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The CB1 cannabinoid receptors involvement in anti-epileptic effect of safranal on penicillin-induced epileptiform activity in rats

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Abstract

Neuroprotective effects for natural products are supported by several studies. In this regard, safranal, a constitute of saffron, has the potential to exert beneficial effects in neurological disorders such as Parkinson's disease, epilepsy, stroke, multiple sclerosis and Alzheimer's disease. Here, we investigated the effect of safranal on penicillin-induced epileptiform activity. Also, the effects of intracerebroventricular (ICV) microinjection of AM251 as a CB1-cannabinoid receptors antagonist to clarify the possible mechanism of safranal were evaluated. Epileptiform activity was induced by intra-cortical administration of penicillin (300 IU, 1.50 µL) in urethane-anesthetized rats. Electrocorticographic recordings were used to analyze the frequency and amplitude of spike waves. Intraperitoneal injections of safranal at doses of 1.00 and 4.00 mg kg^{-1} significantly reduced both the number and amplitude of spike waves. The ICV microinjection of AM251 (0.50 µg 2.00 μL^{-1}) significantly increased the frequency and amplitude of spike waves. In addition, the anti-epileptic effect induced by administration of safranal at a dose of 4.00 mg kg^{-1} was partially prevented by ICV microinjection of 0.50 µg 2.00 μL^{-1} of AM251. The results showed anti-epileptiform activities for safranal. Central CB1 cannabinergic receptors might be involved in the anti-epileptiform activity of safranal.

Introduction

Epilepsy is a brain disorder caused by electrical hypersynchronization of neuronal networks in the cerebral cortex.[1] In order to find out new effective drugs, various animal models have been developed to investigate pathophysiology of epileptic seizures. For example, intra-cortical (IC) administration of penicillin produced focal epileptiform activities that can be observed in electrocorticogram (ECoG) recordings.[2-4] Penicillin selectively antagonizes gamma-aminobutyric acid A-receptor (GABA(A))-receptor which mediates inhibitory post-synaptic potentials in the central nervous system (CNS) and consequently normal excitation/inhibition neuronal balance alters.[5]

Saffron is the most expensive spice derived from stigmas of *Crocus sativus* L. which is cultivated in southwest Asia and eastern Mediterranean. Safranal, a monoterpene aldehyde, is obtained from saffron and is responsible for saffron characteristic aroma.[6] Pharmacological protective property for safranal in some animal models of neurodegenerative disorders such as Huntington's, Parkinson's and Alzheimer's diseases was reported.[7-9] In addition, the anti-convulsant effect of safranal was evaluated in mice using pentylenetetrazole-induced convulsions.[10]

Cannabinoids refer to a group of substances produced in the cannabis plant.[11] Also, two types of endogenous cannabinoids, 2-arachidonyl glycerol and anandamide, have been identified as neurotransmitters.[12] Two members of the G-protein-coupled receptor family mediated biological effects of cannabinoids: Cannabinoid receptors 1 (CB1R) and 2. The CB1R is mainly distributed in the CNS and its role in several brain functions such as mood, pain and memory processing has been documented well.[11]

Unfortunately, a considerable number of epileptic patients do not respond adequately to available anti-epileptic drugs (AEDs).[13] Additionally, many existing AEDs cause neurotoxic adverse effects.[14] Therefore, safer and more effective treatments are needed. Taken together, we designed this study to investigate the effect of intraperitoneal (IP) administration of safranal on penicillin-induced epileptiform activities. Also, the contribution of CB1 cannabinoid receptors was assessed using a CB1 cannabinoid receptor antagonist, AM251, with and without safranal.

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Materials and Methods

Animals. The experiments were performed on healthy adult male *Wistar* rats (280 - 320 g). The rats were housed under a controlled ambient temperature (22.00 ± 1.00 ˚C) in an animal room (illumination from 7:00 to 19:00) with food and water available *ad libitum*. The experiments were carried out between 10:00 AM and 13:00 PM. All experiments involving the use of rat was approved by Laboratory Animal Care and Use Center of the Faculty of Veterinary Medicine of Urmia University, Urmia, Iran (Ethic Code: IR-UU-AEC-3/2-05/04/2023).

Chemicals. Urethane, safranal, AM251 and penicillin G potassium were used in this study. Penicillin G potassium was purchased from Jaber-Ebne-Hayyan Pharmaceutical Company (Tehran, Iran) and the other chemicals were purchased from Sigma-Aldrich (St. Louis, USA). Urethane and penicillin G potassium were dissolved in sterile physiological normal saline 15 min before administration. The AM251 was dissolved in dimethyl sulfoxide (10.00%). In addition, Tween 80 solution with a concentration of 5.00% was chosen for the preparation of safranal.

Experimental groups. In the present study, 48 rats were divided into eight groups of six animals each: Group 1 received an IP injection of the safranal vehicle 30 min before penicillin microinjection. Groups 2, 3 and 4 received IP injections of the safranal (0.25, 1.00 and 4.00 mg kg^{-1}) 30 min before microinjection of penicillin, respectively. Groups 5, 6 and 7 received intracerebroventricular (ICV) microinjection of AM251 vehicle and AM251 (0.125 and 0.50 µg, per 2.00 μL^{-1}), respectively 10 min prior to penicillin microinjection. In group 8, safranal (4.00 mg kg^{-1}; IP) treated rats were received AM251 (0.50 µg per 2.00 μL^{-1}; ICV) 10 min prior to microinjection of penicillin.

Surgical procedure and induction of epileptiform activity. Induction of epileptiform activity and ECoGs were described previously by scholars.[2,3] The animals were anesthetized with IP injection of urethane (1.20 g kg^{-1}), and supplementary doses (0.60 g kg^{-1}) were given if needed. Then, the rats were mounted on a stereotaxic device (Stoelting, Wood Dale, USA). Using a controlled heating pad system, body temperature was kept between 36.00 and 37.00 ˚C. Intra-cortical microinjection of penicillin was adopted to cause epileptic focus. For this purpose, with the scalp incised, a hole with 0.80 mm in diameter was drilled in the skull following coordination according to the rat brain atlas of Paxinos and Watson:[15] Anterior-posterior = 1.00 mm posterior to the bregma, and lateral = 3.00 mm relative to the midline to allow the insertion of a microinjection needle into the right sensory-motor cortex. Penicillin G potassium (300 IU, 1.50 µL) was microinjected 1.00 mm beneath the surface of the skull using a 5.00 µL Hamilton syringe in a period of 60 sec.

Electrocoticogram recordings. The ECoG was recorded using two 5.00 mm length pin electrodes (0.50 mm in diameter) inserted in right frontal and parietal bones according to the following coordinates: Frontal electrode, 1.00 mm anterior to the bregma and 2.00 mm lateral to the midline, and parietal electrode, 5.00 mm posterior to the bregma and 2.00 mm lateral to the midline (parietal electrode). In addition, one common reference electrode was attached to the left pinna. The electrodes were connected to a 4-channel physiograph (Physiograph 4-channels, MK-III-P, NARCO Bio-systems Inc., Houston, USA) *via* a universal coupler (Universal coupler, type 7189, NARCO Bio-systems) for ECoG activity recordings. Recording of ECoG was started 10 min before IC microinjection of penicillin and continued for 180 min. In each of the above-mentioned times, ECoG activity was recorded for a period of 1 min with a speed of 0.25 cm *per* sec. The number and amplitude of spike waves were manually calculated from the recorded ECoGs.

Statistical analyses. Statistical comparisons were performed using the GraphPad Prism (version 8.2; GraphPad Software Inc., San Diego, USA). Two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used to analyze the data obtained from time-points of frequency and amplitude of spike waves. Area under curve (AUC) of spike amplitude and frequency were calculated by the trapezoid area method and analyzed by one-way ANOVA followed by Tukey's test. All the values were expressed as the mean ± SEM. Statistical significance was set at $p < 0.05$.

Results

Figure 1 shows the ECoG recordings samples obtained from 40th min after IC microinjection of penicillin. Baseline ECoG activities of each animal were recorded before IC administration of penicillin and it was confirmed that none of the animals had spontaneous epilepsy (Fig. 1). The IC microinjection of penicillin (300 IU) induced an ECoG epileptiform activity characterized by spike waves (Fig. 1). First spike wave appeared 224.16 ± 16.12 and 229.66 ± 15.87 sec after penicillin microinjection for vehicles of safranal and AM251, respectively. Statistical analysis did not show significant difference between vehicles of safranal and AM251 ($p > 0.05$). The IP administration of safranal at doses of 1.00 and 4.00 mg kg^{-1}, but not at a dose of 0.25 mg kg^{-1}, significantly ($p < 0.05$) increased the latency time to first spike wave to 383.66 ± 18.97 and 518.83 ± 28.65 sec, respectively. In addition, AM251 at dose of 0.50 µg 2.00 μL^{-1}, but not at a dose of 0.125 µg 2.00 μL^{-1}, significantly ($p < 0.05$) decreased the latency time to first spike wave (156.50 ± 12.23 sec). On the other hand, AM251 (0.50 µg 2.00 μL^{-1}) significantly ($p < 0.05$) decreased the increase of latency time to first spike wave of safranal (4.00 mg kg^{-1}) to 337.50 ± 17.46.

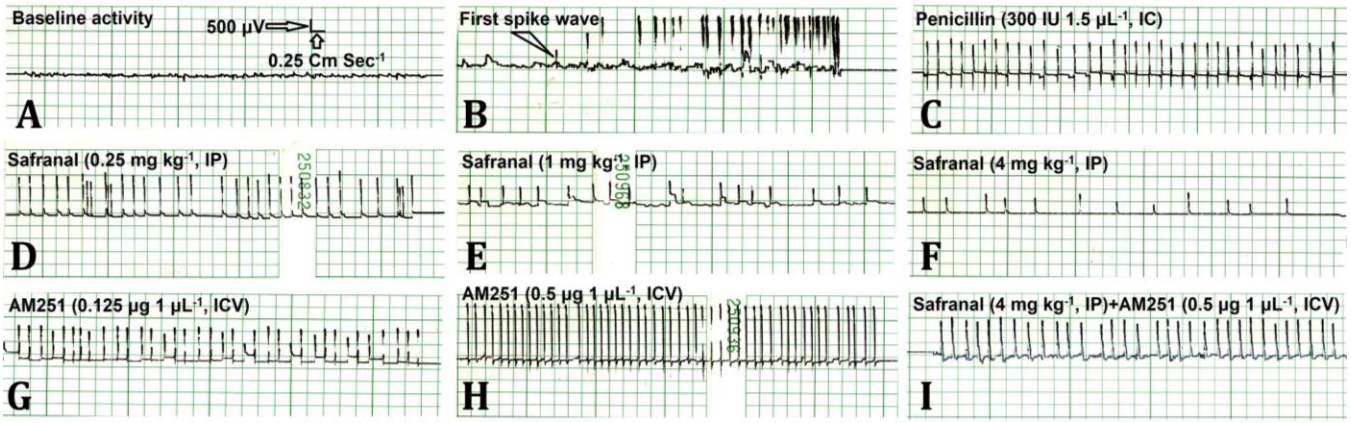

Fig. 1. Electrocorticographic (ECoG) recordings samples obtained from right sensory-motor cortex. **A)** Shows the baseline activity recoded at 10th min before penicillin. **B)** Shows the first spike wave (arrowhead) induced by intra-cortical administration of penicillin. **C)** Shows the epileptiform activity induced by microinjection of penicillin after vehicle administration (Statistical analysis did not show significant difference between vehicles of safranal and AM251; therefore, this panel included only ECoG recordings sample of vehicle of safranal). **D, E** and **F)** Show the effects of safranal at doses of 0.25, 1.00 and 4.00 mg kg^{-1}, respectively, on penicillin-induced epileptiform activity. **G** and **H)** Show the effects of AM251 at doses of 0.125 and 0.50 μg 2.00 μL^{-1}, respectively, on epileptiform activity induced by penicillin. **I)** Shows the effects of combined treatments with effective doses of safranal and AM-251 on the epileptiform activity induced by penicillin. The ECoG was recorded with a speed of 0.25 cm *per* sec under calibration of 100 μV 1.00 mm^{-1} (i.e., 500 μV 5.00 mm^{-1}). IP: Intraperitoneal; ICV: Intracerebroventricular.

Figure 2 shows the effects of IP administration of safranal on the frequency and amplitude of spike waves induced by penicillin. Safranal at a dose of 0.25 mg kg^{-1} did not change frequency and amplitude of spike waves; however, at doses of 1.00 and 4.00 mg kg^{-1} significantly attenuated the frequency ($p < 0.05$; Fig. 2A) and amplitude ($p < 0.05$; Fig. 2B) of spike waves at 20 min time-points. In addition, significant differences were observed between the anti-epileptic effects of 1.00 and 4.00 mg kg^{-1} of safranal. The above-mentioned drug treatment effects were confirmed by the related AUC (Figs. 2C and 2D).

Figure 3 shows the effects of microinjection of AM251 on spike frequency and amplitude. The ICV application of AM251 at a dose of 0.50 μg 2.00 μL^{-1}, but not at a dose of 0.125 μg 2.00 μL^{-1}, significantly increased the frequency ($p < 0.05$; Fig. 3A) and amplitude ($p < 0.05$; Fig. 3B) of spike waves at 20 min time-points. The related AUC supported the above-mentioned drug treatment effects (Figs. 3C and 3D).

Figure 4 shows the effects of microinjection of co-administration of safranal and AM251 on spike frequency and amplitude. Statistical analysis did not show significant difference between vehicles of safranal and AM251 ($p > 0.05$). The ICV application of AM251 at a dose of 0.125 μg 2.00 μL^{-1} did not alter penicillin-induced epileptiform activity; whereas, at a dose of 0.50 μg 2.00 μL^{-1} significantly prevented anti-epileptic effect of safranal (5.00 mg kg^{-1}) in both the number ($p < 0.05$; Fig. 4A) and amplitude ($p < 0.05$; Fig. 4B) of spike waves. In addition, these effects of drug treatments were approved by related AUC ($p < 0.05$; Figs. 4C and 4D).

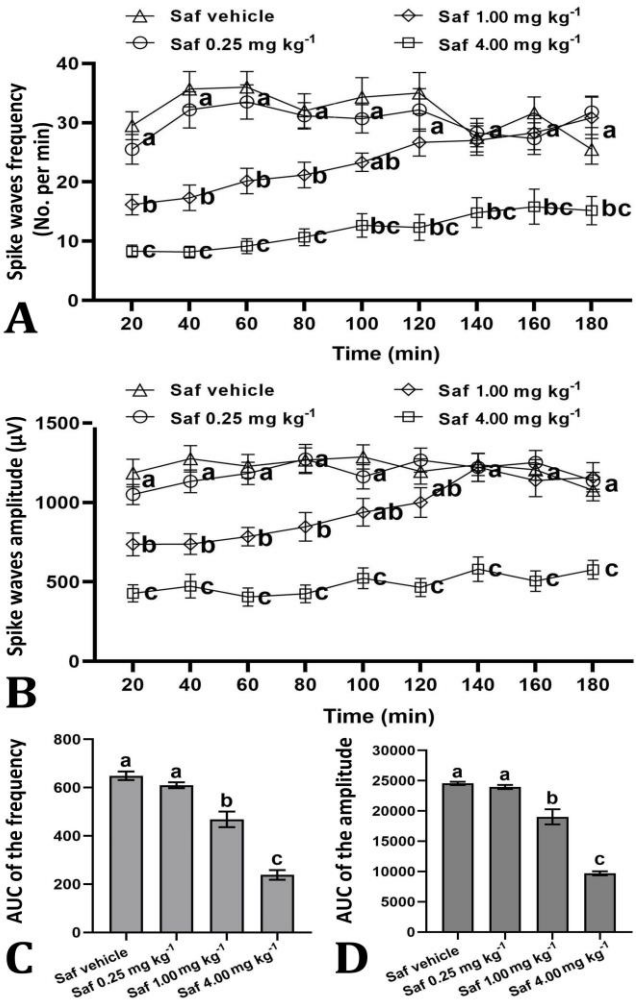

Fig. 2. The effect of intraperitoneal administration of safranal (Saf) and its vehicle on the frequency **(A)** and amplitude **(B)** of spike waves induced by intra-cortical administration of penicillin in rats. Safranal and its vehicle were administered 30 min before microinjection of penicillin. Different letters represent significant differences ($p < 0.05$) for all time-points after administration of safranal at doses of 0.25, 1.00 and 4.00 mg kg^{-1} compared to vehicle-treated group. Related area under curve (AUC) of the frequency **(C)** and amplitude **(D)** of spike waves is shown after microinjection of safranal and its vehicle. Different letters represent significant differences ($p < 0.05$).

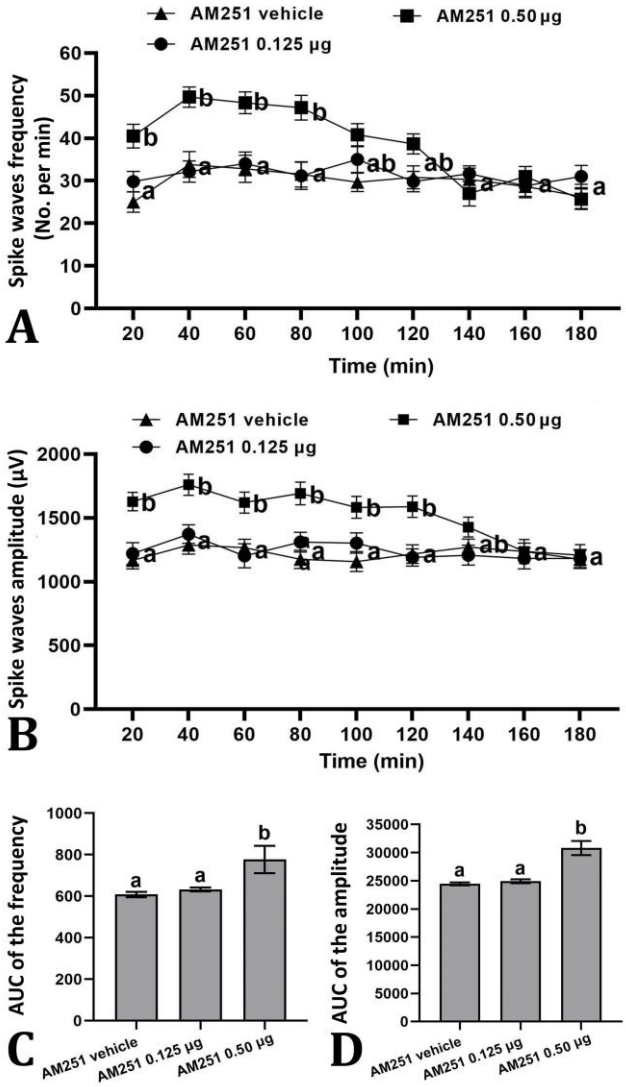

Fig. 3. The effect of intracerebroventricular microinjections of AM251 and its vehicle on the frequency **(A)** and amplitude **(B)** of spike waves induced by intra-cortical microinjection of penicillin in rats. The AM251 was microinjected 10 min before microinjection of penicillin. Different letters represent significant differences ($p < 0.05$) for all time-points after administration of AM251 at doses of 0.125 and 0.50 µg µL^{-1} compared to vehicle-treated group. Corresponding area under curve (AUC) of the frequency **(C)** and amplitude **(D)** of spike waves is shown after microinjection of AM251 and its vehicle. Different letters represent significant differences ($p < 0.05$).

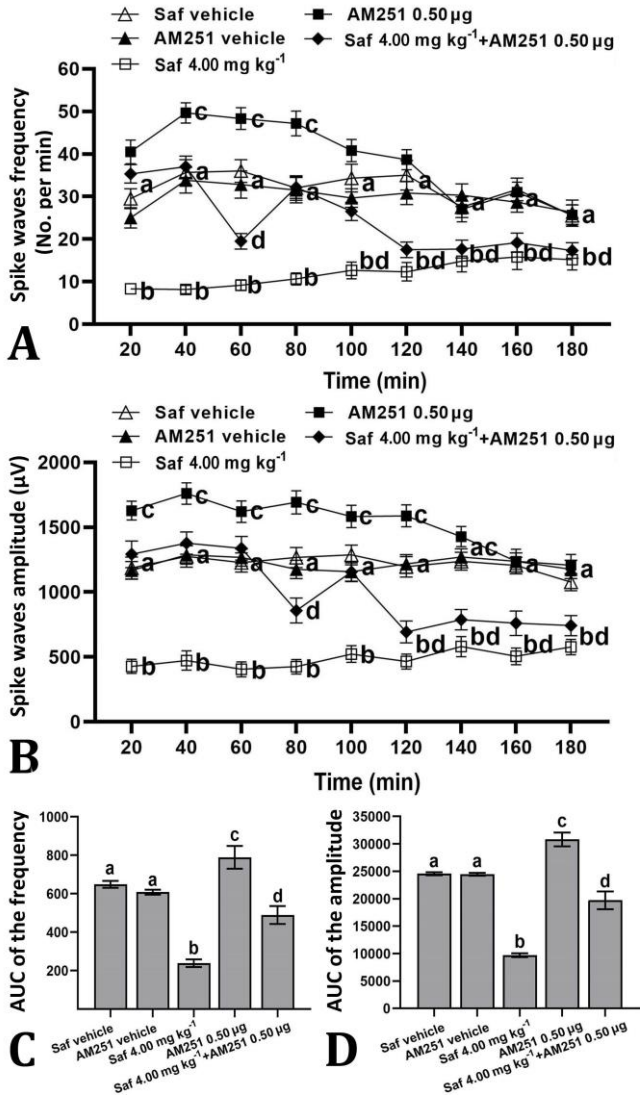

Fig. 4. The effect of administration of effective doses of safranal (Saf) and AM251 on the frequency **(A)** and amplitude **(B)** of spike waves induced by intra-cortical microinjection of penicillin in rats. Safranal and AM251 were administered 30 and 10 min before injection of penicillin, respectively. Different letters represent significant differences ($p < 0.05$) for corresponding time-points. Related area under curve (AUC) of the frequency **(C)** and amplitude **(D)** of spike waves is shown after microinjection of safranal and its vehicle. Different letters represent significant differences ($p < 0.05$).

Discussion

In this study, epileptiform activity was induced by IC injection of penicillin G which was characterized by spike waves on ECoG. Penicillins are one of the oldest groups of antibiotics still widely used in clinical practice.[16] Penicillin G has been widely studied as an epileptogenic agent and our results were approximately consistent with other findings in which the doses of IC injected penicillin were 200 - 500 IU.[2,17-21] Penicillin induced

epileptiform activity began very rapidly and persisted for 3 to 5 hr. Epileptic activity of penicillin is presumably related to its capacity to reduce the neuronal inhibition produced by its GABA antagonism.[16]

Our results demonstrated that safranal alleviated the average number and amplitude of epileptiform spikes. In addition, latency to first spike was increased by systemic safranal injection. The neuroprotective effects of safranal have been documented in animal model of CNS disorders.[7-10,22] Moreover, saffron and its constituents, such as crocin and safranal, have been used in various experiments in controlling certain types of seizures. The aqueous and ethanolic extracts of saffron stigma may have beneficial effects in both absence and tonic-clonic seizures.[23] Also, at the level of the brain, crocin produced anti-epileptic effect in penicillin-induced epilepsy and this effect was antagonized by pre-treatment of a GABA(A) receptor antagonist, flumazenil.[19] However, protective effect of peripheral, but not central, administration of safranal in the rat model of pentylenetetrazol-induced seizures through a GABA(A)-benzodiazepine receptor-mediated mechanism was reported.[24] Moreover, the neuro-protective effects of safranal on experimental absence seizures in mice and kindling model of epilepsy were reported.[24-26] Therefore, the results of the present study regarding the effect of safranal on epilepsy were consistent with other findings. However, the anti-epileptic activity of safranal in penicillin-induced epileptiform activities has not been studied yet.

In the present study, central administration of AM251 increased the average number and amplitude of epileptiform activity and decreased the latency time to first spike. The CB_1 receptor is one of the most abundant receptors in the brain and is highly expressed in the hippocampal formation, neocortex, cerebellum, basal ganglia and brainstem.[27] The involvement of CB1 receptor in the epilepsy processing is documented in several experimental models of seizure activity using different agonists and antagonists.[28,29] In this regard, ICV administration of effective dose of AM251 increased the frequency of penicillin-induced epileptiform activity by producing status epilepticus-like activity when applied alone.[30,31] For example, the CB1 receptor agonist, WIN 55,212-2, produced anti-convulsant effects against both spontaneous recurrent epileptiform discharges and status epilepticus activity, which was blocked by SR141716A (a CB1 receptor antagonist) in *in vitro* models of primary hippocampal neuronal cultures.[32] In addition, application of the SR141716A or AM251 in the hippocampal neuronal culture model of acquired epilepsy caused the development of continuous epileptiform activity, resembling electrographic status epilepticus.[33] Therefore, these findings and our data indicated that cannabinoid system through the CB_1R probably regulates seizure activity in the brain.

In our experiment, AM251 inhibited anti-epileptic effect of safranal. This indicated that central CB_1 canabinoid receptors might be involved in anti-epileptic effect of safranal. Activation of CB1R can prevent seizures and reduce seizure severity in animal models.[28,34] In addition, interaction between various substances such as ghrelin, caspazepine and diazepam with cannabinoid CB_1R in the experimental model of epilepsies was documented.[31,35,36] Although there is not any study indicating an interaction between safranal and cannabinoid receptors, role of cannabinoid receptors in hypoalgesia induced by crocin, another constituent of saffron, in neuropathic pain was reported.[37] Therefore, there is a possibility of a similar interaction between safranal and cannabinoid system.

The results of the present study showed anti-epileptiform effects for safranal. In addition, this effect was probably mediated by CB_1R. Most AEDs cause some degrees of adverse drug reactions. Plant extracts and their isolated constituents such as safranal may provide alternative approaches for epilepsy management.

Acknowledgments

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Conflict of interest

No financial or other conflicts of interest are declared by the authors.

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