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Neuro-protective Efficacy of Apigenin in Induced Oxidative Stress Adult Male Rats

Kahtan Ahmad Almzaien[1], Mohammed Ibrahim Al-Rawi[2], Aous Kahtan Almzaien[3], Mohammed Hadi Ali Al-Jumaily[4]*

[1]*Department of Medical Laboratory Techniques, Dijlah University College, Baghdad, Iraq,* https://orcid.org/0000-0001-9294-5658

[2]*College of medicine, University of Anbar, Al-Anbar, Iraq,* https://orcid.org/0000-0002-1362-1557

[3]*Iraqi Centre for Cancer and Medical Genetic Research- Mustansiriyah University, Baghdad, Iraq,* https://orcid.org/0000-0002-3283-9754

[4]*Department of Anesthesia Techniques, Dijlah University College, Baghdad, Iraq,* https://orcid.org/0000-0002-4666-0886

**Corresponding author: kahtan.ahmed@duc.edu.iq, mohammed.hadi@duc.edu.iq*

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ABSTRACT

This study was conducted to evaluate the promising antioxidant efficacy of apigenin extracted from parsley seeds in glycosidic (aqueous extract) and aglyconic forms (acid hydrolysis and organic solvent extraction), comparing to synthetic antioxidant, (Butylated hydroxytolouene) to ameliorate neurobehavioral disorders in experimentally induced oxidative stress adult male rats. Oral administration of 0.75% H2O2 (exogenic oxidant as reactive oxygen species donar) in drinking water for eight weeks was used for oxidative stress induction. The recorded results showed that oxidative stress has caused significant (P< 0.05) alterations in the neurobehavior of experimental animals in comparison to the control. The animals manifested a significant decrease (P<0.05) in locomotor, and exploration (open field test), defect in vestibular functions (negative geotaxis test), degree of cognitive function, and neuromuscular coordination (rotarod test). Mild (non-significant) changes were recorded in autonomic nervous system activity (number of urination and defecation) in H2O2 treated group, and less or non-significant changes in the other groups. The daily administration of apigenin (150 mg/kg body weight) in glycosidic and aglycon forms protected neurons probably by reducing oxidative damage and attenuated significantly the neurobehavioral disorders and imposed its neuro-protective and sedative effects in comparison to butylated hydroxytoloune (25 mg/kg body weight) treated and control groups.

1. Introduction

Flavonoids and particularly apigenin, which is a dietary flavonoid commonly found in many fruits, vegetables, and parsley seeds, is considered an important source [Wang et al., 2019]. Apigenin considered as a neuroprotective agent protect cells against apoptosis and the dopaminergic neurons [Kim & Park, 2020]. This happens probably by reducing oxidative damage, neuro-inflammations and microglial activation along with enhanced neurotrophic potential [Harry & Kraft, 2008]. However, a clear understanding of the mechanism of action of flavonoids as either antioxidant or signaling molecules is crucial for their application to apply in interventions as neurodegeneration and as brain food [Williams et al., 2004]. Further, because of the ability of apigenin to traverse blood brain barrier, it is considered as a promising candidate for this task.

Many factors contribute to the degeneration of neural cells leading to functional deterioration of neurons and neurogenerative conditions such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson disease, and Alzheimer disease [Orgeta et al., 2020i], with a subsequent behavioral change [Christidi et al., 2018]. It's reported that the number of people worldwide suffering from neurodegenerative diseases is nearly 50 million people and the expected number that by the year 2050 will increase to 115 million people [Livingston et al, 2020]. Current management of oxidative stress involves the use of medicinal plants and herbs for treating various diseases. Neural cells in the brain are more vulnerable to oxidative stress because of their high metabolic activities and oxygen requirements, as well as low antioxidant defense capacities [Cobley et al, 2018]. Oxidative stress and inflammation are the two major factors sharing neurodegeneration and can be established in the brain at different levels of neuronal circuitry, ranging from molecular to systemic [Pereira, et al, 2021]. At the cellular level, neuronal apoptosis in ischemic and neurodegenerative disorders may be triggered mainly by oxidative stress [Chen et al, 2012].

Various experimental and clinical investigations showed that oxidative damage plays a vital role in neuron loss and leads to dementia. As well as, hippocampus disturbance and impairment of learning and memory in male and female rats [Lamtai et al., 2020]. Besides, beta-amyloid a toxic peptide produced by a free radical action is known to be at least in part responsible for neurodegeneration that is observed during Alzheimer's disease progression [Cheignon et al., 2018]. However, the consequences of these conditions are tightly correlated with the development of motor and cognitive disturbances [Halliwell, 2001]. The present study was conducted to investigate the antioxidant efficacy of apigenin extracted from parsley seeds in the glycoside or aglycone form in comparison to the standard antioxidant butylated hydroxytolouene (BHT), in experimentally induced oxidative stress rats. The following behavior alterations: locomotor activity and exploration, vestibular functions degree of cognitive function and neuromuscular coordination, and autonomic nervous system activity were considered.

2. Materials and Methods

Harborne method was used for the extraction of apigenin in aglycone form, while the second method was used to extract apigenin in glycosidic form. Identification of apigenin in both forms (aglycone and glycoside) was carried out on silica gel type G aluminum plates (20x20 cm) at a thickness of 0.25 mm supplied from Fluka Company. Toluene: ethyl acetate: acetic acid at a ratio 36:12:5 used as mobile phase [Medić-Šarić et al., 2004] and UV detectors explore the spots at 254 nm. Standard apigenin 98% purity purchased from Xian Natural Field Bio-techniques Co. Ltda. (Xi`an-China), was used to confirm the extraction method. Rats of each group were subjected to five-neurobehavioral tests at 0, 4, and 8 weeks of the trial period (Figure 1). In this investigation, fifty adult male rats were randomly divided into five groups as follows:

- **Group control – CG**: rats were allowed to ad libitum supply of drinking water and served as negative control group.
- **Group treatment – T1**: animals were allowed 0.75% of H2O2 in drinking water and served as positive control group.
- **Group T2**: rats were allowed 0.75% of H2O2 in drinking water, plus daily oral administration of butylated hydroxytolouene (BHT) 25 mg/kg of body weight using a gavage needle.
- **Group T3 and T4**: rats were allowed 0.75% of H2O2 in drinking water, plus daily oral administration of apigenin glycoside and aglycone (150 mg/kg body weight), respectively.

3. Results – Experimental section

3.1. Open field test

This test is designed to measure the locomotor activity and anxiety of the animals before and after apigenin administration. The subjects were placed in a slightly large square arena, measuring 80 cm × 80 cm, divided into 16 (20 × 20 cm) squares. The animals were placed in the center of the arena to determine anxiety and fear. The number of squares crossed by 4 limbs was recorded in addition to frequency of raring, urination and defecation, during 3 minutes [Moser et al., 1992].

3.2. Negative geotaxis test

A significant ($P<0.05$) increase in the duration period was recorded in T1 after four and eight weeks compared to the control (Table 1). Rats in T2 has shown no significant changes in recorded duration, neither after four nor at eight weeks of the experimental period. A significant ($P<0.05$) increase was observed in T3 after eight weeks, but no significant changes was recorded after fourth weeks, comparing to the control. Animals in T4 did not show significant changes either at fourth or eight weeks of the experimental period comparing to the control.

Negative geotaxis a remarkable parameter in evaluating the orientation and movement direction against gravitational run was used in this study to monitor brain tissues impairment [Ruhela et al., 2019]. Rats in T1 showed an alteration in locomotor activity manifested by a significant elevation in negative geotaxis duration after four and eight weeks of treatment in comparison to the CG, and BHT-treated groups.

Elevated reactive oxygen species and oxidative brain injuries as confirmed in this study by catalase and cholinesterase activity depletion and malondialdehyde level elevation in brain tissues may be considered important factors in locomotor activity alteration [Mottahedin et al., 2017]. Oxidative stress also affects glutamatergic receptors, responsible for the long-term potentiation and synaptic transmission [Rai et al., 2013]. Moreover, although the mechanism by which reactive oxygen species lead to cerebral tissue damage is not clear [Castelli et al., 2019], the result of histological examination of brain tissues (unpublished results) in this study showed a congested blood vessels with inflammatory cells, mainly mononuclear cells lumen in the via matter after four weeks of treatment with H2O2 in drinking water. The lesions in the brain tissues after eight weeks of treatment were more effective. The histopathological results indicated that the damage in the different brain areas due to hydrogen peroxide toxic effect might explain the change in neurotransmitters synthesis and function that may contribute in the neurobehavioral changes in the experimental animals.

3.3. Head pocking test

The mean values of head entrance in pouches recorded by rats in each group during the trial period are clarified in Figure 5. A significant decrease in this parameter was observed in T1 animals after four and eight weeks compared to the control. While, no significant changes were recorded in T2 rats, groups T3 and T4 showed a significant decrease in the number of head entrance in pouches during the experimental period. In addition, no significant differences in this parameter were recorded among T3 and T4 groups.

Lesions of the brain tissues, as confirmed by histological examination and oxidative biomarkers, played a major role in further behavioral alteration, such as significant decrease in head pocking or dipping frequency, and probably in addition to the role of hydroxy radicals in neuron degeneration. Some workers mentioned that head-pocking could be symptomatic of neophiles response that would decrease as animals becomes familiar with the apparatus [Brown, GR., & Nemes, C., 2008]. Other reports have attributed the initial drop in head-pocking frequency in adult male rats to the elevated levels of corticosteroid levels, since the testing apparatus is a stressful event [Márquez et al., 2005].

3.4. Swimming rank test

In this test, there was no significant changes observed throughout the experiment period between all treated groups (Table 1). The swimming rank test showed a non-significant difference between all treated groups and the control, while other behavioral tests confirmed this alteration, particularly in H2O2-treated rats. Some authors have considered oxidative stress as the main cause of the pathogens of major depressive order. Therefore, the non-significant recorded results in this study may be due to the vital role of swimming as an antidepressant [Koob, 2010]. Additionally, it is worth to mention that regular swimming improves brain health, decreased thiobarbituric acid, and impede the generation of reactive oxygen species [Qin et al., 2017].

3.5. Open filed test

The mean values of number of squares crossed by four limbs during three minutes for rats in each group is demonstrated in Table 1. A significant ($P<0.05$) decrease in T1, T3 and T4 were recorded after four and eight weeks of the experimental period. On the other hand, at the end of the trial period a significant ($P<0.05$) differences between T1 and T2 were recorded, while no such differences have shown between T3 and T4. Open field test (number of squares crossed and raring number) is a common test that provides a qualitative and quantitative measurement of exploratory behavior, locomotor activity and neuromotor impairment, have also showed a significant decrease in square crossed numbers as well as in raring number in H2O2 treated group after four and eight weeks of treatment [Gould et al., 2009].

3.6. Raring number

The mean number of ratings recorded in each group during three minutes within open field tests, shown in Table 1. Rats in T1 showed a significant ($P<0.05$) decrease in raring number during the fourth and eighth week of the experiment period compared to control group. No significant changes have shown in T2. Animals in T3 and T4 groups recorded a significant ($P<0.05$) decrease in both time intervals as compared with control group. The results also showed the absence of significant differences between T3 and T4 at fourth and eight weeks. Significant differences were found between T3, T4 and CG, T1, and T2 throughout the experimental period. Raring drop is an indication of degeneration of or elevation in metabolic and oxidative stress in hippocampus [Sturman et al.,2018].

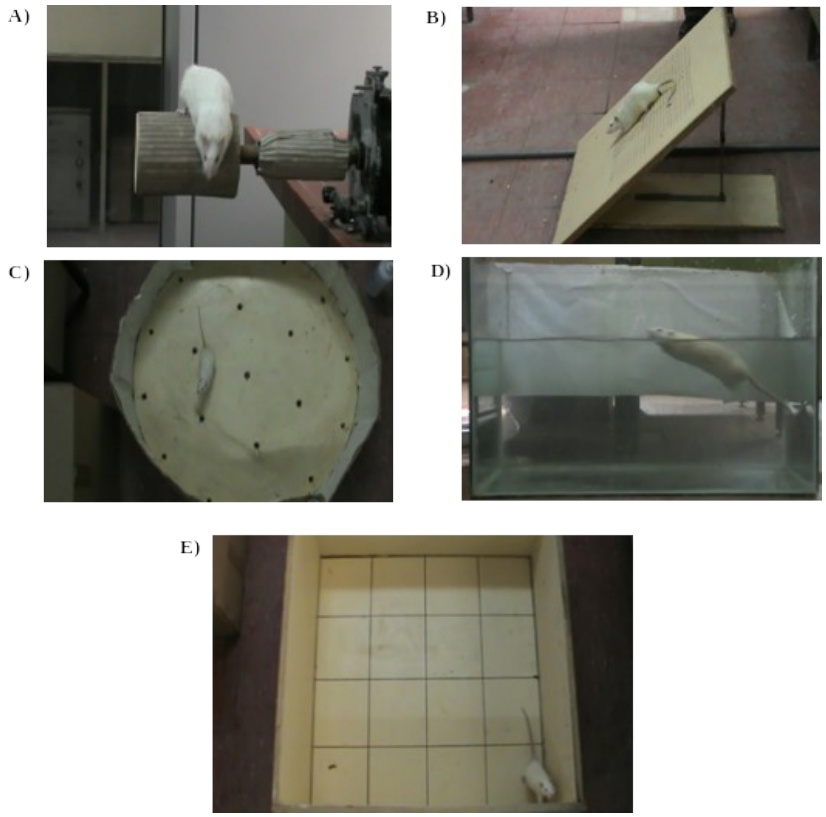

Figure 1 *– Neurobehavioral tests for experimentally induced oxidative stress male rats; A) rotarod test, B) Negative geotaxis test, C) Head pocking test, D) Swimming rank test, E) Open field test.*

4. Results and discussion

The recorded period after eight weeks was the lowest in H2O2 T1 (1.59± 0.3) as compared to the control and other treated groups. Butylated hydroxytolouene plus H2O2 T2 showed also a significant decrease (P<0.05) in persistence duration (3.98 ± 0.1) after four and eight weeks compared to the CG.
Five animals from each group at the end of the fourth and eighth weeks were sacrificed, the head was separated and had immersed immediately in liquid nitrogen to preserve the brain tissue for further analysis. Brain tissue samples were used for the measurement of cholinesterase and catalase activity and malondialdehyde concentration. The study focuses on the neurobehavioral tests that designed to determine different neural and muscular functions. The neurobehavioral tests started after 2 h of oral administration of apigenin, glycoside, aglycone form and BHT and the results were recorded in quite conditions between 5-10 pm using a camcorder (Sony, Compact Cameras, DSLR-A850, Baghdad, Iraq).
A significant difference between treated animals in T1, T2 and CG were recorded at the end of the trial period. On the other hand, no significant changes in persistence duration in T3 rats (H2O2 plus apigenin as glycoside) was recorded, A significant (P<0.05) decrease in this parameter was recorded in T4 (H2O2 plus apigenin in aglycon form) after eight weeks, but after four weeks the decrease in T4 was not significant.

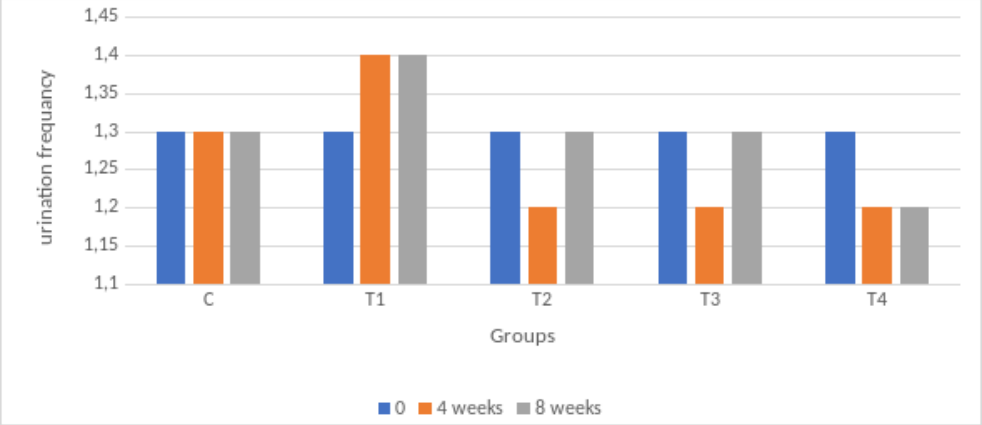

Figure 2 *– Rotarod test (seconds) of rats to oxidative stress via 0.75% hydrogen peroxide in drinking water at the periods zero, four and eight weeks.*

A significant difference between treated animals in T1, T2 and CG were recorded at the end of the trial period. On the other hand, no significant changes in persistence duration in T3 rats (H2O2 plus apigenin as glycoside) was recorded, A significant (P<0.05) decrease in this parameter was recorded in T4 (H2O2 plus apigenin in aglycon form) after eight weeks, but after four weeks the decrease in T4 was not significant.
The brain controls all process that regulates normal body performance such as memory, emotion and functioning of the central nervous system [Phaniendra et al.,2015]. Exogenic H2O2 induced neurodegeneration in the brain tissues of the treated rats manifested by significant elevation in malondialdehyde and depletion in catalase and cholinesterase activity with a consequent alteration in balance, motor coordination and motor skill learning. It is well documented that the two major contributing factors to neurodegeneration in the brain and many different levels of neuronal circuitry are oxidative stress and inflammation [Patki et al., 2013].
Although endogenous hydrogen peroxide has a vital effect on proliferation of cells in live hippocampal neuron and signaling in cell mitosis, but B-amyloid aggregation, dopamine oxidation and brain ischemia reperfusion have the ability to generate H2O2 via Fenton reaction [Guo et al., 2018]. Under stress condition, an excess superoxide (O2.-) release iron from ferritin and the released free iron participates in Fenton reaction and hydroxyl radical (OH.) formation, which play a major role in DNA damage in the presence of transition metal ion [Halliwell, 2000].
Furthermore, a significant decrease in persistence duration (sec) in H2O2 treated group after four and eight weeks of the treatment may be attributed to the neurodegeneration and progressive loss of neuronal cells in specific regions of the brain imposed by oxidative stress, with consequent impairment of neuoro-motor balance and coordination. The brain is vulnerable to the effect of reactive species due to its high oxygen consumption and polyunsaturated acid-rich content as extremely peroxidizable substrate. Polyunsaturated fatty acids have often been implicated as substrates in peroxidative damage because of the abundance of multiple double bonds [Chen et al.,2012]. These double bonds are considered as an ideal site for the attack of free radicals and neuronal degeneration.
Moreover, changes in neuromuscular abilities my also attributed to the effect of free radicals on the acetylcholine, glutamine, dopamine, catecholamine or other neurotransmitters of the central nervous system like serotonin and Gamma amino butyric acid (GABA).

4.1. Frequency of Urination and Defecation

The frequency of urination and defecation during three minutes within open field test is clarified in Figure 3, and 4, respectively. The recorded results of both parameters throughout the experiment period have shown no significant differences among the treatments and the control (P<0.05).

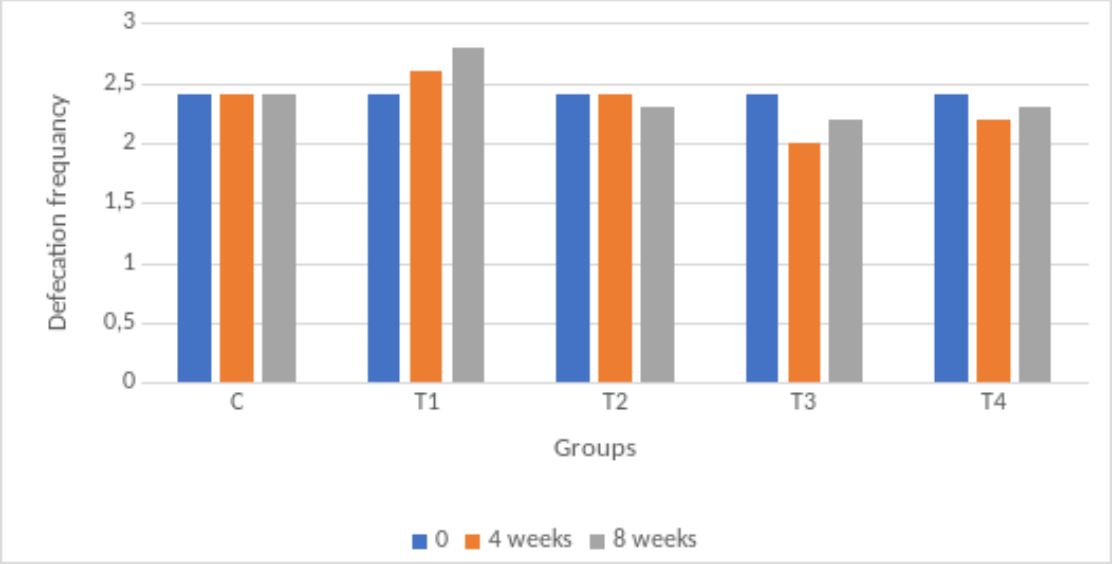

Figure 3 *– Open field test (urination frequency) of rats exposed to oxidative stress via 0.75% hydrogen peroxide in drinking water at the periods zero, four and eight weeks.*

A mild increase in urination and defecation frequency in open field test was recorded in H2O2 treated animals (T1 group), this increment even was not significant but may be due to decrement of cholinesterase activity which may lead to an increase in acetylcholine levels with consequent elevation in urination and defecation frequency, since these two refluxes are mainly under the control of cholinergic activity.
Apigenin (glycoside and aglycon) attenuated significantly the motor and neuromuscular coordination, balances and vestibular functions disorders induced by oxidative stress. The neuroprotective efficacy of apigenin may be attributed to its role in acute transient focal cerebral ischemia reperfusion injury [Pang et al., 2018], inhibiting nitric oxide and prostaglandin E2 production by suppressing the expression of inducible nitric oxide synthase and cyclooxygenase-2 protein respectively. As well as suppression P38 mitogen-activated protein kinase (MAPK), C-Jun N- terminal (JNK) phosphorylation without affecting the activity of extracellular signal-regulated kinase (ERK) [Ha et al., 2008].

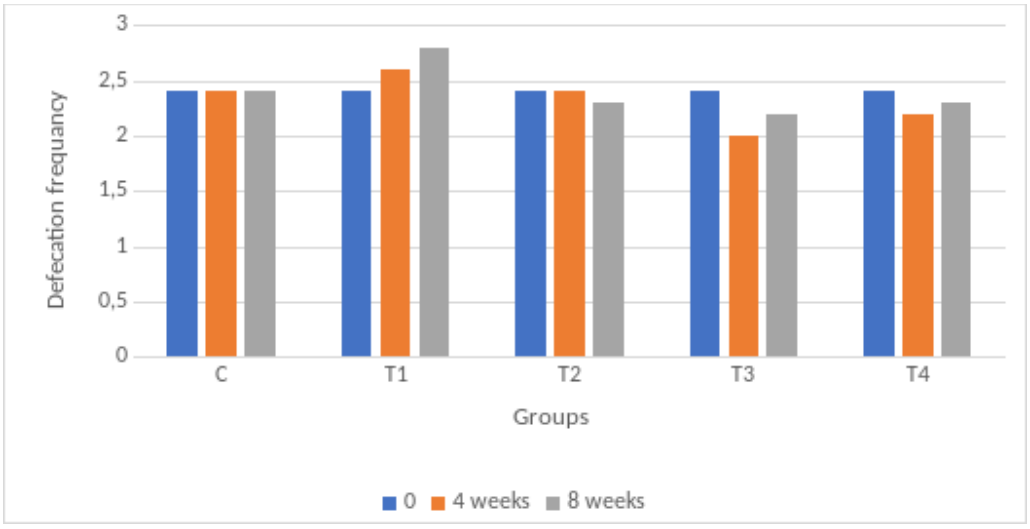

Figure 4 *– Open field test (defecation frequency) of rats exposed to oxidative stress via 0.75% hydrogen peroxide in drinking water at the periods zero, four and eight weeks.*

Apigenin protect neuronal cells from injury in middle cerebral artery occlusion [Liu et al., 2008], suppressed oxidative stress medicated apoptosis and ameliorate learning and memory impairment in Alzheimer's disease through relieving amyloid-B peptide (AB) as well as suppressing the amyloidgenic process [Tu et al. 2017].

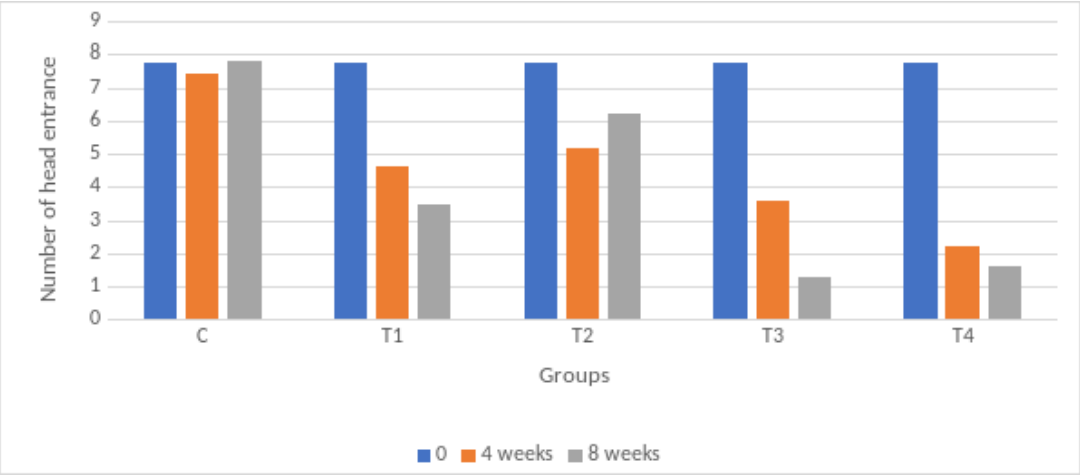

Figure 5 *– Number of head entrance in the pouches of rats exposed to oxidative stress via 0.75% hydrogen peroxide in drinking water at zero, four and eight weeks in different groups of the experiment.*

In spite of the promising role of apigenin as antioxidant, but the significant decrease in the number of squares crossed by rats in open field test and the number of head entrance into the pouches in head pocking test in apigenin treated groups (T3, T4) was probably attributed to apigenin`s sedative effect which approved by a number of workers ($P<0.05$). Apigenin competitively bind to the benzodiazepine binding site of gamma- aminobutyric acid (GABA) type a receptor, and exhibit clear antiolytic activity in mice when administered intraperitoneally [Wasowski & Marder, 2012].
Generally, no significant differences were recorded in all tested behaviors in rats treated with apigenin in glycoside and aglycon forms confirming the capability of both form in penetrating blood brain barrier (BBB) and significantly alternating the harmful effect of hydrogen peroxide by inhibiting toll-like receptor 4 (TLR4)- mediated inflammation and protecting against BBB distribution.
Finally, BHT at a dose of 25 mg/kg body weight shown a significant vital role in suppressing oxidative stress induced by hydrogen peroxide and possibility of the usage of this dose level with no consequences of side effects in adult male rats.

5. Conclusion

In the present study, oral administration of hydrogen peroxide at a level of 0.75% in drinking water to adult male rats induced oxidative stress and neurodegeneration manifested by a significant alteration in oxidative biomarkers. In addition, a significant disorder in motor coordination, balance, motor learning, neuromuscular coordination, vestibular functions, locomotor activity and the degree of cognitive functions and exploration were detected. Parsley seeds apigenin (150 mg/kg body weight in glycosidic and aglycon forms) attenuates to a significant level the consequences of oxidative stress and neurodegeneration in comparison to BHT, and its role in protecting brain tissue was clearly demonstrated in open field and raring number test.

Conflict of Interest: We have no competing interests.

Declarations and Ethics: Our study was done based on the ethical board approval of Baghdad University. The scientific committee in the department of physiological and pharmacology, college of veterinary medicine, university of Baghdad was approved the study of "Neuro-protective Efficacy of Apigenin in Induced Oxidative Stress Adult Male Rats" under (reference number 2570)

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