OPEN ACCESS

ISSN: 2676-7473

RBMS.2021;26(1):e6

ORIGINAL RESEARCH

The effect of *Verbena officinalis* on stress oxidative factors and expression gen of NMDA receptor subunit GluN2B in male rat induced with scopolamine

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Date Received: June, 2020 *Date Accepted: September, 2020* *Online Publication: February 28, 2021*

Abstract

In traditional medicine, the *Verbena officinalis* is used to treat depression, seizure, jaundice, fever, anxiety, insomnia, abdominal problems, throat inflammation and thyroid problems. The aim of this study was to evaluate the effect of *Verbena officinalis* extract on scopolamine-induced memory impairment in rats. In this experimental study, 35 rats were randomly assigned into 5 groups: control group, scopolamine (0.7 mg/kg, i.p., injection) and scopolamine and *Verbena officinalis* recipients at concentrations of 100, 200 and 400 mg/kg. After 3 weeks of treatment, behavioral tests including passive avoidance memory were performed. The level of malondialdehyde and the antioxidant potential of the serum and brain of the rats were also determined. NMDA receptor subunit GluN2B gene expression was also measured. Treatment of rats with *Verbena officinalis* increased the secondary delay in the shuttle box test. Treatment with *Verbena officinalis* also reduced levels of serum and brain tissue malondialdehyde and also increased serum and brain antioxidant capacity. Treatment with 100 doses of *Verbena officinalis* extract increased the expression level of GluN2B gene in the brain. The findings of this study indicate that the extract of this plant can be a neuropharmacologic agent against memory disorders and oxidative stress induced by scopolamine in rats.

Keywords: *Verbena officinalis*, Memory impairment, Hippocampus, GluN2B

Introduction

Over the past decades, the subject of memory and cognitive impairment has attracted many researchers. Pharmacological studies on memory are carried out in hopes that behavioral findings, along with the mechanism of action of drugs, will be studied and clarified the basis of neurobiological memory and learning (Izquierdo et al.,2000). In this context, in much cognitive impairment, medications are used to enhance memory with the intended purpose. Alzheimer, as the most common cause of forgetting, is a progressive neurodegenerative disease, associated with loss of neurons in various brain regions and memory impairment (Dua et al.,2009). Symptoms of Alzheimer's disease include memory loss, loss of judgment and reasoning, changes in mood and behavior, cognitive decline, memory impairment, sleep disorders, and personality and mood changes (Dua et al., 2009). One of Alzheimer's pharmacological treatment strategies is the administration of a bunch of drugs which mechanism is based on the compensation of neurotransmitters (Akhondzadeh et al.,2003). However, the role of medicinal plants in memory and the improvement of symptoms caused by diseases such as Alzheimer's disease have been widely considered (Izquierdo et al.,2000). The increasing emphasis of pharmacology and pharmacy researchers is on the use of herbal medicines and finding more effective and appropriate compounds for the treatment of diseases. Medicinal plants, in addition to having effective ingredients, have other substances that in many cases prevent toxicity and their unwanted effects.

Verbena officinalis is a perennial herb, belonging to the *Verbenaceae* family, commonly referred to as "Vervian". It grows mainly in Europe and Asia, and is commonly found in agricultural land and non-agricultural land and near water stream, and is also cultivated in the north and west of Pakistan (Nasir, 1981).

In traditional medicine, *Verbena officinalis* was used to treat depression, hysteria, seizure, jaundice, fever, anxiety, insomnia, menstrual disorders, abdominal problems, malaria, throat inflammation, edema, cough, asthma, rheumatism and thyroid problems (Khare, 2008). The compounds isolated from the *Verbena officinalis* include verbenin, verbenalin, hastatoside, alpha sitosterol, ursolic acid, oleanolic acid, camphorol, luteolin, verbascoside, aucubin, apigenin and essence of limonene, cineole, spathulenol, AR curcumeme and scutellarein (Khan et al., 2016).

The neuroprotective effects of *Verbena officinalis*, including anti-epileptic and anti-anxiety effects, have been shown in animal models (Khan et al., 2016).Research has also shown that *Verbena officinalis* has analgesic and anti-inflammatory effects in animal models (Calvo 2006). According to the above, it seems that *Verbena officinalis* is effective against cognitive defects caused by neurodegenerative diseases. Therefore, the aim of this study was to evaluate the effect of *Verbena officinalis* extract on scopolamine-induced memory impairment in rats.

Materials and Methods

Preparation of Verbena officinalis extract

After preparation of *Verbena officinalis* and confirmation of the scientific name of plant, sample of plant with number 789056 was kept in Herbarium at Azad University of Izeh. Extraction was done by maceration method. The dried specimen was powdered by electric grinder and 5 times the resulting powder, 70% methanol was added. The liquid was placed on a magnetic rotator with a magnet and stored for 1 week at room temperature, after which the contents of the dish were flattened and the solution was placed in a rotary device to allow water and alcohol to evaporate. Finally, it was placed in an incubator at 37 degrees to dry (Khan et al., 2016).

Dpph radical deletion activity

At first, the extract stokes were prepared at concentrations of 1.0 mg/ml and DPPH at a concentration of 0.1 mmol (in ethanol). Using the obtained stokes, six concentrations of 5-100 µg containing 10, 20, 40, 60, 80, 100 for extract in a volume of 2 milliliters were prepared. 2 mg DPPH was added to each of the six concentrations of extracts and placed in the darkness for 15 minutes. A control tube containing 2 milliliters of ethanol and 2 milliliters of DPPH was prepared alongside the specimens. After 15 minutes, the spectrophotometer was zero at 517 nm with a blank of ethanol and the absorbance of the samples was read. Using the following

formula, IC50 was calculated (Fathi et al., 2015).

$$I(\%) = 100 \times \frac{A_{control} - A_{sample}}{A_{control}}$$

By drawing a chart, a concentration in which 50% of DPPH radicals were neutralized was obtained, in which the Y axis was the percentage of inhibition and X was the concentration of the extract. The concentration in which 50% of the DPPHs are neutralized is IC50 as milligrams of the dry matter of the extract (mmol of the antioxidant) or as an amount of DPPH molecules that are neutralized for each anti-oxidant substance molecule.

Animals

Animals In this experimental study, 35 male Wistar rats weighing 210-240 g were purchased from the Pasteur Institute of Iran. Animals were kept in special cages with 12/12 hour lighting-darkness period and 23 ± 2 °C. There were no restrictions on access to food and water for animals.

Experimental groups

Animals in this experiment were randomly divided into 5 groups (n=7) to study the effect of different concentrations of *Verbena officinalis* extract and determine the effect of effective concentration of extract on rats treated with Scopolamine:

• The control group received saline.

• Extract group with three subgroups, which received 100, 200 and 400 mg/kg concentrations of *Verbena officinalis* extract, respectively.

• A group that received scopolamine 1 mg/kg.

Extracts and scopolamine were dissolved in saline 0.9, and 2ml/kg were injected i.p., to animals for 21 days. After the treatment period, behavioral tests were performed. Then, the rats were placed under deep anesthesia and samples of blood and brain tissue were prepared.

Behavioral test

Passive avoidance memory test

The shuttle box was used for this test. The device consists of a plex box containing two bright and dark sections each measuring 20 x 40 x 20 cm. These two parts are linked by a guillotine door. At the bottom of these two sections, there are metal bars of 1 mm in diameter, spaced one cm apart, and electric shock is applied through the bars in the dark part to the animal's foot. Initially, the animal was placed in the clear section of the shuttle box to get acquainted with the device, and after 30 seconds the door opened to allow the animal to enter the dark area, according to the natural tendency of the dark environment. In this test, the initial delay was recorded at the entrance to the dark room. After the animal arrives in the dark area, a shock is immediately brought to the animal from the leg area (1 mill amperes, 1 second, 1 time), then the mouse was removed from the dark area and returned to the cage. Twenty-four hours later, each mouse was placed in a bright room to continue the test, the time interval between setting in the bright room and the entrance to the dark room was measured and expressed as the secondary delay time (maximum 60 seconds) (Kwon et al., 2010).

Measuring the total antioxidant capacity of the serum and brain tissue

Total antioxidant capacity of serum and homogeneous brain tissue were determined by FRAP method. The FRAP solution was prepared by adding 2.5 ml acetate buffer 0.25 mmol with pH = 3, 2.5 ml of 10 mmol TPTZ. FRAP work solution included 2.5 ml acetate buffer 0.25 mmol with pH = 3, 2.5 ml of 10 mmol TPTZ prepared in 40 mmol hydrochloric acid and 2.5 ml Iron(III) chloride hexahydrate 20 mmol. 25 µl of the serum or homogeneous tissue samples were mixed with 1.5 ml of the FRAP working solution, and after 10 minutes, optical absorption at 593 nm and 37 °C was read by the spectrophotometer (El-Sherbiny et al., 2003).

Measurement of malondialdehyde (MDA) in serum and brain tissue

200 µl of serum/homogeneous brain tissue were mixed with 1.5 ml of acetic acid 20%, 1.5 ml of thiobarbituric acid (TBA) of 0.8 and 200 µl of SDS solution of 1.8%. The samples were then placed in water bath for 60 min. The samples were then cooled, 1 ml of distilled water and 5 ml of n-butanol-pyridine mixture were added and shaken. The mixture was then centrifuged at 4000 rpm for 10 minutes and the optical absorption of the supernatant was recorded at 523 nm (El-Sherbiny et al., 2003).

Measurement of GluN2B gene expression

Samples of rat brain were frozen in an adequate volume of acid guanidium thyocianate solution and kept at -80°C until

RNA extraction. Total cellular RNA was extracted by the method of acid guanidium thyocianate phenol/chloroform extraction. Total tissue RNA concentration was measured by spectrophotometric absorbance (260 nm) and the quality of isolated RNA was verified by agarose gel electrophoresis with ethidium bromide staining. One µg of purified total RNA was used as substrate for reverse transcription. The reaction was performed by incubation of RNA with 1 µM oligo(dT) and 200 units of MMLV reverse transcriptase from a Clontech first strand cDNA synthesis kit. An aliquot (5 µl of a 1/10 dilution) of the cDNA of each sample was used for RT-PCR. The PCR primers used was shown in Table 1. DNA amplification was carried out in 1 x Taq polymerase buffer, 1.5 mM MgCI2 supplemented with 50 µM dNTP, 0.25 µM of 5' and 3'-specific primers, 1 µCi of [α-32p] and 2 units of Taq polymerase (Promega C) in a final volume of 50 µl. The mixture was overlaid with mineral oil and amplified for 30 cycles (each consisting of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1 rain at 72°C) then extension for 7 min at 72°C and storage at 4°C in a Triothermoblock. Ten µl of cDNA products were size-separated by electrophoresis on a 10% acryl/bisacrylamide gel and stained with ethidium bromide (15 µg/ml). Each band was excised from the gel and the quantity of 32p incorporated was measured in a scintillation counter (Rakesh e al., 2015).

Gens	*Forward*	*Reverse*
GluN2B	CTACTGCTGGCTGCTGGTGA	GACTGGAGAATGGAGACGGCTA

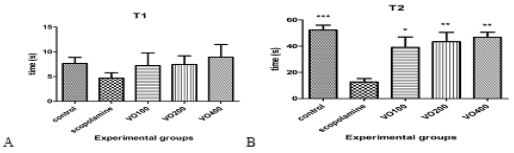
Data analysis

Data were analyzed using SPSS 21 software. One-way ANOVA and Tukey's post-hoc test were used to analyze the data. Data were expressed as mean ± standard deviation and values of P <0.05 were considered statistically significant.

Results

According to the results, IC50 for *Verbena officinalis* was estimated to be 80.421 µg/ml.

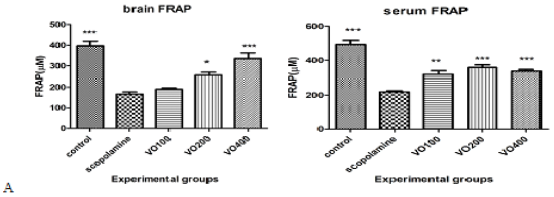
The results of the initial and secondary delay times in the shuttle box test are shown in chart 1 (A, B). According to the results, there is no significant difference between the experimental groups at the initial delay. Secondary delay in the scopolamine group was significantly lower than that in the control group. Treatment with *Verbena officinalis* extract with doses of 100, 200 and 400 mg/kg significantly increased the latency of the entrance to the dark room compared with the scopolamine group.



A B

Chart 1 (A, B): Effect of Scopolamine and Different Doses of *Verbena officinalis* Extract on Initial Delay (A), and Secondary Delay in Shuttle Box Test (B). * Indicates a significant difference at the level of p<0.05. ** indicates a significant difference at the level of p <0.001. VO: *Verbena officinalis*.

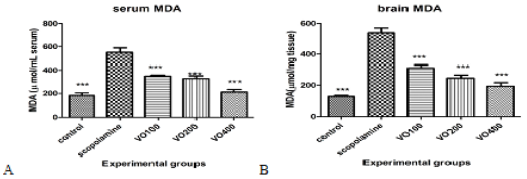
The results of serum and brain antioxidant capacity are presented in chart 2 (A, B). According to the results of animals received scopolamine, the antioxidant capacity of the serum and brain tissue is significantly lower than that of the control group. *Verbena officinalis* extract at doses of 100, 200 and 400 mg/kg significantly increased serum antioxidant capacity in compared to the scopolamine group. Also, *Verbena officinalis* extract at doses of 200 and 400 mg/kg significantly increased the antioxidant capacity of the brain tissue in compared with the scopolamine group.



A
B

Chart 2 (A, B): Comparison of serum (A) and brain tissue (B) antioxidant capacity between scopolamine and extract recipient groups. * indicates a significant difference at the level of p< 0.05. ** indicates a significant difference at the level of p <0.01 and *** indicates a significant difference at the level of p <0.001, VO: *Verbena officinalis*.

The results for serum and brain malondialdehyde in rats are shown in chart 3 (A, B). In rats received scopolamine, serum and brain MDA levels were significantly higher in comparison with the control group. *Verbena officinalis* extract with doses of 100, 200 and 400 mg can significantly decrease serum and brain MDA levels in compared to the scopolamine group.



A B

Chart 3 (A,B): Comparison of serum (A) and brain tissue (B) malondialdehyde levels between scopolamine and extract recipient groups. *** showed a significant difference at the level of p <0.001, VO: *Verbena officinalis*.

Regarding the results of Chart 4, treatment of 100 doses of *Verbena officinalis* extract in rats

increases the expression of GluN2B gene in the brain.

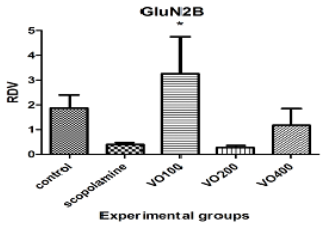


Chart 4. Effect of different doses of *Verbena officinalis* extract on gene expression in the brain. * Showed a significant difference at the level of p <0.05. VO: *Verbena officinalis*.

Discussion

In the present study, treatment of rats with *Verbena officinalis* extract increased the secondary latency in the shuttle box test. Treatment with *Verbena officinalis* extract also reduced levels of serum and brain tissue malondialdehyde and also increased serum and brain antioxidant capacity. Treatment with the dose of 100 of *Verbena officinalis* extract increased the expression level of GluN2B gene in the brain.

Recent evidence suggests that the NMDA receptor activity is associated with learning, memory, and cognition by adjusting the density of the dendritic column, synaptic plasticity, and synaptic power. The NMDA receptors have a heterotetrameric structure, consisting of two subunits of GluN1 and two subunits of GluN2 or GluN3, which together help the receptor function. GluN1 subunit is an essential component of the NMDA receptor, while more variation occurs at the level of GluN2 units. GluN2A and GluN2B are associated with higher brain functions and are expressed primarily in the hippocampus and the cortex (Ling et al., 2012).

GluN2A and GluN2B subunits of the NMDA receptor are involved in the development of LTP in spatial memory associated with the hippocampus and, by increasing the expression of GluN2B type, increase memory in adult mice (Jo et al., 2014). Recent studies confirm the possible role of NMDA receptors in various neurological disorders, such as epilepsy, Alzheimer's disease, Huntington and cognitive impairment (Cull-Candy et al., 2015; Fan et al., 2007). Pharmacological studies have shown that mice that do not have GluN2A / B subunits, have LTP disorders and therefore have memory defects (Huo et al., 2014). Changes in GluN2B expression in mice with memory impairment may indicate the role of NMDAR in learning and memory, and can also act as an evaluation for the molecular mechanism of the effects of herbal neuromodulator drugs. The results of this study indicate that extract of *Verbena officinalis* increases the expression of GluN2B gene in rat's brain and may increase memory in rats received scopolamine.

In this study, scopolamine was used to induce memory deficits in rats. Scopolamine is a muscarinic receptor antagonist, which causes temporary memory impairment and causes a pattern similar to that of Alzheimer's disease in animals (Caine et al., 1981). Studies have shown that memory disorders caused by scopolamine exposure are associated with cholinergic neuronal damage and reduced levels of acetylcholine in the brain tissue. Increased production of reactive oxygen species, followed by oxidative damage to the brain tissue and the structures involved in the learning and memory process are the main mechanisms for the neurological damage caused by scopolamine (Budzynska et al., 2015). Brain tissue is highly sensitive to oxidative damage due to high oxygen consumption, a relatively weak antioxidant defense system, the presence of high amounts of metallic ions producing free radicals, as well as substrates such as glutamate, ascorbate and free fatty acids that quickly enter redox reactions (Markesbery, 1997). Studies have shown that injection of scopolamine accelerates the peroxidation of brain lipids and weakens the activity of its antioxidant enzymes (Markesbery et a;l., 1997). In the present study, injections of scopolamine with a significant reduction in the antioxidant capacity of brain and serum and a significant increase in malondialdehyde were associated with lipid peroxidation, and treatment with *Verbena officinalis* extract significantly modified the changes. Considering the above mentioned, it can be stated that *Verbena officinalis* extract can prevent oxidative damage of neurons by decreasing oxidative stress parameters and subsequently preventing cognitive function degradation. The results of the DPPH test for *Verbena officinalis* extract indicate the high power of this plant to purify the DPPH radicals. Other studies also show that the methanolic extract of *Verbena*

officinalis has a high DPPH radical cleansing ability and can be considered as an excellent source of natural antioxidants (Casanova et al., 2008).

Inflammatory processes play an important role in the pathogenesis of degenerative changes and cognitive impairment associated with Alzheimer's disease (Heneka et al., 2015). Brain inflammatory markers (COX-1, COX-2, IL-1β, and IL10) have been reported to increase in the scopolamine-degraded memory model (Ahmad et al., 2014) Therefore, due to the anti-inflammatory activity of *Verbena officinalis* (Calvo et al., 2006), inflammatory mechanisms can be considered as the major mechanisms involved in plant activity, although in future studies it is necessary to prove this assumption.

The present study indicated the efficacy of *Verbena officinalis* in improving non-active avoidance memory in rats receiving scopolamine. The observed effects of the plant are probably due to its anti-oxidative stress and anti-inflammatory activity. Also, *Verbena officinalis* improvement effects may be due to increased expression of GluN2B gene in the brain.

Acknowledgments

This study was elicited from a master's thesis and thereby the researchers of this study would like to thank the Deputy Director of Research and Technology at the Islamic Azad University of Sanandaj.

Conflict of interest

Authors declare no conflict of interest.

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