Title: Effect of Hydroalcoholic Extract of Satchys Lavandulifolia on Pentylenetetrazole-Induced Seizures in Male Mice: The Role of Gabaergic and Opioidergic Systems

Running title: Effect of Satchys Lavandulifolia Extract on Pentylenetetrazole-Induced Seizures

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Abstract:

Introduction: Epilepsy is one of the most common neurological disorders. Though there are effective medications available for treatment of epilepsy, the use of most drugs is associated with many side effects and drug interactions. Stachys lavandulifolia (SL) used in Iranian traditional medicine show anti-anxiety and sedative actions. The objective of the current study was to evaluate the anticonvulsant effect of hydroalcoholic extract of SL on the pentylenetetrazole (PTZ)-induced seizure in male mice and the role of benzodiazepine and opioid receptors.

Methods: This study was conducted on 100 male mice randomly categorized into 10 groups: Normal Saline, Diazepam groups (0.025 and 0.1 mg/kg), SL extract groups (50, 100 and 200 mg/kg), Diazepam 0.025 mg/kg + SL extract 50mg/kg and three groups that pre-treated with NS, Flumazenil or Naloxone, 5 min before injection of 200 mg/kg extract. After 30 min, PTZ (80 mg/kg) was injected to animals and seizure indices were evaluated.

Results: The SL extract attenuated the PTZ-induced seizures in a dose dependent manner and pre-treatment with flumazenil reversed this effect of SL extract but pre-treatment with naloxone could not reverse this effect, because seizure indices on naloxone pretreated group was still lower than normal saline group. Combination of ineffective dose of diazepam and SL extract decrease PTZ-induced seizures.

Discussion: The results of our study showed the anticonvulsant properties of hydroalcoholic extract of SL. These effects might be due to the impact of the components of this extract on the central benzodiazepine system.

Keyword: Satchys lavandulifolia, PTZ, Seizure, Flumazenil, Naloxone
1. Introduction

Epilepsy is one of the most common neurological disorders after stroke and characterized by recurrent seizures due to abnormal excessive or synchronous neural activity in the brain (Katzung, Masters, & Trevor, 2012). Seizure arises as a result of excessive excitation or loss of inhibition in the brain (Stafstrom, 2010). Although there are many anticonvulsant drugs on the market, the treatment of epilepsy remains still inadequate and one-third of patients suffered from recurring epilepsy despite using different anti-epileptic drugs and multidrug regimens (Mohanraj & Brodie, 2006). Moreover, more than 50% of epileptic patients show side effects of anti-epilepsy drugs during treatment (Krug, Koch, Grecksch, & Schulzeck, 1997). So, there is a necessity to perform more comprehensive studies to develop more effective anti-epilepsy drugs with the minimum side effects. Ethnopharmacology and medicinal plants are considered as new fields of interest.

Utilization of animal models is a proper way to establish epileptic models in order to identify the mechanisms involved in such diseases, evaluate novel anti-epileptic medications and finally reach the efficient therapeutic approaches in epileptic patients (Löscher, 2002; Trojnar, Wojtal, Trojnar, & Czuczwar, 2005). Pentylene-tetrazole (PTZ) is one of the derivate of tetrazol and acts as an antagonist of Gama amino butyric acid (GABA), which can leads to epilepsy in animal models through the inhibition of GABA\(_A\) receptor (Löscher, 2002; Mohammadi-Khanaposhtani et al., 2016) and blockade of chloride inflow (Naseer, Shupeng, & Kim, 2009).

In recent years, a plenty of studies have been conducted on the application of medical plants and Stachys lavandulifolia (SL) is among the Iranian traditional medicine with approved anti-anxiety and sedative features (Kumar & Bhat, 2014; Rabbani, Sajjadi, & Jalali, 2005). This plant has attributed to many therapeutic applications such as: anti-oxidative feature (Saeedi, Morteza-Semnani, Mahdavi, & Rahimi, 2008), sedation and anti-inflammatory function (Delfan, Bahmani, Rafieian-Kopaei, Delfan, & Saki, 2014), anti-anxiety (Rabbani et al., 2005), anti-diarrhea (Bahmani et al., 2014) and analgesic (Hajhashemi, Ghannadi, & Sedighifar, 2007). Some studies have mentioned the sedative and anti-inflammatory function of SL and its significant effects on anxiety have been approved in comparison to diazepam. It is proposed that the mentioned actions may be attributed to the presence of flavonoid, propanoid, and terpenoid components (Monji, HOSSEIN, Halvaei, & ARBABI, 2011; Neshat, Pour, &
Balanejadj, 2017; Rabbani et al., 2005). Nasri et al. showed that the hydroalcoholic extract of SL aerial parts has analgesic and anti-inflammatory effects in male mice (Nasri, Ramezanghorbani, & Kamalinejad, 2011). In addition, Naseri et al. show that chloroformic fraction of SL extract had the spasmylytic effect on ileum contractility of mice that this effect mediated mainly via disturbing the calcium mobilization and partly by opioid receptors' activation (Naseri, Adibpour, Namjooyan, Rezaee, & Shahbazi, 2011). Overall, considering the anti-anxiety, analgesic, and sedative effects of the hydroalcoholic extract of SL, it seems that it might possess anti-convulsive effects too. So, in this study, we explored the effect of intra peritoneal injection of hydroalcoholic extract of SL on the PTZ-induced convulsion in male mice and the role of benzodiazepine and opioid receptors.

2. Methods:

2.1. Animals:

This study was conducted on 100 mature male mice weighing from 25 to 30 gr. The mice were purchased from Pasteur institute (Karaj, Iran) and were subject to 12 h day-night circumstances at 23°C temperature and were fed ad libitum in the animal room of Guilan University of Medical Sciences. After one week accommodation time, the animals were randomly categorized into 10 groups (n=10) as following: normal saline (NS) group, Diazepam positive control groups (0.025 and 1 mg/kg (Rashidian et al., 2017)), 4 & 5 & 6: hydroalcoholic extract of SL groups (50, 100, and 200 mg/kg) (Nasri et al., 2011; Rabbani et al., 2005), 0.025 mg/kg Diazepam + 50 mg/kg SL extract (the simultaneous injection of extract ineffective dose and diazepam), NS + 200 mg/kg SL extract, Flumazenil 2 mg/kg + SL extract 200 mg/kg, Naloxone 5 mg/kg + SL extract 200 mg/kg. In the last 3 groups, normal saline, flumazenil, and naloxone were injected exactly 5 minutes before (Rashidian et al., 2017) the injection of SL extract, respectively (Fig. 1). In all of study groups, convulsion dose of PTZ (80 mg/kg) were injected 30 minutes after the administration of mentioned interventions (Keshavarz, Fotouhi, & Rasti, 2016). Drugs and saline injections were performed intra peritoneal (i.p.).

2.2. Drugs:

PTZ was purchased from Sigma-Aldrich and ampoule flumazenil was purchased from Hmlen, Germany. Naloxone and diazepam were purchased from Tolid daru (Iran) and daru pakhsh
The medications were injected intraperitoneally with 5 ml/kg volume. All drugs and extract were freshly prepared to the desired concentration just before used.

2.3. Plant extraction:

The aerial part of SL was collected from Deilaman-Pirkoo area in summer season. After the taxonomically confirmation of the plants by the herbarium of pharmacology department of Tehran University of Medical Sciences, they were left to be air dried and then were thoroughly pulverized and the extract was extracted by two times percolation method using hydroalcoholic solvent (80% methanol). The concentration of yielded extract was performed by the rotary at almost 50°C. This extract was weighed exactly and 50, 100, and 200 mg concentrations were prepared in the volume of 5 ml/kg for the animal injection.

2.4. PTZ -Induced Seizure:

The anticonvulsant activity of the hydroalcoholic extract of SL was determined with PTZ-induced seizure test. In this model of induced seizure, the ability of the novel compounds to protect mice against convulsion dose of PTZ (80 mg/kg) was evaluated. Vehicle groups received an equal volume of normal saline. After 30 min, PTZ was injected i.p. and then animals were left in a fiberglass chamber with the dimensions of 70×70×50 cm and convulsive behaviors observation were made at least 30 min after the administration of PTZ by video recording. These convulsive parameters included: latency period for initiation of clonic seizure, delay time to start of tonic-clonic seizure and finally the mortality rate in 24 h (Aghaei et al., 2015; Keshavarz et al., 2016; Rostampour, Ghaffari, Salehi, & Saadat, 2014). The latency period for the initiation of clonic seizure shows the time interval between PTZ injection and the start of clonic seizure which was calculated as the needed time for the initiation of clonic seizure (Latency period). In case of not observing tonic-clonic seizure in a thirty-minute follow up, 1800 second was considered in the calculations. The occurrence of seizure was assessed after video recording by two observers blinded to the treatments. Observer reliability was assured via assessment using the kappa coefficient where >80% was considered to reflect a satisfactory level of agreement between observers.
2.5. Statistical analysis:

The results of this study were presented as mean ± standard error of mean (SEM). Statistical analysis was performed using SPSS (version 23). The comparison of data among groups was performed using one-way ANOVA followed by Tukey’s post-hoc test. Kruskal-Wallis test and binary comparison of results were used in case of non-parametric data and for assessment of 24 hours mortality rate. The significance level was considered as $p < 0.05$. This study was approved by the regional committee of ethics in research of Guilan University of Medical Sciences (approval cod: IR.GUMS.REC.1397.4.12).

3. Results:

3.1. Dose dependent anticonvulsant effect of SL extract in comparison to positive control group:

Administration of SL extract (50, 100, and 200 mg/kg) increased the latency period of clonic seizure initiation so that this time in two groups who received 100 and 200 mg/kg of SL extract were significantly more than NS group (74.3±15.51 and 85.3±36.6 versus 45.8 ± 6.28 sec respectively, $p < 0.01$, Fig. 2 A). Also, hydroalcoholic extract in concentrations 100 and 200 mg/kg significantly increased the delay time of tonic-clonic seizure initiation (442.5±159.32 and 669±394.5 sec, respectively), compared to NS group (66.1±31.5 sec, $p < 0.01$ and $p < 0.001$ respectively, Fig. 2 B). However, only 200 mg/kg of plant extract could significantly decrease mortality rate compared to NS group ($p < 0.05$, Fig. 2 C).

In positive control groups (0.025 and 1 mg/kg diazepam), the latency period of clonic seizures initiation were 48.6±5.66 and 113.4±46.66 respectively, and the delay time of tonic-clonic initiation were 126.6±94.81 and 1095±332.39, respectively. However, 0.025 mg/kg dose of diazepam did not have any notable effects on convulsive indices compared to NS group, but one group who received 1 mg/kg diazepam, the latency period of clonic and delay time of tonic-clonic initiation were significantly more than NS group ($p < 0.001$). Moreover, PTZ-induced mortality rate of this group was significantly lower than NS group.
3.2. Exploration of the roles of benzodiazepine and opioid receptors on the anticonvulsant effect of hydroalcoholic extract of SL:

In order to assess the roles of benzodiazepine and opioid receptors on the anticonvulsant effect of hydroalcoholic extract of SL, flumazenil 2 mg/kg (GABA<sub>A</sub> benzodiazepine receptor blocker) and naloxone 5 mg/kg (Non-selective antagonist of opioid receptors) were separately injected 5 minutes before the injection of SL extract (200 mg/kg). The latency period of clonic seizure initiation was 53±10.07 and 75.3±30.75 after pre-treatment by flumazenil and naloxone respectively, so that administration of flumazenil significantly decreased these latency period compared to NS + 200 mg/kg SL extract group (85.88±38.76, p <0.01), but this reduction was not significant compared to NS group. In the other words, pre-treatment with flumazenil decreased the anticonvulsant effect of SL extract but pre-treatment with naloxone could not significantly affect the anticonvulsant effect of SL extract, and latency period was significantly higher than NS group (p <0.01, Fig. 3 A). Pre-treatment with flumazenil and naloxone decreased the delay time of tonic-clonic seizure initiation compared to NS + 200 mg/kg SL extract group (83±36 and 369.3±441.56 versus 656.67±416.68 sec, respectively). Pre-treatment by flumazenil decreased the anticonvulsant effect of SL extract comprised to NS + SL extract group (p <0.001), but pre-treatment with naloxone was not significant reduction of anti-convulsive effect of 200 mg/kg SL extract comprised to NS + SL extract group and the delay time was still higher than NS group (p <0.05, Fig.3 B ).

Furthermore, pre-treatment with flumazenil significantly inhibited the anticonvulsant effect of SL extract so that the 24 h mortality rate was not significantly differ between this group and NS group. But, naloxone pre-treatment could not inhibit anticonvulsant effects of SL extract and mortality rate of the animals significantly decreased compared to NS group (p <0.05, Fig.3 C).

3.3. Anticonvulsant effect of simultaneous administration of Non-effective dose of SL extract and diazepam represented of GABAergic pathway:

The separate administration of diazepam (0.025 mg/kg ) and SL extract (50 mg/kg) had not significant alteration on seizure indices compared to NS group, but simultaneous injection of these ineffective doses could significantly increase the latency period of clonic and delay time of tonic-clonic seizure initiation (76.7±22.19 and 538.2±677.52 sec, respectively), which
were significantly higher than NS group (48.6±5.66 and 66.10±31.53 sec respectively, \( p <0.001 \) and \( p <0.05 \), Fig. 4 A and B). Also, mortality rate significantly decreased after simultaneous administration of ineffective doses of diazepam and hydroalcoholic extract of \( SL \) compared to NS group (\( p <0.01 \), Fig. 4 C).

4. Discussion:

The results of current study revealed hydroalcoholic extract of \( SL \) has anticonvulsant effect that attenuated the PTZ-induced seizures in a dose-dependent manner. Administration of hydroalcoholic extract of \( SL \) increased the latency period of clonic seizure initiation and delay time of tonic-clonic initiation and decreased mortality rate compared to NS group. In addition, the simultaneous administration of ineffective doses of diazepam and hydroalcoholic extract of \( SL \) had anti-convulsive effect too. The blockade of benzodiazepine receptors by pretreatment with flumazenil decreased the anti-convulsive effects of \( SL \) extract, however the blockage of opioid receptors by pretreatment with naloxone could not inhibit the anti-convulsive effect of the \( SL \) extract.

In current study, administration of 200 mg/kg hydroalcoholic extract of \( SL \) increased the latency period of clonic seizure initiation and the delay time of tonic-clonic initiation and decreased mortality rate just like 1 mg/kg dose of diazepam as positive control treatment group. In agreement to our study of Bahramnejad et al showed that peritoneal injection of diazepam could increase the delay time of clonic and tonic-clonic seizure initiation in male mice (Bahramnejad et al., 2018). Also, the mortality rate reached to following the injection of the effective dose of diazepam which was in accordance with the results of Rezvani nejad and colleagues (Nejad, Motevalian, Fatemi, & Shojaii, 2017).

\textit{Stachys lavandulifolia (SL)}, a plant in Iranian traditional medicine, reported that had anti-anxiety and sedative features (Kumar & Bhat, 2014; Rabbani et al., 2005) and spasmolytic effect. (Duke, 2002; Narayan & Kumar, 2003). Some studies reported that \( SL \) extract had included component such as flavonoid, propanoid, and terpenoid, which this component lead to sedative function of \( SL \) and its significant effects on anxiety have been approved in comparison to diazepam (Monji et al., 2011; Neshat et al., 2017; Rabbani et al., 2005). Because, flavonoids have structure similar to GABA\(_A\) receptor ligands (Wasowski & Marder, 2012), thus may be have a modulatory effect on
The current study was conducted to explore anti-convulsive effect of hydroalcoholic extract of SL on PTZ-induced seizure.

In current study, the hydroalcoholic extract of SL (100 and 200 mg/kg) had anti-convulsive effects and the combination of ineffective doses of diazepam and extract showed anti-convulsive effects too. It seems that this effect of SL extract can be mediated by benzodiazepines receptors via hyperpolarization of neural resting membrane potential. In agreement to our study, Rabbani et al. showed that SL extract has sedative and anti-anxiety effects. They proved the significant effects of this extract in case of sedation compared to diazepam and suggested that these effects could be due to the presence of components such as flavonoids, Phenylpropanoids, and terpenoids (Rabbani et al., 2005) but they did not evaluate the role of benzodiazepine receptors.

Furthermore, Nasri et al. showed that hydroalcoholic extract of SL had analgesic and anti-inflammatory effects (Nasri et al., 2011). In another study, Naseri et al. evaluated the spasmolytic effects of chloroformic fraction of SL plant on mouse ileum. They claimed that SL plant had inhibitory effects on the movements and contractions of intestine, which originated from disturbing calcium mobilization and the activation of opioid receptor which antispasmodic effect was reduced by naloxone (Naseri et al., 2011). It seems that anti-anxiety, analgesic and spasmolytic effects of SL extract may be mediated by benzodiazepines and/or opioid receptors that lead to hyperpolarization of pain receptor and visceral smooth muscle. In the current study the role of benzodiazepine receptors (by pretreatment with flumazenil) was evaluated alongside the evaluation of the role of opioid receptors (by pretreatment with naloxone).

Due to the results of our study, the blockade of benzodiazepine receptors prior to the injection of 200 mg/kg SL extract, reversed the anti-convulsive effects of this plant extract, but the blockade of opioid receptors could not significantly diminished the anti-convulsive effects of this extract. Based on these observations, it can be claimed that the anti-convulsive effects of SL extract mainly acts through the impact on GABA<sub>A</sub> receptor because the Benzodiazepines have stimulatory effects on GABA<sub>A</sub> receptor. In this study, pre-treatment with flumazenil, significantly decreased the anti-convulsive effect of hydroalcoholic extract of SL and also co-injection of ineffective doses of diazepam and SL extract caused notable anti-convulsive
activities, while they could not show such effects when injected alone. This simultaneous effect might be partially described by the signal amplification on GABA_A receptor.

Moreover, SL extract has active components such as phenylethanoid, terpenoid, and flavonoid with biological functions (Mohammadhosseini, Akbarzadeh, & Hashemi-Moghaddam, 2016; Monji et al., 2011; Neshat et al., 2017). Flavonoids are an important category of natural antioxidant components (Hajhashemi et al., 2007) with several neuro-pharmacologic features. Some of these features are linked to GABA_A receptors in central nervous system (CNS) (Rabbani et al., 2005; Wasowski & Marder, 2012). While PTZ induces convulsion mainly by antagonizing the GABA_A receptor in chloride channel complex, the brain effects of flavonoids are associated to this receptor too (Dirscherl et al., 2010). Hence, based on this definition and also according to the previous studies such as the study of Pages et al. which showed positive responses to flavonoid components in PTZ-induced convulsion (Abbasi, Nassiri-Asl, Shafeei, & Sheikhi, 2012), it seems that a part of anti-convulsive effects of hydroalcoholic extract of SL can be attributed to its flavonoid components.

On the other hand, the alleviating effect of central opioid system on convulsion is clearly known (Naseer et al., 2009). Many studies have shown that low doses of morphine (µ-opioid receptor agonist) have anti-convulsive effects, while higher doses would make the model animals vulnerable to the convulsion induced by epileptogenic agents such as PTZ (Pages et al., 2010). Naloxone, which is considered as a nonspecific opioid receptor antagonist – claimed by Lauretti et al. (Hong, 1992) –, can mimic these effects of morphine. In accordance, Kazemi Roodsari et al. showed that pre-treatment by different doses of methadone before the injection of PTZ, significantly decreased the threshold of convulsion, while the injection of various doses of naltrexone, as an opioid receptor antagonist, decreased the pre-convulsive activity of methadone in acute phase (Kazemi Roodsari, Bahramnejad, Rahimi, Aghaei, & Dehpour, 2019). In parallel to this observation, we showed that naloxone pre-treatment before SL extract injection decreases the delay time of PTZ-induced seizure; however this reduction was not statistically significant because of the utilization of single doses of naloxone and extract. Therefore, it seems that the effects of hydroalcoholic extract of SL can be related to central opioid system too that needs to be explored in detail.
In the current study, LD50 was not assessed but the toxicity profile of hydroalcoholic extract of SL were evaluated in some study (Monji et al., 2011; Taghikhani, Afrough, Ansari Samani, Shahinfard, & Rafieian-Kopaei, 2014). Monji et al show that acute (24 hrs.), sub-acute (14 days) and sub-chronic (45 days) administration of SL extract (140mg/kg oral gavages) had hepatic and renal toxicity in female mice, so that lead to abnormal changes in kidney and liver weight in treatment groups as well as biochemical parameters after 45 days administration of SL extract were significantly increased who suggested the possible potentials of this extract with doses upper than 140mg/kg. They proposed that dose up 70 mg/kg could be considered as no observable adverse effect level (NOAEL) and could be used in further study(Monji et al., 2011). Therefore, low dose of SL extract can be using with another antiepileptic drugs for treatment of seizure in feature studies.

5. Conclusion:

The results of our study showed the anti-convulsive properties of hydroalcoholic extract of *Stachys lavandulifolia*. These effects might be due to the impact of the components of this extract on the central benzodiazepine system. It seems that hydroalcoholic extract of SL could be used as a proper approach to control the convulsion seizures if more and detailed mechanistic studies take place in this field.

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Competing interests
The authors declare no competing interests.
Figures:

Phase 1:

NS or Diazepam (0.025 & 1 mg/kg) or SL extract (50, 100 & 200 mg/kg) → After 30 min PTZ 80 mg/kg → Assessment with video recording for 30 min

Phase 2:

NS or Flumazenil 2 mg/kg or Naloxone 5 mg/kg + SL extract (200 mg/kg) → After 5 min → After 30 min PTZ 80 mg/kg → Assessment with video recording for 30 min

Phase 3:

SL extract (50 mg/kg) or Diazepam (0.025 mg/kg) or SL extract (50 mg/kg) + Diazepam (0.025 mg/kg) → After 30 min PTZ 80 mg/kg → Assessment with video recording for 30 min

Fig. 1: Flowchart for different groups and drug administration. Phase 1, dose response of hydroalcoholic extract of Stachys lavandulifolia (SL) or SL extract with compression of normal saline (NS) and diazepam (positive control groups) on PTZ-induced seizure. Phase 2, explore of benzodiazepine and opioids receptor roles on antiepileptic effect of SL extract by pretreatment with flumazenil and naloxone. Phase 3, compression of alone and simultaneous administration of sub effective dose of SL extract and diazepam on PTZ-induced seizure. Drugs and saline injections were performed intraperitoneal.
Fig. 2: The effects of hydroalcoholic extract of SL on PTZ-induced seizure parameters and comparing them with the normal saline and diazepam groups. A) The latency period of clonic seizure initiation, B) the delay time of tonic-clonic seizure initiation and C) the mortality rate after 24 hours. *p <0.05, **p <0.01 and ***p <0.001 in comparison to the normal saline group.
Fig. 3: The effects of flumazenil and naloxone pre-treatment on anticonvulsant properties of hydroalcoholic extract of SL. A) The latency period of clonic seizure initiation, B) the delay time of tonic-clonic seizure initiation and C) the mortality rate after 24 hours. *p < 0.05, **p < 0.01 and ***p < 0.001 in comparison to the normal saline group, and ††p < 0.01 and ††††p < 0.001 in comparison to 200 mg/kg extract group.
Fig. 4: The effect of simultaneous administration of hydroalcoholic extract of SL and diazepam on PTZ-induced seizure parameters. A) The latency period of clonic seizure initiation, B) the delay time of tonic-clonic seizure initiation and C) the mortality rate after 24 hours. *p < 0.05, ** p < 0.01 and *** p < 0.001 in comparison to the normal saline group.
References


