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The study on the effect of extract of *Cirtus aurantium* L. on anxiety of male laboratory mice and its interference with alfa2 adrenergic path

Gita Pournik*, Shahrzad Khakpour** and Mohammad Reza Bigdeli***

**M.Sc. Animal of physiology, Islamic Azad University, Zanjan Branch, Zanjan, Iran*
***Ph.D. Animal of Physiology, Azad University, Tehran Medical Branch, Tehra, Iran*
**** Shahid Beheshti University, G.C. Medical Department of Physiology, Iran*

(Corresponding author: Gita Pournik)
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ABSTRACT: The relation between individual's sociability and the amount of epinephrine and nor epinephrine of brain has been known clearly. Present study considered the effect of extract of *Cirtus aurantium* L. on anxiety and its interference with adrenergic paths. To do so, 98 small male mouse were classified in control and experimental groups. The injection of oil extract was done with 0.5, 2.5 and 5% doses for 10 days by intra peritoneal method. Injection of medicines (clonidine with1/1000 mg/kg dose and yohimbine with 1/100mg/kg dose) in 10th day and 30 min before injection of extract was done by intra peritoneal method. Then anxiety of mouse was evaluated by elevated plus-maze test. The data showed that the extract of *Cirtus aurantium* L. with 2.5 and 5% increased the number of entrances to open arms significantly ($p<0.05$); in addition dose of 2.5% increased the passing time in open arms significantly. Simultaneous injection of clonidine along with extract of *Cirtus aurantium* L. with dose of (0/5) increased the number of entrances to open arms significantly ($p<0.05$). Simultaneous injection of yohimbine and extract reduced the number of entrances to open arms. Regarding the results of present study, it is possible that injection of extract reduce anxiety.

Key words: anxiety, clonidine, extract of *Cirtus aurantium* L., elevated plus-maze test, small mice, yohimbine

INTRODUCTION

Anxiety is one of the important psychological concepts that has drew attention of researchers since previous ages. Understanding of interfered mechanisms can help us to find medical solutions, novel medicines and effective treatments for anxiety disorders (Watanabe et al., 1984). In traditional medicine, *Cirtus aurantium* is used as a common medicine for reducing neurological disorders and anxiety. In addition, it is known as calming, hypnagogic and appetizer medicine and can fix a lot of heart poundings (Zargari, 1992). Most of domestic and foreign studies have reported curing, calming, anti-inflammation and anti-oxidant effects of citrus aurantium L. (Pultrini, Carvalho-Freitas, 1999). According to side effects of calming medicines affecting nor adrenergic signals, present studies considered the effect of extract of *Cirtus aurantium* L. on adrenergic path.

Anxiety is one of the mental disorders that has involved lots of youngsters. Advanced tomography of the brain shows that the activity of cingulate cortex is more among anxious people.Anxiety can be classified in to primary or secondary types that is the result of mental or physical disorders specifically depression. Various neurological structures have interfered in anxiety, some of the most important structures are amygdala, hippocampus, septum, Locus Coeruleus and raphe nucleus. In anxiety trait a neurotransmitter and some parts of brain are not involved, but some neurotransmitters and some parts of brain are interfered. In most of the cases medical treatment can be helpful. The most common medicines for curing anxiety are: beta blocker adrenergic, benzodiazepines and agonists HT1A-5. Prescription of these medicines varies based on the severity of illness.

Although most of the drugs have side effects such as heart rhythm disorders, sexual dysfunctions, sleep disorders and hypertension. Therefore the curing direction should change toward effective drugs with lower side effects. According to production and market of herbal medicines, people welcome using the natural and herbal medicines.

Cirtus aurantium is one of the most important traditional medicine. The drug that is achieved from this plant is calming and eliminate tensions. This drug is the best for those who have anxiety and sleep disorders. Its consumption can be helpful in curing depression. Its skin and fruit have some materials such as synephrine-p and octopamine-p that are introduced as agonist of nor adrenergic signals.

The components of *Cirtus aurantium* are: glycoside and glycan flavors, poly myxin, coumarin, aldehyde, amino and monoterpene. The results of previous researches have reported the components of *Citrus aurantium* skin and the differences between components of ripe and unripe fruits. Synephrine-p and octopamine-p both are energetic agonists which are found in *Citrus aurantium*.

There are methods of evaluating the quantity of synerphrine in citrus skin. Limonene is the main component of *Citrus aurantium* skin. The contents of energetic amino such as tiramin, synephrine-p and octopamine-p have been determined by HPLC along with UV in fruits, extracts and herbal products. *Citrus aurantium* belongs to southern Asia and is a wild product of Venezuela. It distributes in Northern provinces of Iran, Fars and Kerman. In traditional medicine, dried fruit of *Citrus aurantium* is used for curing neurological disorders as a calming medicine. Therefore, according to the wide use of chemical medicines as anti-anxious drugs which affect alfa2 adrenergic signals and their side effects, the present study considered the anti-anxious effects of *Citrus aurantium* on alfa2 adrenergic signals. As a first step, this experiment was done on laboratory animals.

The aim of present study is considering the effect of intra peritoneal injection of extract of Citrus aurantium on anxiety and its interference with Alfa 2 adrenergic system. Eventually, it is tried to find whether the extract of *Citrus aurantium* can reduce anxiety or not?

MATERIALS AND METHODS

Study type- understudied population and maintaining conditions of mice

The present study is an experimental study on male …. Mice from NMRI family in the weight range of 20-30gr. The animals were bought from randomly of Baghiatalah University. They were classified in to 14 groups of 7 each. Each group were kept in clear plastic cages with dimensions of (45*30*15 cm) in standard environmental conditions (22-24°C) with lighting/darkness circle of (12/12h). Lightening started from 7A.M and they had enough water and food except experiment time; their location was changed every 3 days by sawdust. Mice were transferred to the keeping place one week earlier than injection time to habituate them. During the keeping, testing and injecting time, all of the regulations were observed. Each mouse was used once and then they were dismissed. Behavioral tests were done in lightening phase.

Experimental time and location

This study was done in animal physiology laboratory of Baghiatalah University of Tehran that lasted from November to December 2012.

Experimental method

Animals were kept in separate cages in groups of 7, a label was attached on each cage to show injection type, and then each animal was weighted and numbered by water proof marker. Then according to the weight of each animal, extract and drug were injected as intra peritoneal method and recorded. This lasted for 14 days and after that each mouse was tested by elevated plus-maze test; the time and number of entrances to open and close arms were recorded by clock and camera. Experimental time and conditions were similar for all groups. The results were compared after statistical analysis on graphs.

Elevated Plus-Maze (EPM) test

This type of animal is important to study the anxiety of rodents. The main advantage of this type of animal is its cheapness, simplicity and validity. This model was designed based on two types of instinct: seeking sensation and their avoidance from open, elevated and lightened environments. They spent most of their time in close arms and tended to enter to close arms rather than open ones. As Pellow et al described, this tool was made of wood and has 4 open arms. The dimension of each arm is 50*10cm. Two opposing arms had elevated walls with 40cm height called close arms. Two other arms that hadn't wall called open arms. To prevent from falling the mice glassy edges with the height of 1cm were located at two sides and at the end of open arms. Four open arms led to a central area with dimension of 10×10cm. The maze was located on the height of 50cm from the ground. Mice were located at the central area infron of open arm and required light was provided by a 100W lamp on the height of 120 cm from center of maze. During 5min that the mouse could move freely, it moved in different parts of maze. Following parameters were measured through observation:

1. The number of entrances to open corridors.
2. The number of entrances to close corridors.
3. The length of time that animal remained in open corridors.
4. The length of time that animal remained in close corridors.

The purpose of entrance to open and close corridor is the time when all four legs of animal is located in the corridor. The passing time is also calculated in this way. The evaluated traits of recorded parameters for each laboratory animal are listed as bellow:

1. The percentage of passing time in open arms in comparison with total passing time in open or close arm * 100 called OAT%.
2. Close Arm Time (CAT) means passing time in close arm.
3. Open Arm Entries (OAE%) means percentage of entries number that mice has entered to open arm.
4. Close Arm Entries (CAE %) means percentage of entries number that mice has entered to close arm.

Plant preparation:

In present study dried flower of Shiraz *Citrus aurantium* was used. This was bought from Kohestan grocery store in Tajrish bazar; it was milled and was used as powder. It should be mentioned that dried plants should be kept away from sun light to prevent its oxidation and evaporation of its extract.

Extracting Method

Water distillation method by Clevenger Apparatus instrument was used. This instrument belongs to chemistry department of Islamic Azad University, north Tehran branch.

First, dried powder was put in the water of distillation balloon by distilled water, so that 2/3 of balloon's volume was water and it was heated for 2h. Produced vapors containing extract molecules were transformed to liquid and gathered in receiver part after passing the condenser tubes. In addition, it is better that water covers the plant to have no contact with the body of instrument; because direct heat of balloon burns the plant and makes unfavorable smell, it also damages the main components of extract. To avoid such action, it is better to heat the balloon indirectly, for example a fireproof net can be put between flame and distillation balloon or the heat can be provided by chouf balloon. In this method some components of extract are hydrolyzed with water, therefore their quality can be affected, and its yield increases significantly. The water vapors condensate in condensation part, these vapors have lots of extract molecules. When the water is cooling, aforementioned molecules are cooling as well and separate from water in normal temperature. These molecules are oily so they can't combine with water molecules. The oily phase is light yellow. This extract was separated after 2h distillation (it usually takes 2-5 h). When it is determined that the volume of extract is fix, then distillation can be stopped. The extract should be poured to beaker carefully with Na2So4 without water to absorb water and moisture, then liquid extract should be passed from cotton or glass wool, Na2So4 absorbs water particles and transforms to a tough solid, the rest remains at the end of beaker as a white powder. Eventually it is washed by hexane and is poured to extract dish. The achieved extract should be poured in to a dark glass in 2-4°C temperature.

The preparation method of different doses of used drugs for injection

The drugs (clonidine and yohimbine) were solved in saline and proper dose for clonidine (1/1000 mg/kg of animal's weight) and yohimbine (1/100mg/kg of animal's weight) were prepared. The proper concentrations of *Citrus* aurantium in olive oil were 0.5, 2.5 and 5%. The proper doses of drugs were calculated based on animal's weight, then the injection was done as intra peritoneal method with insulin needle for 14 days. Then in 14th day, one hour after the last injection, the elevated plus-maze test was done and it was filmed to record the stop time and number of entries to open and close arms.

RESULTS

According to the result of variance analysis and figure 1, it is shown that yohimbine intra peritoneal injection increases the number of entrances to open arms and this had significant difference with control treatment.

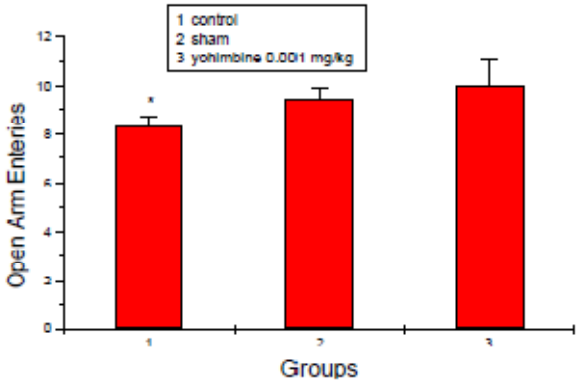

Fig. 1.

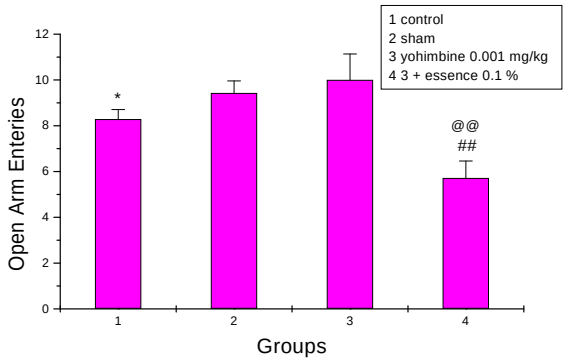

Fig. 2. Yohimbine intra peritoneal injection increases the number of entrances to open arms and this had significant difference with control treatment. Simultaneous consumption of yohembine and extract of Citrus aurantium with dose of (0/5) reduced the number of entrances to open arms and this had significant difference with control treatment.

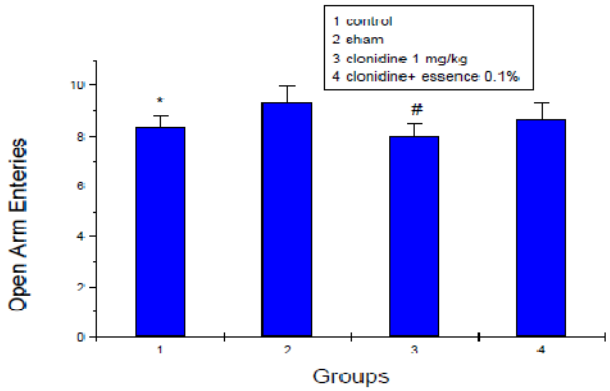

Fig. 3. Clonidine intra peritoneal injection decreases the number of entrances to open arms and this had significant difference with control treatment. Simultaneous consumption of clonidine and extract of *Citrus aurantium* with dose of (0/5) increased the number of entrances to open arms and this had significant difference with control treatment.

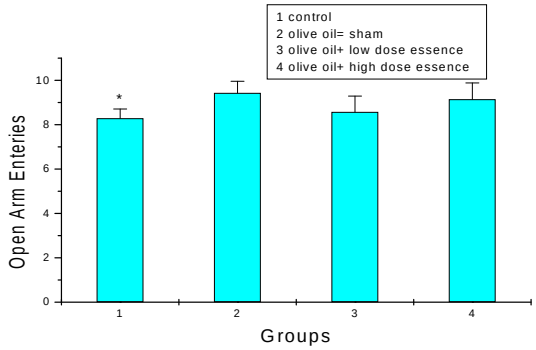

Fig. 4. Intra peritoneal injection of extract of *Citrus aurantium* with 5% dose increased the number of entrances to open arms that had significant difference with control treatment.

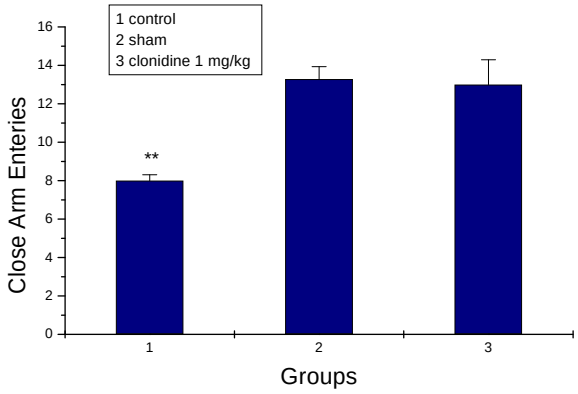

Fig. 5. Clonidine intra peritoneal injection decreases the number of entrances to close arms and this had significant difference with control treatment.

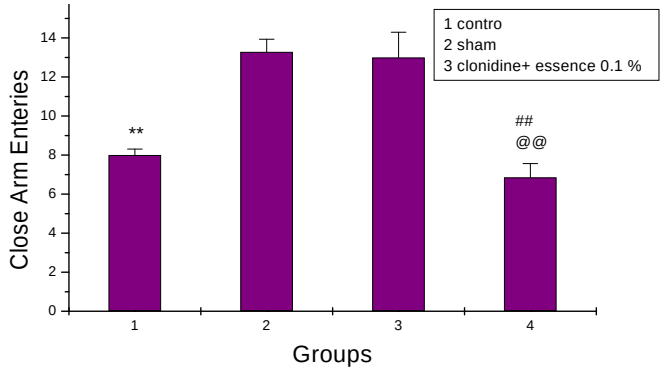

Fig. 6. Simultaneous intra peritoneal injection of clonidine and extract (0/5) reduced the number of entrances to close arms that had significant difference with control treatment.

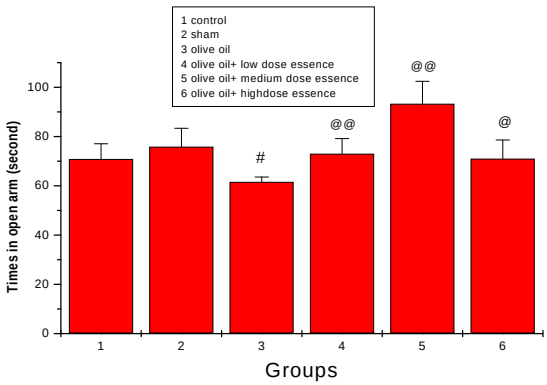

Fig. 7. Intra peritoneal injection of extract of *Citrus aurantium* with 2.5% dose increased the elapsed time in close arms that had significant difference with control treatment.

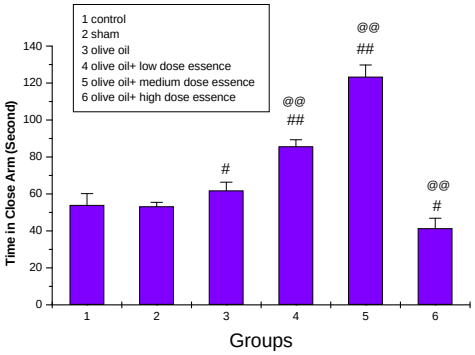

Fig. 8. Intra peritoneal injection of extract of *Citrus aurantium* with 5% dose decreased the elapsed time in close arms that had significant difference with control treatment.

DISCUSSION

Anxiety is an alarming sign that informs about an imminent danger and prepares the creature to confront with that threat. Anxiety increase individual's ability to confront with a problem and potential danger, this is an evolutionary act. In the other hand sever anxiety doesn't increase confliction ability, this kind of anxiety doesn't have a real source and is intensive. It may appear as an intangible to significant and sever form (Pultrini *et al.*, 2006). Present study considered the interaction effects of clonidine and yohimbine on adrenergic system about anxious behavior. To test the anxiety, elevated plus-maze test was used that is an acceptable test for studying anxiety of rodents, this test can be helpful to study the calming effect of drugs.

Citrus aurantium is an effective plant using in traditional medicine to cure depression and anxiety. Iran has a long history in using essences and decoction to cure various neurological disorders.

Hosseini *et al.* (2011) induced electronic shock and made depression to study anti-anxious effects of hydro alcoholic extract of *Citrus aurantium*, the showed that this extract reduced depression. Manchini *et al.*, (2011) showed anti-inflammatory, antioxidant and hypoglycemic effects of hydro alcoholic extracts of citrus. Khorio *et al.*, (2006) studied the effect of extract of *Citrus aurantium* on electro physiologic features of atrioventricular node of rabbit and indicated that citrus has anti-arrhythmias effect. Abbas Nejad *et al.*, (2012) showed anticonvulsant effect of *Citrus aurantium*. Previous studies have considered different effects of extract of *Citrus aurantium*. The components of extract of *Citrus aurantium* and its leaves are linalool, linalylacetate, limonene, limonoid, alkaloid and significant amount of flavonoids, these components have more concentration in shoots of citrus rather than leaves (Ghasemi Dehkordi, 1997; LM Lopes *et al.*, 2011). The extract of *Citrus aurantium* can act as giver of electron such as hydrogen and control free radicles. This extract can clear free radicles till 53.98%. Gamma-terpinene, geranyl, beta-pineneandmyrcene have high antioxidant activity and are present in this extract.

In a study it was determined that linalool can be effective in the incidence of induced seizures among mice (Elisabetsky *et al.*, 1999; Fukumoto, 2008). In a research that was carried out by Komiya *et al.*, (2006) it was determined that limonene has anti-anxious effects in mice. In addition Limonene has calming and anticonvulsant effects on animal model (Elisabetsky *et al.*, 1999); moreover, limonene of extract of *Citrus aurantium* enters to brain through peripheral circulation and reduces simultaneous and collective activity of neurological neurons and has anti-anxious effects (Re *et al.*, 2000).

One of the other effects of linalool reduces CNS activities and has calming effect by inhibiting the release of acetylcholine from synaptic terminals (Dwckers *et al.*, 2003).

Shabanian *et al.,* (1998) compared the effect of *Citrus aurantium* and diazepam in reduction of anxiety before surgery, they found that *Citrus aurantium* can be used as a prodrug that is effective in reduction of anxiety of patients before surgery. In a research anti-anxious and calming effects of *Citrus aurantium* showed that *Citrus aurantium* can increase sleeping time; and it is an anti-anxious compound (Carvalho-Fretas & Costa, 2002). Reinforcement the activity of heart and neurological system, prevention of creation of convulsion and anti-hysteria effects are other medical usage of this plant in traditional treatment (Guyton, 2000).

A thin layer (TLC) of components of Citrus aurantium was extracted through chromatography that showed octopamine-p and cinephrine-p that are biogenic amino both are alpha and beta agonists (Scott, 2004).

In an experiment that cinephrine-p (50 mg) was used as one dosage, it hadn't significant effect on blood pressure or heart beats.

Cinephrine-p is a beta agonists the same as ephedrine; this component can increase the amount of metabolism by increasing lipolysis and basic metabolism levels. In most of the cases these are not related to nutrition and can reduce the weight in long term (Airriess, 1997). Song *et al* considered anti-anxious effects of cinephrine on mice and showed oral administration of this drug (0.3-10 mg/kg) reduces the mice's immobile time. Lehrner (2000) indicated that distribution of extract of *Citrus aurantium* in waiting room of dental office reduces anxiety of patients.

According to aforementioned issues and the focus on the role of nor adrenaline and *Citrus aurantium* for curing anxiety, the effect of extract of this plant and alpha 2 adrenergic system were considered by using clonidine, agonist and yohembine drugs of alpha 2 adrenergic signals on laboratory mice through elevated plus-maze test.

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