April 30, 2021

PharmacologyOnLine

Archives • 2021 • vol.1 • 472-479

THE EFFECT OF *APIUM GRAVEOLENS* SEEDS METHANOLIC EXTRACTS ON ANXIETY AND THE *GABA*$_A$ GENE EXPRESSION IN MALE MICE.

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Abstract

Background: Anxiety is the unpleasant sensation of fear and distress. Excessive anxiety may be considered as an anxiety disorder. ***Apium graveolens*** seed has been found to have anti-anxiety role by producing a combing effect on the central nervous system. Data showed that both methanolic extract of apium graveolens and diazepam which is used as primary medicine in treatment of anxiety can bind to GABA receptor and thereby enhance its action to prevent the excitation.

Objectives: This study was conducted to examine the effect of methanolic extract of *apium graveolens* on anxiety and expression level of *GABA* gene in male mice. Also, to compare that with the effect of diazepam.

Methods: In comparison to diazepam (1mg/kg) a standard anxiolytic drug the efficacy of methanolic extract of *apium graveolens* (200mg/kg) was assessed. Anxiogenic and anxiolytic activity were measured by using open field test. $GABA_A$ receptor gene (2and 5 subunits) levels were measured by using Quantitative real time PCR after 30 days treatment with methanolic extract of apium graveolens.

Results: According to open-field test, the extracts significantly decreased the number of squares crossed, and number of grooming ($p<0.001$), while the duration of grooming was increased ($p<0.001$) in comparison to control (d.w) group. $GABA_A$ subunit 2 and 5 showed significant increase in the group which treated with *apium graveolens* (200mg/kg) when compared to control group.

Conclusion: methanolic extract of *apium graveolens* can treat anxiety and produce sedation by producing change in the molecular level of $GABA_A$.

Keywords: *anxiety, celery seeds, diazepam, open field, $GABA_A$ gene.*

Introduction

Anxiety disorders including obsessive-compulsive disorders, generalized anxiety, post-traumatic stress and phobias are quiet common and can be regarded as major cause of disability (1). Anxiety affects 1/8th of the total population worldwide and becomes a hot topic for psychopharmacology researchers during last decade (2). Nowadays, varying degrees of anxiety have become increasimgly common. This might be attributed to complexity of daily life in modern societies. Medical patients with chronic pain were found to have evident mood and anxiety disorders in both developed and developing countries (3). GABAergic, serotonergic receptors have been implied in anxiety disorders. Similarly, adrenergic and dopamanergic system could be potential factors in the development of anxiety disorders. (4). Currently, benzodiazepines (BZDs) are the most commonly prescribed medications for treatment of variable types of anxiety disorders. Because of BZDs adverse effects including dependence liability, psychomotor impairment and potentiation of other central depressant drugs, their clinical uses are limited (5). Diazepam act by targeting *GABA* $_A$ and the greater anxiolytic effect of benzodiazepines is mainly attributed to its binding to *GABA* $_A$ (subunit2) (6).

Apium graveolens is classified as one of the Apiaceae family and is famous as celery (7). *A. graveolens* extracts have different useful biological activities concerning its antibiotic activity (8). *A. graveolens* root and leaves extracts can act as antioxidants because they act as free OH and DPPH radicals scavenger besides liposomal peroxidation inhibitor (9). Celery seed by its combing effect can control nervous system. It produce sedative effect and stimulates sees drives (10). Celery seeds (apium graveolens) extract contain a 3n butylphthalide or (3nB) compound which is considered as powerful healing factor which explains its effect in lowering BP & cholesterol (11). Beside that, the ethnomedical evidence show that Apium graveolens seeds can elicit sedative activity. Moreover, Apium graveolens showed an important effect on the expression level of some genes like Sox17, *Pax6, Ins1, Ins2* in rats (12).

$GABA_A$ receptor channels are universal in the central nervous system of mammalian by increasing chloride ions intery that mediate fast inhibitory neurotransmission in response to GABA. There is a variety of GABA(A) receptor subtypes that are consist of a diverse subunits proteins. Alpha1 subunit preferring agents like benzodiazepine give sedative effects only with no anxiolytic effect. Many herbal medicines active ingredients like flavonoids and terpenoids are used to promote sleep and many of them have GABA(A) receptor modulation function. (13)

Primitive data showed that methanol water extract of whole parts of apium graveolens with a dose of 200 mg/kg is the best fraction that has the strongest sedation effect (19). It exert its role by binding to the GABA receptor so that GABA receptor activity increases, then causes the chloride channel to open. Opening the channel causes chloride to enter the cell, leading to hyperpolarization and decreasing excitation (14). Up to our knowledge, no previous work has evalauted the effect of methanolic extract of apium graveolens seeds on the level of $GABA_A$. Our study for the first time examine the effect of this extract on $GABA_A$ (mRNA) expression level in male mice with induced anxiety.

Methods

This study was performed under the supervision and approval of hammurabi medical collage research ethicas committee, with reference number 4 at 30th December 2020.

Preparation of Extract:

Celery seeds from local market were carefully washed, then left for air dried at room temperature, after that fine powder was gained by grinding. The plant methanolic extract was prepared by using Soxhlet apparatus for 36 hrs at 50-60 °C till colorless solvent in the siphon tube was appeared. Pellets of the extract were obtained by evaporation. MAG is the methanolic extract of *Apium graveolens* seed. The required dose for treatment was administered by stomach tube to each animal after preparation by dissolving the pellets in distilled water at a dose of 200 mg/kg daily for 30 consecutive days (15).

Animals: a 21 swiss adult male mice (25- 30 g weight) were used. In standard cages the animals were housed in the Babylon Medical College animal house, under 25 ℃ and 12 hours' light-dark cycles. A standard diet with tap water ad libitum.

Experimental design: The animals were randomly divided after 2 weeks of adaptation into 3 groups (in each group 7 mice) as follows:

Assessment of anxiolytic activity:

Group (1): They were treated with vehicle (0.3 ml d.w po) for 30 days.

Group (2): The MAG in a dose of 200 mg/kg as a single daily dose for 30 days treatment. Open field test done at day 31.

Group (3): animals were treated with 1 mg/kg oral diazepam 30 days. Open field test done at day 31.

Open field test

Under identical situations this test explore behavioral changes, and detect anxiogenic-anxiolytic activity in mice exposed to novel environments. A floor space of 100 cm x 100 cm with 50 cm walls hight were used. The black color floor area was divided by white lines into 100 equal squared. At the center of the field a mouse was placed and by using video camera (SONY/ Cyber-shot) for 5 minutes the following parameters were distinguished:

a. No. of crossed squares.
b. No. of times the animals stand on the near paws (Rearing against the wall).
c. Grooming No. and duration. (face washing, scratching, body and paw licking, body and genital grooming).
d. Time (seconds) spent in the central square

With 70% ethanol the floor of the open field was cleaned and allowed to dry between tests (16).

Primers Design

The primers of $GABA_A$ receptor alpha 2&5 subunits were designed as mentioned in previous papers [17]

RNA extraction from the brain tissue and cDNA conversion

After a month and following behavioral testing, the mice were scarified. Carefully, the brains were collected and washed in normal saline. The prefrontal cortex is dissected out immediately. The tissues were kept in RNAlater (Qiagen Inc., Valencia, CA, USA) and stored at 4℃ for one week. RNeasy Lipid Tissue Mini Kits (Qiagen Inc., Valencia, CA, USA) was used to isolate total RNA from brain tissues according to the manufacture's protocol. Purity of RNA was obtained by checking the optical density (OD) ratio and concentration of RNA was noted in ng/µl. RNA was converted to cDNA using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The sample was stored at -20°C until the polymerase chain reaction (PCR) analysis.

Quantitative Real-time polymerase chain reaction

The expression of $GABA_A$ mRNA from the brain tissue was tested by using a IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The reaction was performed in a total volume of 20 µl reaction mixture according to the manufacturer steps procedure. All the samples were run in triplicates. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the amplification efficiencies. The fold change in the target gene for the results of quantitative amplification was calculated according to the method used before [18].

Statistical analysis:

A 22.0 SPSS version was used for the statistical analysis, for multiple sample analysis (ANOVA) was used. Results were expressed as mean ± SD (19).

Results

Effects of methanolic extract on locomotion and exploratory activities in open field test.

The MAG group show extremely significant decrease in the number of squares crossed, and number of grooming ($p<0.001$), while the duration of grooming increased ($p<0.001$) compared to control (d.w) group. Number of Rearings, and time

spend in central squares were not changed (p>0.05). while the diazepam group showed highly significant increase in the number of squares crossed and duration of grooming (P<0.01), rearing against a wall, and time spend in central squares were extremely significant increased (P<0.01). While number of grooming showed extreme significant reduction (P < 0.001) compared to control (d.w) group (Table 1).

The effect of methanolic extract on the expression of mRNA *GABA*$_A$

Quantitative Real Time PCR showed that methanolic extract of of apium graveolens increases the expression level of *GABA*$_A$ (subunit 2& 5) in the mice, the same effect as that of diazepam on this gene.The fold change of *GABA* $_A$ (subunit 2 and 5) in the brain tissue treated with MAG are 2.65± 0.842and 2.44 ±0.846 higher than the level of control respectively, (P <0.05) (figure 1). The tissues of animals treated with diazepam also show higher fold change for *GABA*$_A$ (subunit 2 and 5) when compared to the control group (2.67± 0.854, 2.71±0.83. P<0.05) (figure 2).

Discussion

Evaluation of the effect of the extract on paradigms of depression and anxiety in the open field showed reduction in locomotor and exploratory activities (MAG decreased the number of squares crossed) which might be attributed to reduced excitability of the central nervous system, most likely due to central depression (20). Also the observed reduction in central square movements time could be due to impairment of the locomotor activity (21). Grooming activities is an expected displacement response in a new environment. Consequently, the grooming frequency reduction and duration increment may indicate stress reduction reliable with anxiolytic effect (22). It has been accepted that grooming behavior is linked to the lowering arousal following the stressful event (23). Our study revealed that number of grooming decreased in both the diazepam and MAG groups. This indicate that the MAG has the same anxiolytic effect of diazepam in stress conditions. Emotional activity parameters such as rearing in mice were not significantly affected by treatment with MAG.

From above results, we find that MAG have sedative effect presented by decreased locomotor activity and the time spend in central squares, in addition to reduced grooming, which indicates reduction of stress consequences and of the concomitant arousal (24). These effects may be due to *A. graveolens* phytoconstituents such as luteolin which is a naturally occurring flavonoid and has anxiolytic effects (25).

Current study agreed with Tanasawet *et al.* (2017) who evaluated different doses of *chinese celery* methanolic extracts of different doses (125, 250, 500 mg/kg orally). They found an anxiolytic effect in all doses with peak effect in a dose of 125 mg/kg due to the free radicals inhibition and MAOA activity modulation by which number of survival neurons may be increased as a result (26).

The dose of 1 mg/kg diazepam orally in the present study possess anti-anxity effect that is relatively different from a Tanasawet S *et al.* (2017) study in which the 2 mg/kg diazepam orally show anxiolytic effect (26).

Previous research showed that celery seeds extract can increase the expression of several genes (18). In their research, AlMalaak *et al.*2018 showed that Methanolic extract produce (produces) overexpression of Sox17, Pax6, Insulin1, Insulin2, and Glucagon genes which are already downregulated in rats suffering from Diabetes (12). Here, we proposed that methanolic extract can have an effect on the molecular level of *GABA* $_A$. Our idea is supported by the fact that methanolic extract of apium graveolens can bind to the GABA receptor to produce its effect as anti-anxiety substance (13). The results of this study for the first time show that methanolic extract increase the expression of *GABA* $_A$ (subunits 2& 5). Furthermore, it support that the effect of metathanolic extract on the GABA $_A$ receptor is the same as Diazepam. However, the effect of the methanolic extract on this gene is still unclear and further investigation are needed to identify wether this substance has direct or indirect influence on GABA gene expression level.

Conclusions

This study displayed that methanolic extract of *Apium graveolens* hold anxiolytics like effects and may have direct or indirect role on the GABA gene

expression. Its effect is similar to the effect of Diazepam.

Acknowledgments

Special thanks to the department of pharmacology and the animal house at the college of medicine for their assistance.

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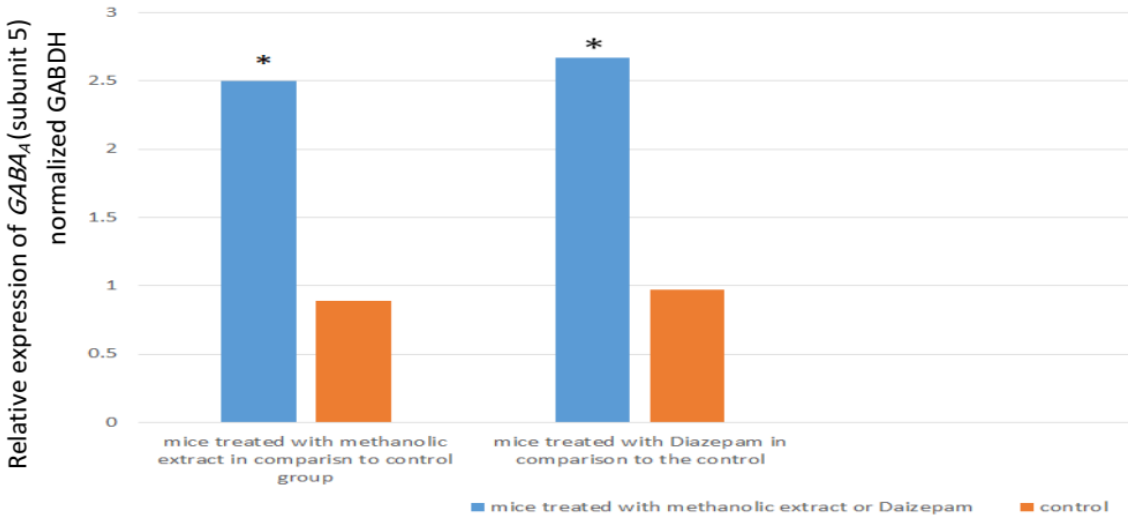
Table 1. changes in open field test seen by *A. graveolens* methanolic extract (200mg/kg p.o).

Groups	No. of Rearings	No. of squares crossed	Central squares time spend (sec.s)	No. of grooming	Grooming duration of (sec.s)
Group (1)	26 ±2.7	295±21.4	9±2.3	18.1±6.49	13.1±5.8
Group (2)	22.1±1.9	165.7±32***	9.2±11.6	7.00±2.16***	83.1±52.1***
Group (3)	33.4±3.4***	347.8±39.9***	152±30.3***	5.00±2.16***	72.1±19.7**

** p<0.01 *** p<0.001



* means P<0.05

Figure 1. Show significant increase in the level of *GABA$_A$* (subunit 5) in the brain tissues of mice treated with methanolic extract of apium graveolens or diazepam when compared to the control group.

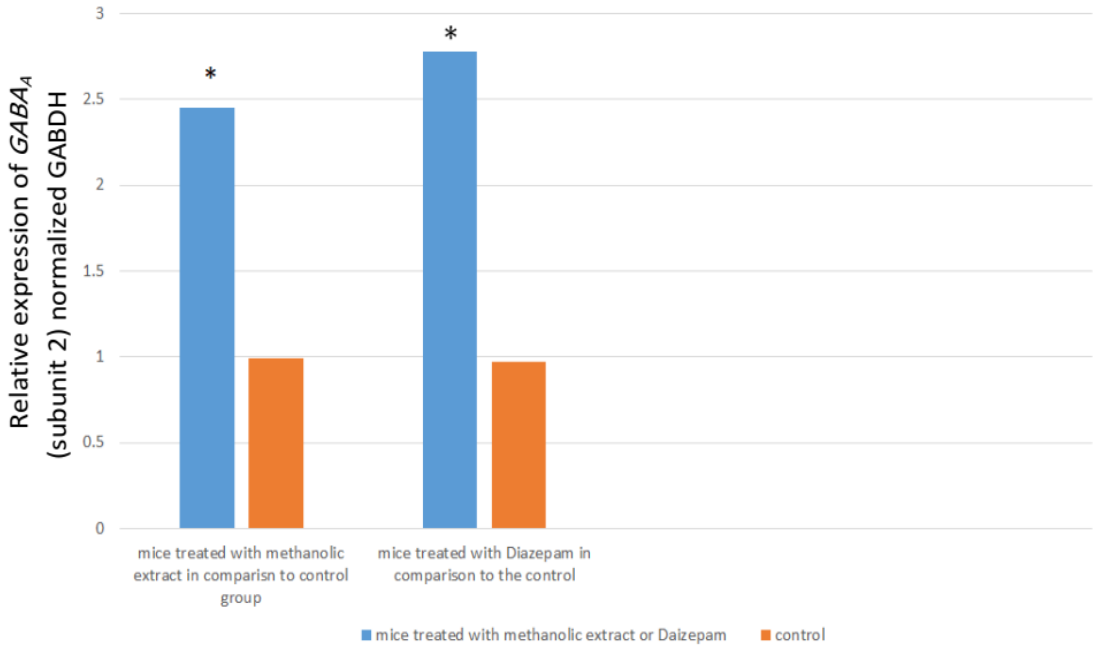


Figure 2: The fold change of GABAA (subunit 2) shows significant increase in both MAG and diazepam group when compared to the control group (P<0.05).