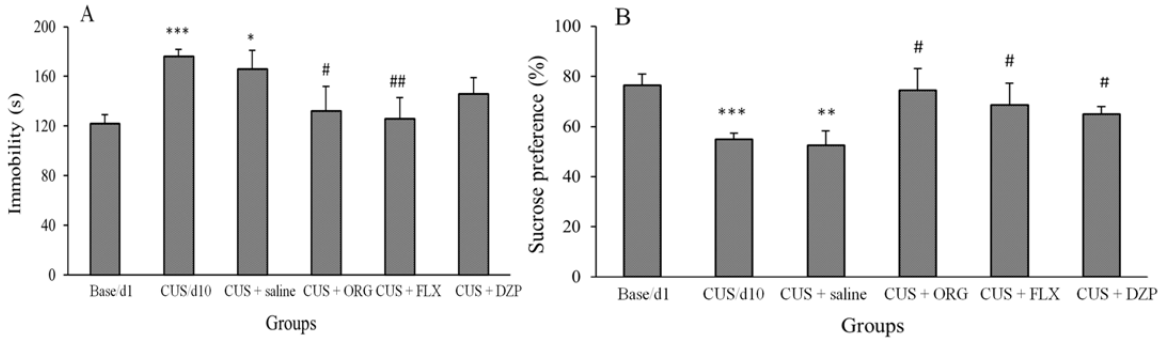


**Fig 1.** Effects of CUS and intraperitoneal treatment with saline, oregano essential oil (0.2 mL/kg), diazepam (3 mg/kg), and fluoxetine (10 mg/kg) on the immobility time in (A), forced swimming test and (B), sucrose preference test. Each point represents mean ± SEM (n = 7). *, **, and *** indicate significant differences ($P < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively) from baseline values; # and ## indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) from CUS day 10 in both experiments. (Base), baseline values; (CUS), chronic unpredictable stress; (CUS d10), measurements at the time point 10 days after beginning of stress exposure; (Saline), saline; (OEO): oregano essential oil; (FLX), fluoxetine; (DZ), diazepam.

***Effects of oregano essential oil on TLR2/4 mRNA expression levels***

TLR2, TLR4, and GAPDH mRNA amplified and quantitatively detected by the SYBR green I real-time PCR. Melting curve analysis demonstrated that each primer pairs amplified a single product with a distinct melting temperature as shown in Fig. 2A. Also, all primer pairs produced a single band of the expected size on agarose gel electrophoresis including TLR2, 182; TLR4, 419 and 392 bp for GAPDH; as depicted in Fig. 2B. There were no significant differences between molecular data in saline (control) and intact animal groups (data not shown). Therefore, the data from these two groups were averaged and used as the control (saline) group.

As it is shown in Fig. 3 a higher levels of TLR2 mRNA was seen in the CUS group compared to control rats in the prefrontal and hippocampus ($P < 0.05$). In the hippocampus and prefrontal, the chronic stress induced increases in the TLR2 mRNA levels were not reversed by administration of OEO. Thus, there was no difference in the TLR2 mRNA expression levels between rats treated with CUS + OEO and the CUS group. A significant difference was observed between CUS + OEO group compared to control group in hippocampus ($P < 0.05$) and in prefrontal cortex ($P < 0.01$).

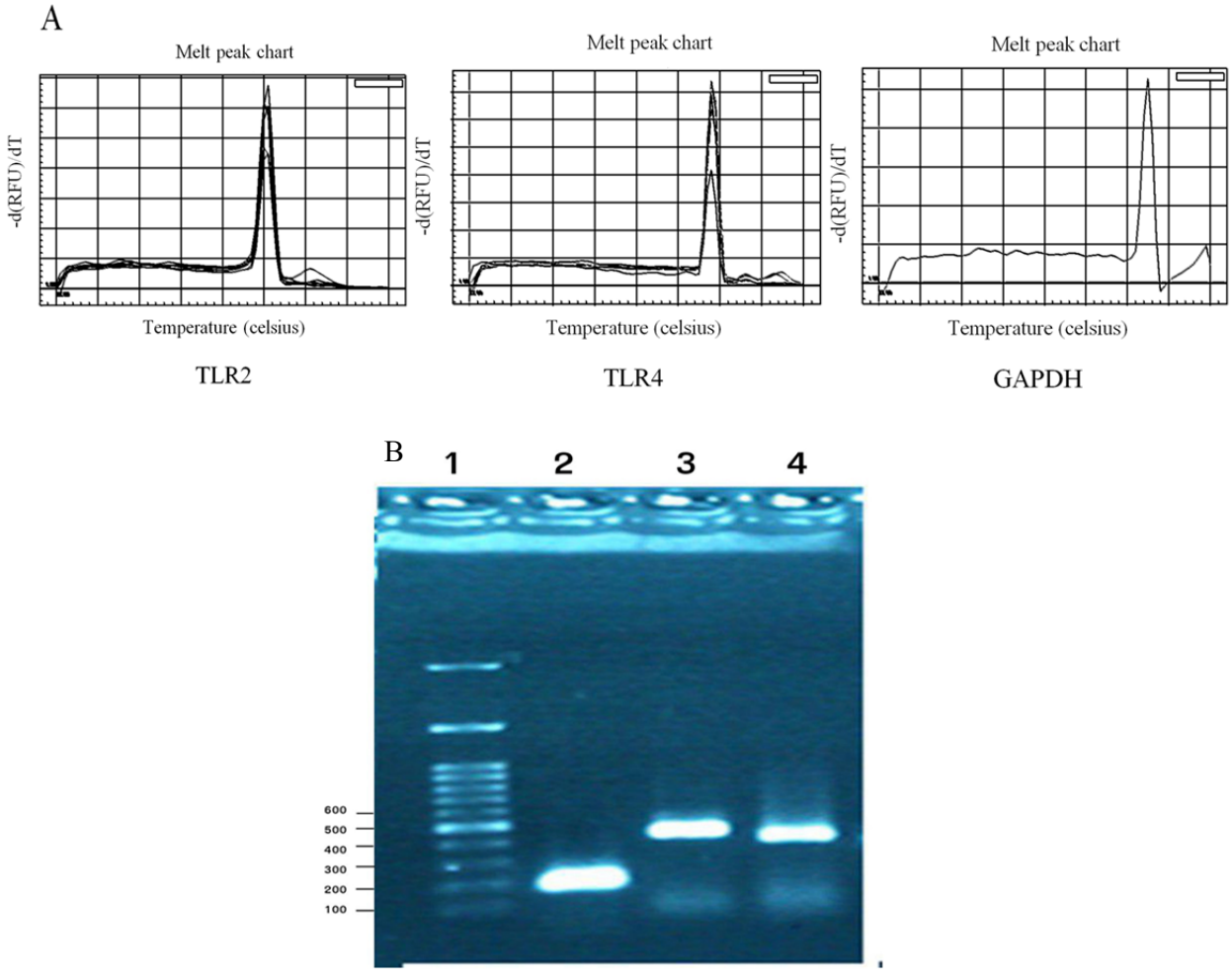


**Fig. 2.** (A): Melting curve of (1), TLR2; (2), TLR4; (3) GAPDH cDNA amplification products. (B): Agarose gel electrophoresis of TLR2, TLR4 and GAPDH cDNA amplification products under UV light: (lane 1), Marker 100 bp + 3 kb DNA Ladder (Smobio. Taiwan); (Lane 2), TLR2 (182 bp); (Lane 3), TLR4 (392 bp); (Lane 4), GAPDH (419 bp).

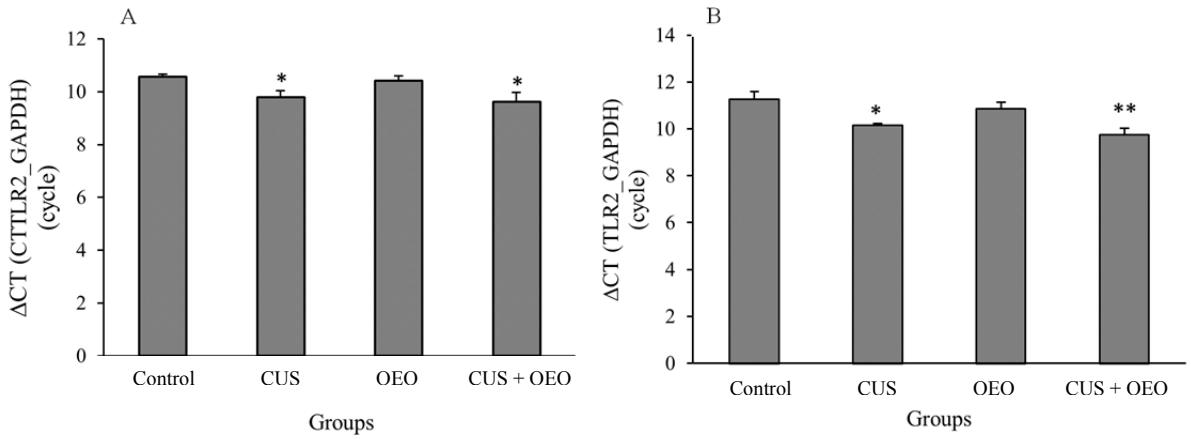


**Fig. 3.** Effects of chronic unpredictable stress and intraperitoneal administration of essential oil of oregano (0.2 mL/kg) on TLR2 mRNA levels in the (A), hippocampus; and (B), the prefrontal. Each point represents mean ± SEM. (n = 6). * and ** indicate significant ($P < 0.05$ and $P < 0.01$, respectively) differences from control according to one-way analysis of variance. (OEO), oregano essential oil; (CUS), chronic unpredictable stress.

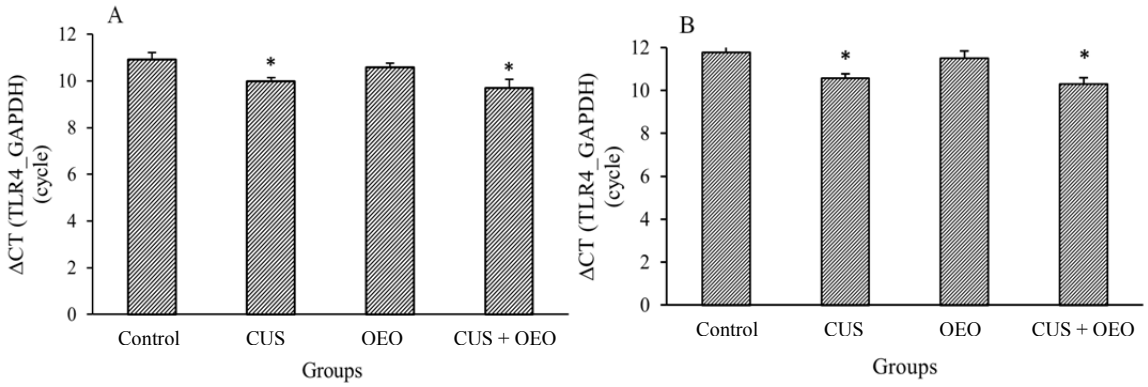


**Fig. 4.** Effects of chronic unpredictable stress and intraperitoneal administration of essential oil of oregano (0.2 mL/kg) on TLR4 mRNA levels in the (A), hippocampus; and (B), the prefrontal. Each point represents mean ± SEM. (n = 6). *, indicates significant ($P < 0.05$) difference from control according to one-way analysis of variance. (OEO), oregano essential oil; (CUS), chronic unpredictable stress.

Fig. 4 showed the effects of the CUS and injection of OEO on expression of TLR4 in the hippocampus and prefrontal cortex. As shown in Fig. 4 A and B, CUS increased the expression of TLR4 mRNA in the prefrontal and hippocampus ($P < 0.05$) compared to control group. These increases were not reversed by chronic administration of OEO. Thus, a significant difference was observed between CUS + OEO compared to control group ($P < 0.05$) in prefrontal cortex and hippocampus.

TLR4 mRNA expression levels in rats treated with oregano subjected to CUS were higher than CUS groups in the hippocampus and prefrontal cortex but these differences were not statistically significant.

## DISCUSSION

In this study, we investigated the effect of OEO against CUS-induced depression, using FST and SPT. The CUS animal model has good validity and seems to represent unpredictable pressure faced by people every day. Previous studies have demonstrated that exposing rodents to unpredictable stress procedures that use different stressors, 1-2 times a day for 7-54 days leads to a behavioral model of long-term human stress exposure (28-30). The current study found that after exposure to 10 days of unpredictable stress, the animals develop variety of symptoms similar to symptoms of human suffering from depression. According to these data, CUS

induced depression like behavior as it is evident from increased immobility time in the FST and significant drop of sucrose preference and weight loss. The FST and SPT have widely been utilized to assess depression-like behavior (26,31).

Based on our results, 24 days chronic unpredictable stress exposure up regulates TLR-2 and TLR-4 mRNA expression levels both at the hippocampus and prefrontal cortex of the male rats. Although there is some positive correlation between mRNA and protein expression, a combination of genomic and proteomic experiments leads to definite results. There is increasing attention to TLRS due to their potential role in neuropsychiatric disease.

The possibility that TLR is also involved in the pathophysiology of stress-related mood disorders is supported by the results of recent studies (6-9). The findings of the present study support evidence of the important role of TLR4 in stress-induced depression. To our knowledge, direct evaluation of TLR-expression changes have been done by a few studies in the context of depression. In animal studies, lack of TLR4 is associated with the reduction of alcohol withdrawal-related anxiety behaviors (32). However, further investigations are still needed, to provide more evidence on the association between TLR gene expression and chronic stress induced disorders. Recently several studies have focused trying to link TLR with neuropathology. McKernan, *et al*. demonstrated that there are specific alterations in TLR2 and TLR4 activation in schizophrenia and bipolar disorders (33). In a recent report by Wu, *et al*., it was suggested that anxiety has a significant association with peripheral TLR4 mRNA expression (34). Preclinical studies also indicate that TLR4 expression has witnessed a rise in the prefrontal cortex in a stress-based model of depression (35,36). Besides alterations in the central nervous system expression, changed peripheral expression of TLRs appears to be closely connected with depression and is perhaps responsible for the heightened inflammatory state (37).

Altogether, the findings of our study suggest that the OEO possesses an antidepressant-like activity and reversed behavioral alterations observed in the CUS model. A previous study has demonstrated that the oregano extract inhibits the reuptake and degradation of the monoamine neuro-transmitters in a dose-dependent manner (38). In the present study, we attempted to find out whether OEO can reduce the stress-induced expression of TLR-2 and TLR-4 in hippocampus and prefrontal cortex. In spite of the antidepressant-like activity, stress-induced TLR-2/4 upregulation is not prevented by the chronic OEO treatments. We did not find any remarkable effect of oregano on the stress up regulated TLR2/4. To the best of our knowledge, this is the first study to evaluate the effects of oregano on the chronic stress induced TLR gene expression in the nervous system. However, further investigations needed to provide more evidence on the mechanism of OEO effect on stress induced depressive like behavior and neuroinflammation.

## CONCLUSION

In conclusion, our study shows 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 brain. In spite of the antidepressant-like activity, stress-induced TLR-2/4 upregulation is not prevented by the OEO treatments. It seems that further research is required to clarify the effect of OEO on neuroinflammations.

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