

Effects of Tarragon and Coumarin on Memory and GABAA Gene Expression in Rat Hippocampus

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Abstract

Objective(s): GABAA receptors in the hippocampus are effective in learning and memory mechanism. The present study effect of tarragon and coumarin on memory and GABAA gene expression in rat hippocampus.

Methods: 56 Wistar male rats were used and random placement in 7 groups (N = 8) including: Intact, receiving DMSO, tarragon hydroalcoholic extract (25, 50, 100 mg/kg) coumarin (3, 5 mg/kg) intraperitoneal once a day for two weeks. The shuttle box was used for the memory retention test. Brains of rats were removed, 4 brains in each group for the histological test using Nissl staining. In the other four brains, the hippocampus was removed immediately, a gene expression test was performed using real-time PCR. All data are expressed as mean \pm STD. Statistical analysis was performed using one-way analysis of variance (ANOVA).

Results: significant decreased in the memory retention test and the number of healthy pyramidal neurons as well as a significant increase in GABAA- $\alpha 5$ and GABAA- $\alpha 2$ genes expression compared to the group receiving solvent.

Conclusion(s): Tarragon hydroalcoholic extract and coumarin affects memory impairment through increasing the GABAA- $\alpha 5$ and GABAA- $\alpha 2$ genes expression and decreasing the number of healthy hippocampal neurons.

Keywords: memory retention, GABAA gene expression, hippocampus, tarragon hydroalcoholic extracts, Coumarin, male rats.

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INTRODUCTION

Learning is the behaviour changes due to gaining experience, and memory is the keeping of such information. One of the most significant performing levels of the central nervous system is learning and memory. Hippocampus is of the main brain structures involved in learning and memory.¹

Previous studies have shown the high concentrations of GABAA receptors in the hippocampus, which indicates the fact that activating GABAA receptors can control the process of learning and memory.² Gamma-Aminobutyric Acid (GABA) is the brain's major inhibitory neurotransmitter. GABA attaching to the GABAA receptors opens the chlorine channel and causes hyperpolarization.³ Approximately 80% of all GABAA receptors contain an attaching place of benzodiazepines located in between the related subunit of $\gamma 2$ and subunit of α ($\alpha 2$, $\alpha 5$).⁴

Tarragon (*Artemisia dracuncululus* L.) is a perennial herbaceous plant of the Asteraceae family.⁵ Tarragon aerial parts include a broad array of phytochemicals such as monoterpenoids, selenocoumarins, phenols, polyethylenes, alkaloids, coumarins and flavonoids.⁶ Tarragon flavonoids contain quercetin and eriodictyol.⁷ Researches carried out on Tarragon have revealed that this plant contains bioactive compounds such as α -pinene, coumarin, trans-anethole,

methyl eugenol and γ -terpinene.⁷ There are some reports indicating the effect of flavonoids on the benzodiazepine position of the GABA receptor, which ultimately leads to the increasing of GABA activities.⁸ GABAA (α) subunits are the dominant subunits in the central nervous system and are considered essential in attaching to benzodiazepines as well as in memory.⁹ Studies showed that the two subunits of $\alpha 2$ and $\alpha 5$ are playing a more important role in memory.¹⁰ Some phenolic substances in tarragon hydroalcoholic extract such as coumarin produce anticonvulsant, anti-anxiety, and tranquillizing effects through binding to the GABAA receptors.¹¹ Coumarin is a combination of various physiologic features such as anticoagulant, antimicrobial, antioxidant and complexes carrying the features of anti-cancer that has been taken into consideration by many chemists and pharmacists.

Coumarin is effective in decreasing seizures by binding to the benzodiazepin GABAA receptors.¹¹ Muke and colleagues had carried out research in 2018 that showed the tranquillity and anti-anxiety effects of Coumarin probably through affecting GABAA receptors.¹² No research or study has been published on such substance regarding learning and memory using molecular methods. Thus, due to the importance of molecular and tissue methods on retentioning memory and through benefiting from the modern method, the present study is going to molecularly express the effects of tarragon hydroalcoholic extract and coumarin on memory in the hippocampus.

MATERIALS AND METHODS

56 male rats weighing 220 to 230 g were procured from the Pasteur Institute of Iran for the purpose of the present study. They were kept in standard temperature condition ($23 \pm 2^\circ\text{C}$) with a relative humidity of 40-45% and lib access to pellets and freshwater.¹³ The rats were randomized into 7 groups ($n = 8$); these groups included the intact, the control+ receiving 0/5 cc solvent, the receiving tarragon hydroalcoholic extract with doses of 25, 50 and 100 mg/kg and coumarin with doses of 3 and 5 mg/kg. The treatment was administrated intraperitoneally once a day for 15 days.¹⁴ Tarragon was purchased from an agricultural farm in Qom that grew vegetables. Then, tarragon was matched with a sample plant preserved in the Faculty of Pharmacy of Shahid Beheshti University of Medical Sciences No. 861 (voucher specimen). The hydroalcoholic extract was provided using dried tarragon, 70% alcohol and Soxhlet apparatus.⁸ Violet crystal and coumarin were obtained from Sigma Aldrich. Paraformaldehyde, gelatin, chloroform, xylazine, alcohol 96%, absolute alcohol, xylene, formamide, distilled water, and DMSO were purchased from the Merck Company in Germany. accuprep genomic dna extraction kit (Cat No: K-3032) and AccuPower® 2X Greenstar qPCR Master Mix were procured from BIONEER Company. The present research protocol was approved by the Ethics Committee of the University (ethic code: IR.IAU.SRB.REC.1397.127) based on the International Laboratory Animals' Regulations.

A shuttle box contains two light and dark chambers ($30 \times 20 \times 30$ cm) and a guillotine lid (9×7 cm) was used to measure the memory retention. The bottoms of both chambers were made of stainless steel (2.5 mm diameter) and were connected to a stimulator. The animal was put in the light side of the box for preliminary training, then after 5 seconds, the guillotine lid opened. The time required for the rat to enter the dark box was recorded. Then, it was released. This procedure was repeated 30 minutes later, this time the rat was exposed to foot shock with the frequency of 50 Hz and voltage of 0.5-0.6 mA for 9 seconds just after entering the dark side of the box, then after 5 seconds the rat was taken out of the device. The rat was located on the light side of the box 24 hours after training for the retention test. 24 hours after training, the rat was located on the light chamber of the box for taking the test of retention. 30 seconds later, the guillotine lid opened and the duration for entering the dark chamber of the box was recorded as the memory retention time and and the duration of the delayed entry of male rats into the dark compartment of the shuttle box was calculated and used on the test day. The whole test was taken in 600 seconds.¹⁵

After the memory retention test using shuttle box, in order to test histology, the rats were immediately anaesthetized with chloroform to remove their brain. In each group, 4 brains were used for histological study and the other 4 were allocated for molecular study. The 4 brains were fixed in formalin solution (10%) and then dehydrated and moulded. 10 slides with serial cuts of 5-7 μm were provided and stained with Nissl. Then, 5 sections from brain tissue of different groups were selected randomly and the area CA1 studied microscopically. OLYMPUS, AX70 microscope was used for cell counting and the sample images were recorded at 40X magnification. Cells were counted using Olysia bio report software. Considering the Paxinos atlas, pyramidal neurons in 3.3-3.6 sections (relative to Bregma) were selected.¹⁶

To perform RT-PCR, the hippocampal region was firstly removed and then, immediately frozen in liquid nitrogen. Hippocampal samples were refrigerated at -84°C and stored till RNA was extracted. In order to determine the expression of $\alpha 2$ and $\alpha 5$ genes in hippocampal GABAA receptors, the selected tissue was firstly homogenized and then the whole RNA of the tissue was extracted using +RNX extraction solution and chloroform- alcohol protocol. The RNA concentration test was conducted to determine the acceptable ratio of purity. Concentration was measured by a spectrophotometric method using a nano-drop device (Nanolytik company, Germany), followed by cDNA making.

Polymerase chain reaction (PCR) was used to amplify the cDNA of the above genes. The PCR process was expressed by AceQ™ RT_PCR SYBR Green Master using Taq polymerase enzyme and specific primers for GABAA- $\alpha 2$ (5'-GAACAGAGAATCGGTGCCAGCAAGA-3')F (5'-TGCAAATTCAATTAGGGCAGAGAACACAA-3')R gene, GABAA- $\alpha 5$ (5'-TGCTATGCATTTGTCTTCTCTGCTCTGATT-3')F, (5'-GATTAGACCCGTTACCATCGAGAT-3')R gene, β -Actin (5'-CCCAGCCATGTACGTAGCCA3')F (5'-CGTCTCCGGAGTCCATCAC-3')R gene. The condition in a laboratory was separately optimized. Then, the condition was assessed in terms of primer binding temperature, number of cycles, and PCR rate using PCR program; and afterwards, it was assessed by using Stepone software. The thermal cycle features are as follows: 15 seconds at 95°C , 60 seconds at 60°C for 40 cycles. The mean Ct of reference and target genes in each sample was calculated. The difference between the threshold cycle of target and reference genes in the control sample was calculated as ΔCt and the difference between them was expressed as $\Delta\Delta\text{Ct}$.¹⁷

Statistical Analysis

All data are expressed as Mean \pm STD. The analysis of memory retention and histology tests were conducted for statistical analysis, using One-way, analysis of variance (ANOVA). In all calculations, $P < 0.05$ was considered as a significant difference. Gene expression data were calculated and analysed by $\Delta\Delta\text{Ct}$. A level difference of greater than 1 was considered as the level of significance.

RESULTS

The group receiving solvent (control+) showed no significant difference in memory retention compared to the intact group. The groups receiving tarragon hydroalcoholic extract with doses of 50 and 100 mg/kg and coumarin with a dose of 3 mg/kg showed a significant reduction in memory retention in comparison with the control+ group (Figure 1). The control+ group reflected no significant differences in the number of hippocampus healthy neurons compared to the intact group. Those groups that received tarragon hydroalcoholic extract with doses of 50 and 100 mg/kg and coumarin with doses of 3 and 5 mg/kg showed a significant decrease in the number of healthy neurons in the CA1 area of the hippocampus compared to the control group. They gave (Figures 2, 3). The control+ group was not show significant difference in GABAA- $\alpha 5$ and GABAA- $\alpha 2$ expression compared to the control+ group. The group that received tarragon hydroalcoholic extract with a dose of 25 mg/kg reflected a

significant increase in GABAA- α 2 gene expression compared to the control+ group (Figure 4). Those groups that received tarragon hydroalcoholic extract with doses of 50 and 100 mg/kg showed a significant increase in GABAA- α 5 gene expression compared to the control+ group (Figure 5).

The graphs show the mean and standard deviation (Mean \pm STD). Using variance analysis, data was one-sided and afterwards, the Toky test was analysed ($n = 8$, $p < 0.01^{**}$, $p < 0.001^{***}$).

The histology research results indicated that the group received tarragon hydroalcoholic extract with doses of 50 and 100 mg/kg and coumarin with a dose of 3, 5 mg/kg showed a meaningful decrease in the number of hippocampus healthy neurons compared to the control+ group (Figures 2, 3).

The graphs show the mean \pm standard deviation (Mean \pm STD). Using variance analysis, data was one-sided and afterwards, the Toky test was analysed ($n = 8$, $p < 0.1^{*}$, $p < 0.001^{***}$).

- a. The tissue section of CA1 region of Hippocampus of 40X magnification in the untreated group that received no substance (intact). In this group no decline or necrosis of neurosis cells was reported. Lots of pyramidal healthy cells (white cell) can be seen with a bright core and black arrow.
- b. The tissue section of the CA1 region of the Hippocampus of 40X magnification in the treated group with solvent (control+); in this group, an insignificant decline or neuron cells necrosis was reported comparing to the intact group.
- c. The tissue section of the CA1 region of the hippocampus in the healthy group treated with a 25 mg/kg dose of tarragon hydroalcoholic extract (extract 25); in this group, the decline or nervous cells necrosis was insignificant in comparison with the control+ group. Meanwhile, the pyramidal cells necrosis (dark Cell) with picnose nucleus using red arrow can be observed.
- d. The tissue section of CA1 region of the hippocampus in the group treated with 50 mg/kg dose of tarragon hydroalcoholic extract (extract 50); in this group the decline or nervous cells necrosis was significantly observable in comparison with the control+ group.
- e. The tissue section of CA1 region of the hippocampus in the healthy group treated with 100 mg/kg dose of tarragon hydroalcoholic extract (extract 100); in this group the decline or nervous cells necrosis was significantly observable in comparison with the control+ group.
- f. The tissue section of CA1 region of the hippocampus in the healthy group treated with 3 mg/kg dose of coumarin (coum 3); in this group the decline or nervous cells necrosis was significantly observable in comparison with the control+ group.
- g. The tissue section of the CA1 region of the hippocampus in the healthy group treated with a 5 mg/kg dose of coumarin (coum 5); in this group the decline or nervous cells necrosis was insignificant compared to the control+ group.

The results of gene expression revealed that the group received tarragon hydroalcoholic extract with a dose of 25 mg/kg showed significantly increase in GABAA- α 2 gene expression compared with the control+ group (Figure 4).

The graphs show the mean and standard deviation (Mean \pm STD). The level difference $\Delta\Delta C_t$ was considered more than one level ($n = 4$).

The results of the gene expression study revealed that the group that received tarragon hydroalcoholic expression with doses of 50 and 100 mg/kg showed a significant increase in GABAA- α 5 compared to the control+ group (Figure 5).

The graphs showed the mean and standard deviation (Mean \pm STD). The level difference $\Delta\Delta C_t$ was considered more than one level ($n = 4$).

DISCUSSION

Memory is an important cognitive function without which life is not possible. Information is saved through harmonic neural networks in the brain.¹⁸ Hippocampus composition plays an important role in the calculation and saving of spatial information. The pyramidal cells of the hippocampus CA1 region are too sensitive and among the first neurons that would be destroyed in pathologic conditions and some brain degenerative diseases.¹⁹ There are dominant GABAA subunits receptors of $\alpha 2$ and $\alpha 5$ in the hippocampus that exists in whole regions of CA1 and CA3.²⁰ GABAA- $\alpha 2$ and GABAA- $\alpha 5$ receptors are high-affinity binding sites for benzodiazepines.⁴ These two subunits are crucial in learning and memory.²¹

The shuttle box of the behavioural test has been conducted for the present research to investigate memory, and the results revealed that the group that received solvent showed no significant differences regarding on the delayed entrance of the male rats to the dark chamber of the shuttle box amount compared to the intact group. Therefore, the extracting solvent DMSO had no effect on the process of memory in male rats. Meanwhile, the on the delayed entrance of the male rats to the dark chamber of the shuttle box amount in the group that received tarragon hydroalcoholic extract with doses of 50 and 100 mg/kg and coumarin with a dose of 3 mg/kg significantly decreased compared to the control+ group. Probably such decrease was due to direct or indirect impact on GABAA receptor which is aligned with the previous studies and researches.

Ding et al.²² introduced methyl eugenol as a new agonist of ionotropic GABA receptors. Methyl eugenol has a strong inhibitory activity against nerve stimulation in hippocampal neurons. Methyl eugenol inhibits voltage-gated sodium channels, voltage-gated potassium channels and voltage-gated calcium channels. Intraperitoneal injection of methyl eugenol mitigates anxiety behaviours in rats, which is likely to be accompanied by the activation of GABAA receptors. Thus, compounds of tarragon such as estragole, methyl eugenol and coumarin represent a new agonist of GABAA receptors.²³

Kavvadias et al. indicated in a research conducted in 2000 that the tarragon hydroalcoholic extract contains benzodiazepines such as temazepam and lorazepam (100-200 ng/g).²⁴ Through binding to the GABAA receptors, benzodiazepines produce anticonvulsant effects. Such compounds as the GABAA agonist receptors increases the frequency of opening chlorine channel related to the receptors.²⁵ Moreover, the injection of quercetin to the rats exposed to chemical kindling increases oxidative stress and produces dose-dependent anticonvulsant effects.²⁶ Naringenin passes through the blood-brain barrier and affects the central nervous system via the GABAA receptors.²⁷ The injecting alpha-Pinene to the rats and recording of electroencephalographic curves of the brain also illustrates the binding of the benzodiazepine site of GABAA receptors to alpha-Pinene, which is used as insomnia medication.²⁸ Terpinene induces effects similar to those of pentobarbital and diazepam including strong sedative and anticonvulsant effects.²⁹ Ahmad Tarmizi et al showed in research that trans-anethole through binding to the GABAA receptor increase its activity and could be used as an anti-epileptic drug.³⁰ Also coumarin injection to the rats produced sedative and anti-epileptic effects that control the brain's neurons through increasing GABAA release. Coumarin attached to the benzodiazepine site of GABAA receptor in three points and increases the activities of this receptor as an anti-epileptic drug.⁹

Jarogniew et al. used electronic kindling to create convulsion and through injecting intraperitoneal coumarin to rats, they found that convulsion threshold has extremely decreased; and probably such result was due to attachment of coumarin to the GABAA receptor and the increase of its activity.³¹

The comparison of the mentioned results with the present research indicates that probably the tarragon hydroalcoholic extract having different substances attached to the benzodiazepine sites in GABAA receptors such as coumarin increases the activities of this receptor and by opening the chloride channel of such receptor fortifies hyperpolarization and finally intensifies the memory disorder in male rats.

In the present study, a histology test has been performed using Nissle colouring for measuring the density of the pyramidal healthy neurons cells of the hippocampus. The results show that the decrease of pyramidal healthy cells in the treated group with 50 and 100 mg/kg doses of tarragon hydroalcoholic extract and 3 and 5 mg/kg coumarin indicated the destruction of hippocampus pyramidal cells compared to the control+ group probably through substances in the tarragon hydroalcoholic extract which confirms the results of Guo et al. research conducted in 2020. The findings of the research showed that the intraventricular injection of methyl eugenol to rats ended in the increase of tension in rats. Meanwhile, methyl eugenol extremely caused hippocampus functional disorder at behavioural, cellular and molecular levels and this indicated potential dangers of methyl eugenol to the central nervous system.³² Moreover, Pawel Kowalczyk et al researches showed the anti-cancer and antimicrobial effects of coumarin; yet, excessive consumption of coumarin might negatively affect the live cell and even destroy lung and liver's cells. In addition, the toxic effect of coumarin destroys the live cells including bacteria.^{33, 34} Thus, it seems the toxic feature of coumarin depending on its dose has affected the hippocampus pyramidal cells.

A real-time PCR test was used in the present study to express the gene. The results of the RT_PCR test indicated that GABAA- α 2 and GABAA- α 5 gene expression in the control+ group showed no significant difference compared to the intact group. So, the solvent had probably no effect on the two genes expression. The GABAA- α 5 gene expression in the group treated with 50 and 100 mg/kg dose of tarragon hydroalcoholic extract significantly increased in comparison to the control+ group. The two subunits of α 2 and α 5 of GABAA receptors play important roles in memory.¹⁰ GABAA receptors have also binding sites for compounds of benzodiazepine, barbitol and steroids; these receptors at the presence of GABA and in some cases directly, induce GABAA and activate the receptors' channel.³⁵ Since the high density of GABAA receptors in the hippocampus indicate that activating the GABAA receptors can control the process of learning and memory³⁶, it is probable that the memory disorder reported in this group is due to the increase of GABAA- α 5 gene expression, the increase of GABAA inhibitory synapses, the increase of time duration of such synapses and finally because of the more sensitivity of such receptors to the GABA. The present research result shows the overlapping of histology and RT_PCR tests, which is consistent with previous findings.

Zhao et al. found in research that injecting a low dose of isoflurane (anaesthesia substance) can decrease the GABAA- α 5 gene mRNA expression in hippocampus nerve cells in old ages, yet it is increased in young animals. These findings showed that L-655708 injection (agonist GABAA- α 5) inhibits spatial learning, memory disorder and spatial memory due to isoflurane in Wistar old rats.³⁷

Magnin et al. found in research that inhibiting GABAA- α 5 in rats controlled can also improve spatial learning compared to the rats having the inactive vasoactive intestinal peptide in the CA1 region.³⁸

Etherington et al. carried out a study that S44819 is a tricyclic peptide that can act as the chosen agonist of GABAA- α 5 Receptor, thus, S44819 causes the hippocampus synopsis flexibility and increases pre-recognition effectiveness. Regarding such features, S44819 might improve the recognition functions in neurological disorders and make it easier to recover after a stroke.³⁹

Vinnakota et al. conducted research indicated that α 5IA (3-(5-Methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3-a]phthalazine) injection, a reversed agonist of GABAA- α 5 receptors to Alzheimer induced rats could prevent cell destruction due to ($A\beta_{1-42}$) in a laboratory condition that might hold neuroprotective features.⁴⁰

Magnin et al. considered a relation between memory disorder and the increase of GABAA- α 5 receptor gene in hippocampus CA1 pyramidal cells.³⁸ Moreover, GABAA- α 2 gene expression in the group treated with 25 mg/kg tarragon hydroalcoholic extract showed a significant increase compared to the control+ group. Since the high density of GABA receptors in the hippocampus indicates that activating GABA receptors controls the process of learning and memory², it is probable that memory disorder in this group was due to the increase of GABAA- α 2

gene expression, the increase of GABA inhibitory synopsis, the increase of the synopsis time duration and the more the sensitivity of such receptors to GABA. These findings are consistent with the previous results.

Julian I. Hofmann et al. showed in research that prescribing diazepam to wild rats with GABAA- α 2 mutant gene resulted in changes in the automatic electronic activity patterns in brain cortex through using benzodiazepine so that the low-frequency increase of produced γ by diazepam might reflect the increased inside cortex harmony. By increasing the high-frequency band, diazepam decreases the frequency power through GABAA- α 2 receptors.⁴¹

Toshihiro Nomura et al. conducted research and found that enhancing subunits α 2 holding GABAA receptors in Dravet (severe Myoclonic epilepsy in infants) syndrome model can protect rats against convulsions.⁴²

CONCLUSION

Having different substances attached to the benzodiazepine sites in GABAA receptors, tarragon hydroalcoholic extracts might increase the activities of GABAA receptors as well as hyperpolarization through increasing GABAA- α 5 and GABAA- α 2 gene expression, and coumarin causes memory disorder in the hippocampus of male rats through decreasing the number of healthy pyramidal neurons. It should be noted that there is a need for extra studies.

Data Availability: The data will be provided if required.

Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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FIGURES

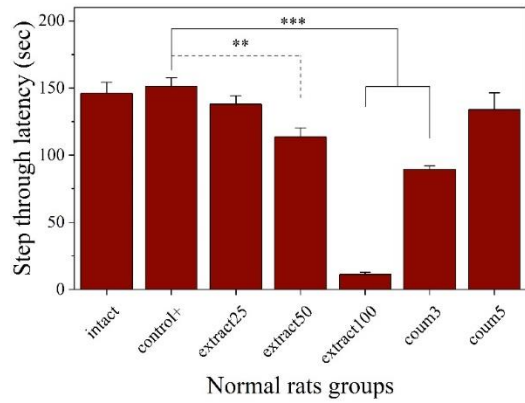


Figure 1. The effect of tarragon hydroalcoholic extract and coumarin on the delayed entrance of the male rats to the dark chamber of the shuttle box on the test day. The error bars represent the standard deviation of measurements for 8 particles in seven separate sample runs (n = 56).

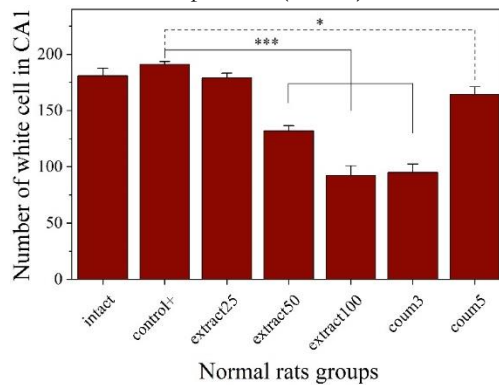


Figure 2. The effect of tarragon hydroalcoholic extract and coumarin on the number of pyramidal healthy neurons in. Cornu Ammonis (CA1) region hippocampus on Male rats (quantity study of histology). The error bars represent the standard deviation of measurements for 8 particles in seven separate sample runs (n = 56).

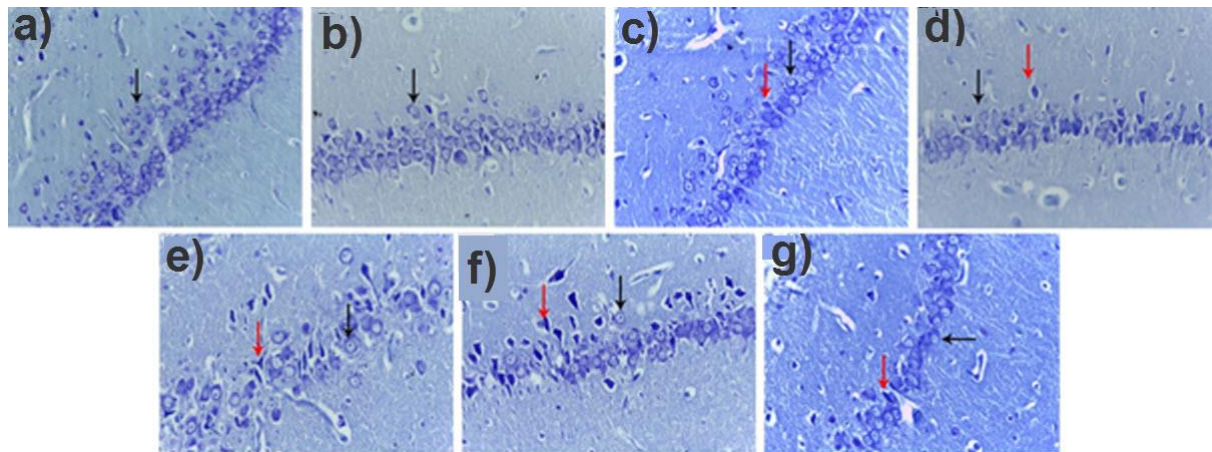


Figure 3. Histological images (quantity research) of the Nissl from the CA1 area of the hippocampus in the male rats.

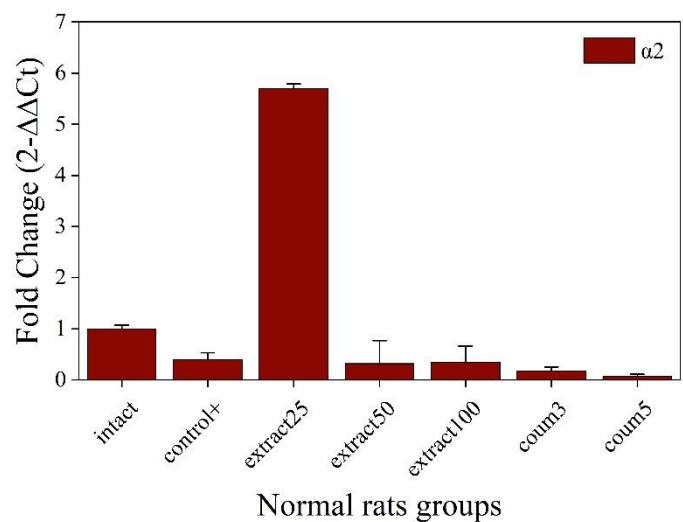


Figure 4. The relative level of mRNA GABAA- α 2 gene in the hippocampus of male rats treated with tarragon hydroalcoholic extract of 25, 50 and 100 mg/kg doses and coumarin with doses of 3 and 5 mg/kg compared to the control+ group. The error bars represent the standard deviation of measurements for 4 particles in seven separate sample runs (n = 28).

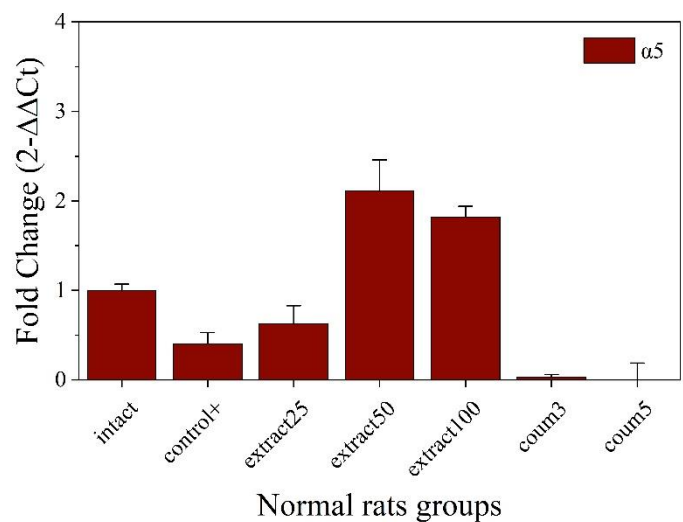


Figure 5. The relative level of mRNA GABAA- α 5 in male rat hippocampus treated with tarragon hydroalcoholic extract with doses of 25, 50 and 100 mg/kg and Coumarin with doses of 3 and 5 mg/kg compared with control+ group. The error bars represent the standard deviation of measurements for 4 particles in seven separate sample runs (n = 28).