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Research Paper

The Effect of Hydroalcoholic Extract of Salep On Pentylenetetrazole-induced Seizure in Adult Male Rats

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ABSTRACT

Background and Aim: Epilepsy as a neurological disorder is characterized by unpredictable and periodic seizures. In the present study, the anticonvulsant effect of different doses of Salep hydroalcoholic extract on pentylenetetrazole (PTZ)-induced seizures were investigated.

Methods and Materials/Patients: The animal model of seizure was established by the intraperitoneal injection of pentylenetetrazole (PTZ-85 mg/kg). Hydroalcoholic extract of Salep was administered to the animals in 3 doses (80, 160, and 320 mg/kg). The parameters of the onset and duration of tonic-clonic seizure, total seizure time, balance (falling), and jumping during seizure mortality rate was measured. The results were analyzed by analysis of variance (ANOVA) test at a significant level of $P \leq 0.05$.

Results: The results of the current study indicated that Salep extract increased the delay in the onset of seizure in the PTZ+extract group compared with the PTZ group. The duration of tonic-clonic seizure and the number of falling, jumping, and total seizure times were significantly decreased by salep extract compared with the PTZ group. In the seizure+salep extract, the number and percentage of 24-hour mortality among animals decreased with increasing dosage. All changes were dose-dependent, with 320 mg/kg showing the greatest effect.

Conclusion: Extracts of salep can probably have anticonvulsant properties. However, further studies are needed to clarify the exact mechanisms.

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Highlights

- Salep can probably have an anticonvulsant effect.
- Salep has a beneficial effect on seizures as an antioxidant herbal medicine.

Plain Language Summary

Epilepsy is one of the crucial neurological disorders recognized via seizures. In this case, oxidative stress was induced in brain cells during attacks. While reactive oxygen species (ROS) were produced, they could cause a lot of injuries to the cell, such as inflammation, or instability of cell membrane which was ruptured after a time course. Finally, the inflammatory mediators were released and transferred into interstitial space and disturbed other cells. We can protect them using herbal antioxidants. This idea is probably used as a defense mechanism against oxidation and to prevent the inflammatory effects of ROS. In this study, we tried to explore the effect of herbal therapy on seizures. Because of having side effects, chemical drugs can be more protective, therefore we created a seizure model using laboratory animals (rats) and pretreated them with Salep extract (orally) as an antioxidant. After 4 weeks, we considered some of the criteria of seizure attack between these groups of animals pretreated with different doses of Salep extract. We found that using this extract delayed the seizure attacks and decreased their duration. Falling, jumping, total time of seizure, and mortality rate also decreased.

1. Introduction

Epilepsy is a neurological and common disorder characterized by unpredictable and periodic seizures. About 1%-2% of the world's population experience seizures as the second most common cause of neurological diseases [1]. The prevalence of epilepsy is also estimated at 1%-3% of the total population in Iran [2].

Epileptic patients do not respond to one-drug treatment and multi-drug therapy has many side effects. In addition, 30% of patients with recurrent seizures are resistant to anticonvulsant medications [3]. Therefore, it is necessary to find some anti-epileptic medications with lower side effects. For many years, herbal medications were used to treat diseases [4], such as pulmonary fibrosis, epilepsy, and others due to their therapeutic aspects and lower side effects [4, 5].

One of the useful plants for the treatment of disease is the Salep plant which contains compounds, including nitrogenous substances, starch, protein, sugar, hydroxybenzaldehyde, ferulic acid, quercetin, daucosterol, cirsilineol, steroids as well as glucomannan [6-8]. Previous studies have shown that the hydroalcoholic extract of root-tubers of Salep (*Dactylorhiza maculata*) increases total antioxidant capacity (TAC) while decreasing lipid peroxidation indicators, such as malondialdehyde and total oxidant capacity (TOC) in the rat [9]. Pentylenetetrazole (PTZ) as an antagonist of the γ-aminobutyric acid type A (GABAA) receptor induces epilepsy [10, 11].

Epilepsy is characterized by an increase in neuronal excitability, which increases energy consumption at the synapses and increases oxidative stress [12, 13]. Neuronal discharges in epilepsy consume a lot of energy, therefore inducing oxidative stress.

Oxidative stress resulting from a deficit in peroxides and antioxidants balance leads to the accumulation of toxic free radicals, therefore it can damage lipids, proteins, or nucleic acids and finally results in mutation and cell death [3]. Previous studies also indicated that treatment with anti-epileptic drugs had no significant difference in the methyl-D-aspartate level [14].

On the other hand, recent studies investigated that the increase of internal antioxidants and the prescription of external antioxidants such as lipoic acid, maxidol, tocopherol, melatonin, resveratrol, and vitamins C and E have a protective effect against some damages induced by epilepsy on the brain [15, 16].

Previous studies on animal models of epilepsy showed an increase in oxidative stress in the epileptic rats, and antioxidant effects of Salep hydroalcoholic extract. Therefore, the present study assessed the effect of Salep hydroalcoholic extract on symptoms of epilepsy induced by PTZ in male rats.

2. Materials and Methods

Sampling

In this experimental study, 32 male Wistar rats with a mean weight of 19010 g were randomly divided into 4 groups of 8 according to Table 1.

These animals were taken from the Laboratory Animals Reproduction and Breeding Center in Jahrom University of Medical Sciences, Jahrom, Fars.

Rats were kept in the animal breeding room of Jahrom University of Medical Sciences for one week to adapt to the condition. Light-dark cycle consisted of 12 hours of lightness and 12 hours of darkness and ambient humidity was about 50% to 55%. The temperature was 23± 2°C. The prescribed concentration of Salep was specified in the amounts of 80, 160, and 320 mg per kg of body weight, according to previous articles and a pilot project [9].

In Vivo treatments

Different concentrations of Salep extract in a volume of 0.5 cc were injected intraperitoneally only once. In the evaluation process of seizure, a solvent or extract was injected intraperitoneally. After 30 minutes, 85 mg/kg of pentylenetetrazol was injected intraperitoneally into the animal. Rats were transferred to a separate cage immediately after the injection, and within 30 minutes of observation (onset time of a clonic seizure, onset time of a tonic seizure, duration of tonic-clonic seizure, total time of seizure, balance-falling position) and jumping during seizure up to 30 minutes. The mortality rate of rats in each group was assessed up to 24 hours later. The seizure stage was determined using the definitions given for each seizure below [17]:

Tonic phase: Severe stiffness of the muscles and stretching of arms and legs to the sides.

Clonic phase: A short period of seizure with twisting movements of the head and neck and movements of the hands and intense jumps and turns.

Tonic-clonic phase: Generalized seizures with sudden tonic spasms and stable epilepsy positions with very short jumps.

Seizure duration: Duration of seizure from the beginning to the end of seizure after PTZ injection.

Falling: Losing rats' balance due to the seizure and lying on one side of the body.

Jumping: Sudden jumps higher than 20 cm above the ground.

Mortality: This quantity of seizure is discrete and recorded after observation. If the seizure is excessive, some rats die.

Salep extract preparation

We combined 100 grams of fresh and chopped root of Salep plant with 500 cc of ethanol 96 degrees and then put the mixture in a rotary machine for 24 hours at room temperature to achieve a homogeneous mixture. Then, the filtered solution was centrifuged at 3000 rpm for 5 minutes to ensure the separation of suspended particles in the plant. The obtained solution was placed in ambient conditions for 48 hours to convert into a solid, dry, alcohol-free extract. The obtained dry extract was dissolved in distilled water [9].

Determination of extract purification

Phenol determination

First, 1 mL of phenol solution was combined with 200 µL Salep extract. Then maintained in a dark place for 6 minutes. After that, 2 mL of $Naco_3$ (7%) was added and kept for 120 minutes. The solution absorbance was measured at a wavelength of 765 nm. Finally, the total phenolic amount of extract was calculated using the standard curve of gallic acid and reported based on mg/(GAE)$^{4/g}$. [18] (Table 2).

Table 1. Groups of animals

Control-PTZ	Experimental Group 1 (PTZ+Sa 80 mg/kg)	Experimental Group 2 (PTZ+Sa 160 mg/kg)	Experimental Group 3 (PTZ+Sa 320 mg/kg)
Normal saline+pentylenetetrazole at a dose of 85 mg/kg	(80 mg/kg of Salep extract+pentylenetetrazole at a dose of 85 mg/kg)	160 mg/kg of Salep extract+pentylenetetrazole at a dose of 85 mg/kg	(320 mg/kg of Salep extract+pentylenetetrazole at a dose of 85 mg/kg

Table 2. Total antioxidant activity determination. assessment of phenol, anthocyanidin, and flavonoid concentration in Salep extract. Salep concentration was 100 mg/mL

Anthocyanidin (mg/L)	Phenol (mg/mL)	Flavonoid (µg/mL)
15.32	8.81	40.96

Flavonoid determination

The total flavonoid measurement was calculated based on the method of Dharmadasa et al. and reported using the quercetin standard curve as mg(QE)$^{5/g}$ of extract [19] (Table 2).

Anthocyanidin determination

The measurement of anthocyanidin was done according to the Tali Gene Pars kit and its concentration was reported based on mg/L in the extract (Table 2).

The antioxidant activity of the extract was assessed using reactive oxygen species (ROS) inhibitory method called 2,2-diphenyl-1-picryl-hydroxyl-hydrate (DPPH). First 1 mL of Salep extract was combined with 3 mL of methanolic solution of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH). Then it was kept in a dark and warm place for 30 minutes. The absorbance of the solution was read at a wavelength of 517 nm. Methanol was also used as a blank sample [20] (Table 2). Finally, the inhibitory activity was calculated based on the Equation 1:

1. % Inhibitory activity=$[(A_{control}-A_{sample})/A_{conntrol}]\times100$

Drugs

PTZ was purchased from Sigma-Aldrich Co. The drugs were dissolved in a saline solution (0.9%) and injected intraperitoneally (i.p.) in a volume of 1 mL/kg of the rat's body weight.

Statistical analysis

SPSS software, version 25 was applied for statistical analysis. Data are shown as Mean±SEM. P<0.05 is considered significant. One-way analysis of variance (ANOVA) with the Duncan post hoc test was used to compare the groups.

3. Results

Effect of Salep extract on latency of tonic seizures in PTZ-induced rats

The Salep extract manifested dose-dependent effects against PTZ-induced seizures. The latency of tonic seizure (LTS) can reduce convulsing animals in the control group. Pretreatment with extract (160 and 320 mg/kg) significantly increased latency and attenuated PTZ-induced seizures (Figure 1).

Histograms represent Mean±SEM for eight animals. Different letters showed P<0.05, versus the PTZ group by ANOVA with Duncan's test.

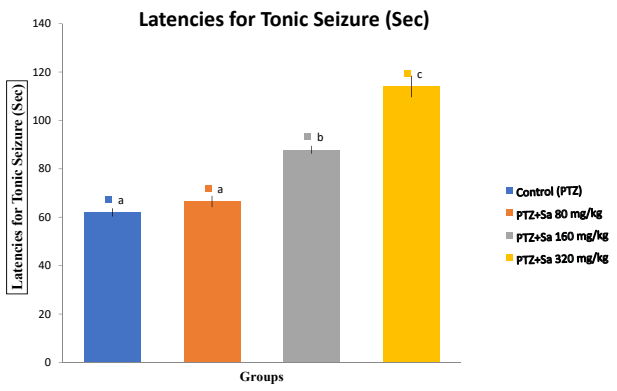

Figure 1. The effect of Salep extract on the latency for tonic seizure in PTZ-induced rats

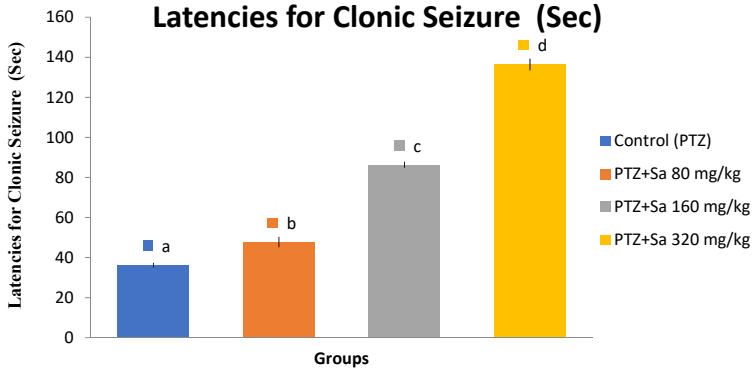

Figure 2. The effect of Salep extract on the latency for clonic seizure in PTZ-induced rats

Effect of Salep extract on the mean time changes of the onset and the latency of clonic seizure (LCS)

The extract at doses of 80, 160, and 320 mg/kg can significantly alter the latency for clonic seizures compared with PTZ-treated rats (Figure 2).

Histograms represent Mean±SEM for eight animals. Different letters showed P<0.05, versus the PTZ group by ANOVA with Duncan's test.

The onset and duration of tonic-clonic seizure time (DTCS)

The PTZ injection increased the onset of tonic-clonic seizure time which was significantly attenuated by extract (80, 160, 320 mg/kg) (Figure 3).

Data represent Mean±SEM (n=8). Different letters showed a significant difference, (P<0.05) compared with the PTZ-injected group according to Duncan's test.

Evaluation of the (balance-falling) position

The PTZ-treated rats showed a reduction in falls. Alternatively, it was significantly increased in the PTZ-induced group (Figure 4).

Data represent Mean±SEM (n=8), and different letters showed P<0.05 compared with the control.

The effect of Salep extract on jumping activities in the PTZ-treated animals

The rate of jumping activities increased following the PTZ administration. However, the extract (160 and 320 mg/kg) can significantly prevent the effect of PTZ on jumping activities (Figure 5).

Data represent Mean±SEM (n=8). Different letters showed P<0.05 compared with the control non-treated group according to Duncan's test.

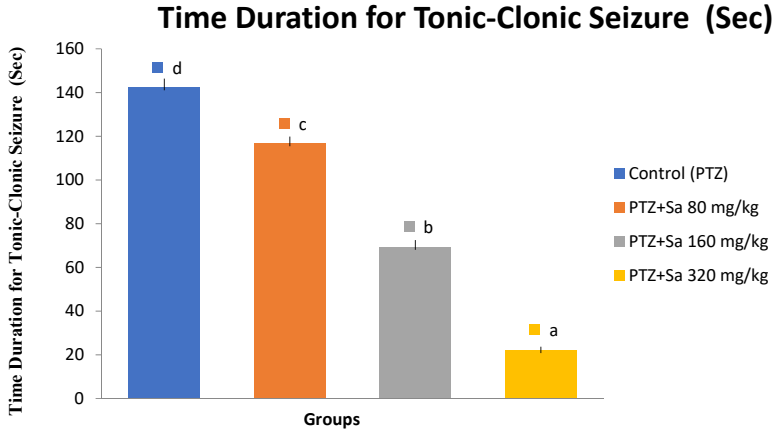

Figure 3. The effect of Salep extract on the mean time changes of tonic-clonic seizure duration in the PTZ seizure models

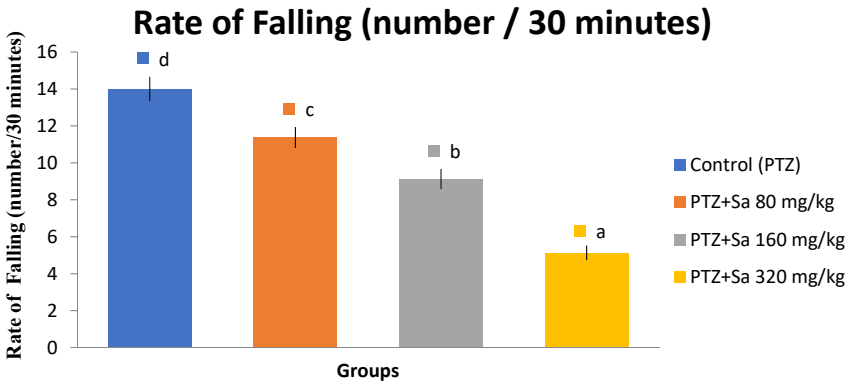

Figure 4. The effect of Salep extract on the falling levels in the rat PTZ seizure models

Time changes of seizure duration (Mean±SD)

According to the obtained results of Salep extract, an increase in duration times (min) of seizure was observed. The reduction was observed after pretreatment with Salep (Figure 6). The means in each column, which have at least one common letter, do not have any significant difference, according to Duncan's test.

The effect of Salep extract on mortality rate in the PTZ seizure model

Compared with the control group, all groups receiving Salep extract represented a reduction in mortality rate. The highest effect belonged to the concentration of 320 mg/kg (Figure 7).

The averages in each column, which have at least one common letter, did not have any significant difference, according to Duncan's test.

4. Discussion

The results of the present study showed that Salep extract has beneficial properties for seizure. Salep extract delayed the occurrence of tonic-clonic seizure and decreased the duration of the tonic-colonial phase and the total time of the seizure. It also decreased the rats' mortality rate up to one day after inducing a seizure and decreased the number of jumps and falls in rats. Decreasing the duration of seizure after injection of Salep extract was one of the crucial findings in this study.

Oxygen free radicals appear to be involved in epilepsy induction and post-seizure neurological disorders. It had previously been indicated that PTZ induced epileptic seizures via oxidative stress by decreasing antioxidant defense systems and increasing lipid peroxidation in erythrocytes, the liver, and the brain. Decreasing oxidative stress and preventing the activity of antioxidant enzymes leads to reducing the death of brain cells during seizure [21]. Therefore, Salep extract can play a role in protecting neurons against oxidative stress and preventing their death, through its antioxidant properties.

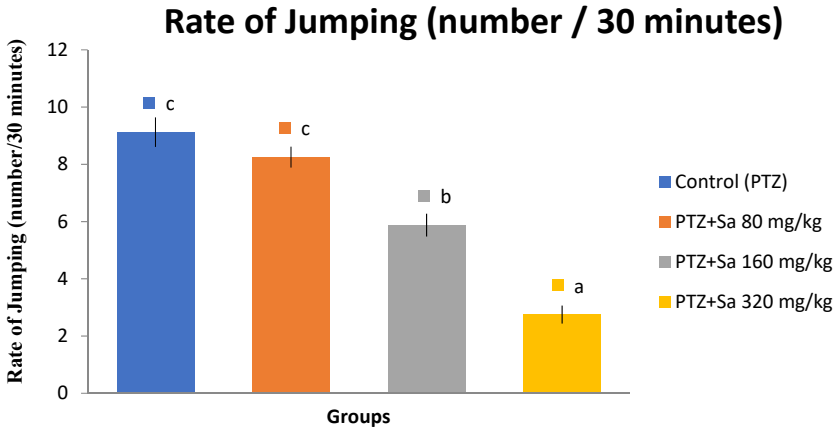

Figure 5. The effect of Salep extract on jumping rating activity in the PTZ seizure model

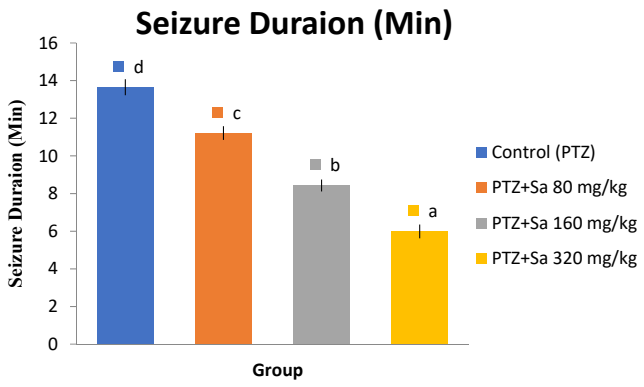

Figure 6. Comparison of the mean of the different groups about Mean±SD based on Duncan's test

nan fiber, nitrogenous substances, starch, protein, sugar, hydroxybenzaldehyde, ferulic acid, quercetin, dao cholesterol, garlic, and steroids [6]. Some of these components have antioxidant activity.

Previous studies indicated that quercetin is vital as an anti-carcinogenic, anti-inflammatory, antiviral, and antioxidant agent [17, 18]. For example, quercetin administration can decrease histological aspects of acute inflammation in animals by inhibiting the release of chemokine and lipid peroxidation and increasing antioxidant enzyme activity [19]. Quercetin also shows a neuro-protective function in several central nervous system disorders, such as seizures and Huntington's disease [20, 21] (ameliorating effect of quercetin on epilepsy by inhibition of inflammation in glial cells).

On the other hand, a study on the antioxidant properties of Selinum vaginatum (Edgew) Cl has shown that ferulic acid in this plant contains phenolic compounds and is used in traditional medicine to treat some nervous system diseases, including epilepsy [22]. The rate of glu-

Salep plant contains compounds including glucoman- comannan has been observed between 7% to 61% in dif-

ferent species [23]. Glucomannan polysaccharide acts as an analgesic component in animal models of acute and chronic pain due to its antioxidant and anti-inflammatory properties [24]. According to the researchers' report, the analgesic effects of glucomannan are associated with the inhibition of inflammatory reactions as well as the inhibition of neurotransmitters involved in the pain pathway, such as glutamate and N-methyl-D-aspartate (NMDA) [25]. In a study of the compounds of Satureja khuzestanica Jamzad, it is shown that this plant contains Cirsilineol, which is a derivative of flavonoids and has antioxidant, anti-inflammatory, and anti-diabetic effects [26].

5. Conclusion

The present study confirms that the induction of epilepsy by PTZ leads to a decrease in seizure onset delay and an increase in the duration of tonic-colonic seizures and the number of falling, jumping, and total seizure times. Salep extract appears to have positive effects on these impairments. Though possible mechanisms of

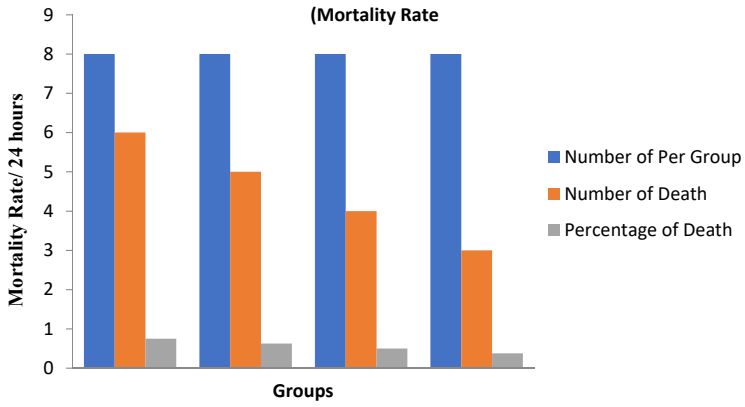

Figure 7. Comparison of the mean of the different groups about mean of 24-Hour mortality rate based on Duncan's test

this process were not evaluated in the current study, according to previous studies, it seems that the protective effects of Salep extract are probably due to its antioxidant effect which requires further studies to determine its exact mechanisms in the future.

Ethical Considerations

Compliance with ethical guidelines

In this study, all ethical issues regarding working with laboratory animals were considered. The research was approved by the Research Ethics Committee of Jahrom University of Medical Sciences (No.: IR.JUMS.REC.1395.151).

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Authors' contributions

Conceptualization and study design: Hossein Kargar Jahromi; Data collection: Shekoufeh Atashpour and Maryam Jalali; Data analysis and interpretation: Arman Khurram; Writing the original draft, review, editing and critically revising: All authors.

Conflict of interest

The authors declared no conflict of interest.

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