Anticonvulsant Effect of C*itrus Bigaradia* Duh. Leaves Extracts in Mice

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Summary

Citrus bigaradia Duh. is used in traditional medicine for its anticonvulsant property. To confirm the traditional use of this plant, the anticonvulsant effects of the aqueous and ethanolic extracts of *C. bigaradia* leaves were studied in mice.The anticonvulsant effect of both extracts of *C. bigaradia*, was investigated using pentylenetetrazole (PTZ) and maximal electroshock (MES) induced seizure models. Also, phytochemical screening of both extracts was performed. LD50 values of the aqueous and ethanolic extracts were 5.74 g/kg and 6.59 g/kg and the maximum non-fatal doses were 3.5 g/kg and 3 g/kg, respectively. In the PTZ test, the i.p. injection of aqueous extract (0.35, 1.4, 2.45 and 3.5g/Kg) and ethanolic extract (0.3, 1.2, 2.1 and 3 g/Kg) delayed the onset of tonic convulsions. The protective effect against lethality was 75% and 25% in the highest dose of both extracts, respectively. In the MES test, both extracts showed mild to moderate anticonvulsant activity. The effects of both extracts were dose dependent and aqueous extract was more effective than ethanolic extract.The phytochemical analysis showed the presence of flavonoids, alkaloids, saponins and tannins in the aqueous extract and tannins in the ethanolic extract.The present study indicated that *C. bigaradia* has anticonvulsant activity in PTZ-induced seizures, in mice, more than in maximal electroshock (MES) model. It is concluded that *C. bigaradia* may be useful in petit mal and grand mal epilepsy.

Keywords: *Citrus bigaradia*, *Citrus aurantium*, Anticonvulsant, Pentylenetetrazole (PTZ) induced seizure, Maximal electroshock seizure (MES) test.

Introduction

Citrus bigaradia Duh., *Citrus aurantium*, (also called Seville orange, Bitter orange or sour orange) is a plant that belongs to the Rutaceae Family (1). It is a small tree, about five meters tall, with pleasant odor and white flowers. It is too sour to eat, but the ripe fruit is eaten in Iran. The peel of *C. bigaradia* is often used in marmalade and the flowers are used in tea, too. The medicinal uses of this plant are common (2). Several components such as phenethylamine alkaloids (octopamine, synephrine,tyramine, *N*-ethyltyramine and hordenine), flavonoids, including limonene, hesperidin, neohesperidin, naringin, myrcene and tangaretin and furanocoumarins are present in this plant (3). The peel, flower, leaf, and fruit of *C. bigaradia* are used in both traditional and modern medicine (4). *C. bigaradia* has been utilized in folk remedies for the treatment of upset stomach, infections, inflammation of the eyelid, bruised skin, muscle pain and as an appetite stimulant (2, 4). Also it is reported that *C. bigaradia* has been used for the treatment of obesity (2, 3, 5), gastric, mammary, pulmonary adenoma and liver cancers and oesophageal reflux disorder recently (6). It has been demonstrated that *C. bigaradia* because of its flavonoids with anti-oxidative activity has radioprotective effect (7). In addition it is used for treatment of mild insomnia, anxiety and the flowers are often utilized as a mild sedative and leaves infusion of this plant alleviated seizure disorder in folk and modern medicines (2, 8, 9). Epilepsy is the most common neurological diseases in the world and up to 5% of the world population develops epilepsy in their lifetime (10). Despite different anticonvulsant drugs are available to control epileptic problems in patients, there are still around 30% of patients medicated incompletely who have seizures, ttherefore investigation of new therapeutic agents is necessary for alleviating of seizure attacks (11). The plants such as *Hypericum perforatum*, *Crocus sativus* and *Nigella sativa* and or their constituents have been shown anticonvulsant activity (10, 12-16). Due to the use of *C. bigaradia* in folk medicine for alleviating of seizure attacks and reported evidences of this plant for treatment of central nervous system disorders this study was initiated to evaluate the anticonvulsant activity of this plant.

Materials and methods

Animals

The study was performed on male and female BALB/c, 25 ± 2 g. Animals were housed in a ventilated room under a 12/12 h light/dark cycle at 24 ± 2°C and had free access to water and food. All animal experiments were carried out in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

Plant

C. bigaradia leaves were collected from Tabas (Yazd province), Iran and were identified by Ferdowsi University and voucher samples were preserved for reference in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashhad (Voucher no. 29804).

Preparation of Extract

C. bigaradia leaves were cleaned, dried in shadow and powdered by mechanical grinder. Then powder of leaves (100 g) was defatted with petroleum ether (40–60 °C) using the Soxhlet apparatus. For the ethanolic extraxct, powder (100 g) was subsequently macerated in 1000 ml ethanol (80%, v/v) for 3 days and the mixture was subsequently filtered and concentrated in vacuum at 40 °C. The residue was suspended in saline normal with twin 80. For the aqueous extract, 400 ml distilled water was added to 100 g leaves powder and filtered through cloth. The extract was then concentrated in vacuum to the desired volume.

Acute toxicity

Different doses of extracts were injected intraperitoneally into groups of six mice. The number of deaths was counted at 48 h after treatment. LD50 values and corresponding confidence limits were determined by the Litchfield and Wilcoxon method (PHARM/PCS Version 4).

Anticonvulsant activity

Pentylenetetrazole (PTZ)-induced seizure

The mice were divided into fourteen groups of eight animals each. In the first and second groups, the mice were given PTZ at an intraperitoneal dose of 90 mg/kg, 30 min after administration of vehicle (normal saline + Tween 80) and normal saline as negative controls respectively in order to compare them with treatment groups and also to determine the influence of Tween 80 in our experiments. Animals in the next four groups were treated with phenobarbital as a positive control in the increment doses of (10, 20, 30 and 40 mg/kg, i.p.) 45 min before PTZ challenge (90 mg/kg, i.p.). Groups 7 to 10 received aqueous extract at the doses of 0.35, 1.4, 2.45 and 3.5 g/kg, i.p., 30 min before the injection of PTZ (90 mg/kg). Groups 11 to 14 received ethanolic extract at the doses of 0.3, 1.2, 2.1 and 3g/kg, i.p., 40 min before the injection of PTZ (90 mg/kg). The animals were individually placed in plastic boxes and observed immediately after PTZ injection for a period of 30 min. The latency and duration of myoclonic jerks, as well as the percentage of protection against incidence of mortality were recorded (17, 18).

Maximal electroshock seizure (MES) test

The animals were divided into 10 groups of eight mice each. The negative and positives controls and different doses of aqueous and ethanolic extracts (above doses) 30, 45, 30, 40 min before the induction of MES were given to the separate groups of mice, respectively. Then, the stimulus train was applied via ear-clip electrodes (sinusoidal pulses 150 mA and 60 Hz for 0.2 s) using a constant current stimulator (Digital Electroshock Model 150, EghbalTeb Co., Mashhad, Iran). A drop of 0.9% saline solution was poured on each ear of animals prior to placing the electrode. The latency and duration of tonic seizure (tonic hind limb extension) were determined. The percentage of protection against the incidence of seizure and mortality were also recorded (17, 18).

Phytochemical screening

Phytochemical screening of both extracts were performed using the following reagents and chemicals, alkaloids with Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with 1% gelatin and 10% NaCl solutions and saponins with ability to produce suds and hemolysis reaction (19).

Statistical analysis

The data were expressed as mean values ± S.E.M. and tested with analysis of variance followed by the multiple comparison test of Tukey–Kramer. Discrepancies with $P< 0.05$ were considered significant.

Results

Acute toxicity

LD50 values of the aqueous and ethanolic extracts were 5.74 g/kg body wt. (95% CL: 5.05, 6.53) and 6.59 g/kg body wt. (95% CL: 5.54, 7.84), and the maximum non-fatal doses were 3.5 g/kg body wt. and 3 g/kg body wt., respectively.

PTZ-induced seizure

In the PTZ-induced seizure, the administration of all doses of aqueous extract 30 min before the injection of PTZ, prolonged the latency of myoclonic seizures. Also, ethanolic extract with the doses of 2.1 and 3 g/kg 40 min before the injection of PTZ reduced the duration of myoclonic seizures. Both extracts exhibited its anticonvulsant activity through a dose-dependent manner. The protective effect against lethality was 75% and 25% in the highest dose of aqueous and ethanolic extracts, respectively. There was no significant difference between seizure parameters of mice in control group (normal saline + Tween 80) and those treated only with normal saline. Also, phenobarbital prolonged the duration and shortened the latency of myoclonic seizures dose-dependently (Tables 1 and 2).

Table 1. Effects of aqueous extract of *C. bigaradia* on PTZ-induced seizure in mice.

Treatment	Latency (Sec)	Protection against mortality (%)
Control (10 ml/Kg)	45.875 ± 1.394	-
Phenobarbital 10 mg/Kg	85.75 ± 3.121 ***	-
Phenobarbital 20 mg/Kg	173.125 ± 3.221 ***	37.5
Phenobarbital 30 mg/Kg	302.625 ± 4.567 ***	62.5
Phenobarbital 40 mg/Kg	376.875 ± 3.009 ***	100
Extract 0.35 g/Kg	74.5 ± 2.442 ***	50
Extract 1.4 g/Kg	94 ± 2.659 ***	62.5
Extract 2.45 g/Kg	116.375 ± 3.359 ***	87.5
Extract 3.5 g/Kg	131.125 ± 4.286 ***	75

Phenobarbital was administered 45 min before the injection of PTZ. The aqueous extract was administered 30 min before the injection of PTZ. Values are the mean ± SEM for 8 mice; ***$p < 0.001$, as compared to control (saline), Tukey-Kramer.

Maximal electroshock seizure (MES) test

It was shown that doses 2.45 and 3.5 g/kg of aqueous extract indicated moderate effect on tonic seizure in MES and mild effect of ethanolic extract on tonic seizure in the highest dose was observed (Tables 3 and 4).

Phytochemical screening

Phytochemical screening of the aqueous extract indicated the presence of alkaloids, flavonoids, tannins and saponins and also the presence of tannins in ethanolic extract.

Discussion

The present study indicates that aqueous and ethanolic extracts of leaves of *C. bigaradia* have moderate anticonvulsant activity. Our results demonstrate that both extracts of *C. bigaradia* leaves show anticonvulsant activity in the PTZ-induced seizure model.

Table 2. Effects of ethanolic extract of *C. bigaradia* on PTZ-induced seizure in mice.

Treatment	Latency (Sec)	Protection against mortality(%)
Control (10 ml/Kg)	52.5 ± 2.053	-
Phenobarbital 10 mg/Kg	89.625 ± 2.162 ***	-
Phenobarbital 20 mg/Kg	178.25 ± 3.277 ***	25
Phenobarbital 30 mg/Kg	308.5 ± 2.452 ***	75
Phenobarbital 40 mg/Kg	379.125 ± 4.24 ***	100
Extract 0.3 g/Kg	65 ± 2.299	0
Extract 1.2 g/Kg	71.25 ± 2.883 **	12.5
Extract 2.1 g/Kg	78.125 ± 2.979 ***	12.5
Extract 3 g/Kg	97.75 ± 2.678 ***	25

Phenobarbital was administered 45 min before the injection of PTZ. The ethanolic extract was administered 40 min before the injection of PTZ. Values are the mean ± SEM for 8 mice; **p < 0.01, ***p < 0.001, as compared to control (Normal saline + Tween 80), Tukey-Kramer.

Generally, compounds with the anticonvulsant activity in the petit mal epilepsy, are effective in PTZ-induced seizure model (20). Thus, *C. bigaradia* may be useful in petit mal epilepsy. On the other hand, as *C. bigaradia* extract exhibit mild to moderate anticonvulsant property in MES model, therefore it can exert a protective activity against the grand mal epilepsy, too, because compounds which possess anticonvulsant activity in MES, may be considered as an effective compounds against the grand mal epilepsy (20). As this plant showed more anticonvulsant effect in the PTZ-induced seizure model than MES test, thus it may be more effective against petit mal epilepsy. Also the effect of aqueous extract was more than ethanolic extract.

Table 3. Effects of aqueous extract of *C. bigaradia* on MES-induced seizure in mice.

Treatment	Duration (sec)	Protection against seizure (%)	Protection against mortality (%)
Control (10 ml/Kg)	13.025 ± 0.3579	-	75
Phenobarbital 10 mg/Kg	8.7 ± 0.2911 ***	12.5	87.5
Phenobarbital 20 mg/Kg	7.2 ± 0.2928 ***	12.5	100
Phenobarbital 30 mg/Kg	4.8 ± 0.1472 ***	50	100
Phenobarbital 40 mg/Kg	0 ± 0 ***	100	100
Extract 0.35 g/Kg	12.8 ± 0.4062	12.5	100
Extract 1.4 g/Kg	12.25 ± 0.4145	-	100
Extract 2.45 g/Kg	9.65 ± 0.3412 ***	-	100
Extract 3.5 g/Kg	7.75 ± 0.2550 ***	-	75

Phenobarbital was administered 45 min before the MES model. The aqueous extract was administered 30 min before the MES model.Values are the mean ± SEM for 8 mice; ***p < 0.001, as compared to control (saline), Tukey-Kramer

In respect to LD50 values, the aqueous extract was more toxic than the ethanolic extract. Compared with a toxicity classification (21), these extracts are relatively non-toxic.

As presence of contents such as flavonoids, alkaloids and saponines have been indicated by preliminary phytochemical results in aqueous extract, so the anticonvulsant activity of the this plant may be related to them. Other studies have demonstrated that various flavonoids such as limonene and mircene (22) as well as alkaloids (23, 24) and saponins (25)

produce significant anticonvulsant activity. It was shown that limonene is one of the flavonoids that present in this plant abundantly (9). Thus, the anticonvulsant activity may be probably more via flavonoids. Tannins that have been found much more in ethanolic extract, probably have lower anticonvulsant activity than other contents in this plant.

Table 4. Effects of ethanolic extract of *C. bigaradia* on MES-induced seizure in mice.

Treatment	Duration (sec)	Protection against seizure (%)	Protection against mortality (%)
Control (10 ml/Kg)	13. 2 ± 0.4175	-	75
Phenobarbital 10 mg/Kg	8.67 ± 0.3092 ***	12.5	87.5
Phenobarbital 20 mg/Kg	7.53 ± 0.6009 ***	25	100
Phenobarbital 30 mg/Kg	4.85 ± 0.2661 ***	50	100
Phenobarbital 40 mg/Kg	0 ± 0 ***	100	100
Extract 0.3 g/Kg	13.01 ± 0.5998	12.5	87.5
Extract 1.2 g/Kg	12.14 ± 0.53	12.5	87.5
Extract 2.1 g/Kg	11.825 ± 0.3954	-	87.5
Extract 3 g/Kg	10.2 ± 0.4351 ***	-	100

Phenobarbital was administered 45 min before the MES model. The ethanolic extract was administered 40 min before the MES model. Values are the mean ± SEM for 8 mice; ***$p < 0.001$, as compared to control (Normal saline + Tween 80), Tukey-Kramer.

The present study indicated that extracts of *C. bigaradia* have anticonvulsant activity in PTZ-induced seizures, in mice, more than in maximal electroshock (MES) model. Therefore, *C. bigaradia* may be useful more in petit mal epilepsy. The phytochemical analysis showed the presence of flavonoids, alkaloids, saponins and tannins in the plant material.

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