ANTICONVULSANT EFFECT OF *ORIGANUM MAJORANA* L.

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Summary

Origanum majorana Linn. (Family- Labiatae) is a herb, commonly grows in Mediterranean regions. The plant has been used in the treatment of diseases related to the nervous system as an antiepileptic and sedative drug in traditional medicines. In this study, anticonvulsant and sedative activities for different extracts of aerial parts (leaves and stems) of *O. majorana* are evaluated. An anticonvulsant effect of *O. majorana* was investigated using the Pentylenetetrazole (PTZ) and maximal electroshock (MES) test The pet ether, chloroform, acetone, methanol and aqueous extracts (PEOM, CEOM, ACEOM, MEOM, and AQEOM respectively) of *O. majorana* exhibited anticonvulsant effect in both the PTZ and MES induced seizure models at the doses of 250 and 500 mg/kg, i.p. The extracts of *O. majorana* delayed the onset of seizures and reduced the duration of seizures in PTZ test and decreased the duration of seizures in MES test compared to the control group. The CEOM exhibited maximum reduction (58.47 and 44.83% in PTZ and MES test respectively) in the duration of seizures, hence it was processed to isolate triterpenoic acid fraction (TAF) which contained substantial amount of ursolic acid. The TAF exhibited maximum reduction (64.54 and 59.31% in PTZ and MES test respectively) in the duration of seizures compared to the other extracts of *O. majorana.* Also, the test extracts decreased the latency and increased the duration of total sleeping time significantly. The antagonism of chemically and electrically induced seizures that *O. majorana* extracts possess anticonvulsant activity. Presence of flavonoids, steroids, triterpenoids and essential oil may be responsible for the anticonvulsant activity of this plant.

Keywords: Anticonvulsant, Labiatae, maximal electroshock, *Origanum majorana*, Pentylenetetrazole, Sedative, sodium pentobarbitone.

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Introduction

Marjoram, *O. majorana* is a tender perennial herb of the mint family (Labiatae), which was formerly classified as *Majorana hortensis* Moench (Vagi et al,2002). Majorana is a fairly small slightly sprawling, soft grey-green leafed plant, to 2 feet/ 60 cm (smaller if grown as an annual) with 'knots' of fairly insignificant pink or white flower or weiry erect stems in mild summer. It is widely used in traditional medicines as well as in the food and cosmetic industries. The herb has carminative, antispasmodic, diaphoretic and diuretic properties (Vagi et al, 2005). In particular sweet marjoram herb contains upto 3% volatile oil, flavonoid glycosides, tannin, steroids (e.g. ß-sitosterol) and triterpenoids (oleanolic acid and ursolic acid) (Leung et al, 1996). It also contains linalool, sabinol, terpinen-4-ol, thujanol, camphor, carvacrol, eugenol, pinene, alpha terpenene (Shirley, 1993).The different extracts of *O. majorana* possess antioxidant, antimicrobial and antiinflammatory activities (Heo et al, 2002; Deans et al, 1990; Ezzeddine et al, 2001). The herb is used in some parts of Punjab as pot herb like mint. It is a good general tonic, with carminative, diaphoretic, diuretic, expectorant and stimulant effect (Nadkarni et al,1999). It has a stronger effect on the nervous system however no experimental evidence reported in the literature (Chevallier**,** 1996)

The epilepsy belongs to the most encountered neurological conditions since it affects approximately 1% of the world population. About 75-80% of epileptic patients may be provided with adequate seizure control with the help of conventional allopathic antiepileptic drugs. There is therapeutic failure in 20-25% of patients which stimulated exhaustive research on novel antiepileptic drugs (Piotr et al, 2005). For centuries, herbs have been used in traditional medicines to treat a wide range of ailments, including central nervous system disorders such as epilepsy. However, the mechanism of action by which these botanicals exert their therapeutic effects has not been completely elucidated. Use of *O. majorana* in the traditional medicine for the treatment of epilepsy and lack of experimental evidence motivated us to undertake the present investigation.

Methods

Drugs and chemicals

Pentylenetetrazole was purchased from Sigma-Aldrich chemical company, USA. Diazepam and Phenytoin were obtained from Ranbaxy, Delhi, India. Sodium pentobarbitone was purchased from BDH chemicals, Kandivli, Mumbai, India. The solvents and chemicals used were of analytical grade. Ursolic acid standard was received from Sami Labs Limited, Banglore, India.

Plant material and extraction procedure

The dried aerial parts (leaves and stems) of *O. majorana* were collected from the local market, Mumbai and were authenticated by Dr. A. M. Mujumdar, Head Plant science division, at Agharkar Research Institute, Pune, India, in September 2005. The dried aerial parts of *O. majorana* were powdered. The powder was extracted successively with solvents of increasing polarity viz. pet ether (60-80^{o}C), chloroform, acetone, methanol and water using soxhlet extraction apparatus. The extracts were concentrated under vaccum at (60±5^{0}C). The chloroform extract was processed to get a triterpenoic acid fraction (TAF). The extracts were suspended in 3% w/v acacia solution before administration to animals.

Fractionation of Triterpenoic acid fraction

The TAF was generated from chloroform extract. In brief, 8 g of dried chloroform extract was dissolved in 40 ml of chloroform, which was subsequently treated with 40 ml of saturated sodium bicarbonate solution twice. The sodium bicarbonate fractions were separated and pooled. Triterpenoic acids were regenerated by acidifying the solution with concentrated hydrochloric acid followed by extraction in chloroform. The triterpenoic acids were obtained by evaporating chloroform under vacuum. The TAF was quantified for ursolic acid using HPTLC technique.

Animals

Swiss albino mice of either sex (18-22 g) were used for the present study. Male mice are preferred for the electroshock test. The animals were housed in standard environmental conditions of temperature (22 ± 5^0C) and relative humidity (55 ± 15 %) with 12-h light-dark cycles. They were fed on conventional laboratory pelleted diet and water *ad libitum*. All the procedures were performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under Ministry of Animal Welfare Division, Government of India, New Delhi, India (CPCSEA, 2003). The Institutional Ethical Committee and in-charge of animal house of Sarawathi Vidya Bhavan's College of Pharmacy, approved the experimental protocol.

Quantification of ursolic acid using HPTLC

Ursolic acid content of the test extracts of *O. majorana* were determined by HPTLC on 20x20 cm silica gel 60, F254 (Merck, Germany) plate, using chloroform: methanol (95:5) as the mobile phase and ursolic acid as reference standard. Application of samples was carried out using Linomat 5 applicator and CAMAG 3 densitometer was used to analyze the plate (λmax-366 nm).

Induction of Convulsive seizures

Pentylenetetrazole (PTZ) induced convulsions

Convulsive seizures were induced by intraperitoneal injection of PTZ (70 mg/kg, i.p.). The mice were divided into 14 groups of 6 each. Control group received vehicle (0.5 ml of 3% w/v acacia solution), other standard group received diazepam (10 mg/kg, i.p.) as reference standard and the remaining 12 groups were administered PEOM, CEOM, ACEOM, MEOM, AQEOM and TAF in doses of 250 and 500 mg/kg, i.p., 30 min prior to the administration of PTZ. The onset of general clonus was used as the end point. The general clonus was characterized by fore limb clonus followed by full clonus of the body. The time of onset and duration of clonic convulsions, and mortality protection were recorded (Vogel et al, 1997). Percentage reduction in duration of seizures is calculated by considering the duration of seizures in control group as 100%.

Electroshock induced convulsions

The procedure was carried out as described by Taman (Toman et al,1964) and swinyard et al (Swinyard et al,1952) The electrical stimulus (50 mA, 0.3 s) was applied 30 minutes after i.p injection of the *O. majorana* extracts through ear-clip electrodes using stimulator apparatus (KI 9531, Dolphin, Mumbai, India). Tonic hind limb extension was considered as the end point. Anticonvulsant activity was evaluated by determining two parameters viz: the electroconvulsive threshold and percentage reduction in seizure duration by MES test. The convulsive threshold was determined using electroshocks of various intensities. The convulsive threshold was determined and the protective effect of test extracts was assessed against maximal electroshock. (50 mA,0.3 s) induced convulsive seizures.

In both cases, the animals were divided into 14 groups of 6 each, among which the 12 groups of mice were administered PEOM, CEOM, ACEOM, MEOM, AQEOM and TAF in doses of 250 and 500 mg/kg, i.p. each. Standard group received phenytoin (20mg/kg i.p.) and control group received vehicle (0.5ml of 3%w/v acacia solution). The time for the extract to reach the maximum effect was determined as 30 minute after i.p. injection. The threshold, duration of tonic convulsion and mortality protection were recorded (Hosseinzadeh et al,2003). Percentage reduction in duration of seizures is calculated by considering the duration of seizures in control group as 100%.

Statistical analysis

The data were expressed as the mean ±S.E.M. obtained from the number of experiments (n=6). One-way ANOVA with Dunnett's post test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The Statistical significance was accepted at $p<0.05$.

Results

Preliminary phytochemical screening

The preliminary qualitative phytochemical analysis revealed that the extracts of *O. majorana* contained flavanoids, triterpenoids and steroids.

HPTLC analysis

In HPTLC analysis, ursolic acid was identified and quantified from each extract having RF value in the range from 0.4 to 0.45. The plot of the peak area of the ursolic acid Vs concentration (mcg) was straight line shown in the figure 1. R^2=0.9966. The presence of ursolic acid in the extracts is shown in the figure 2 and its percentage in each extract is mentioned in table 1.

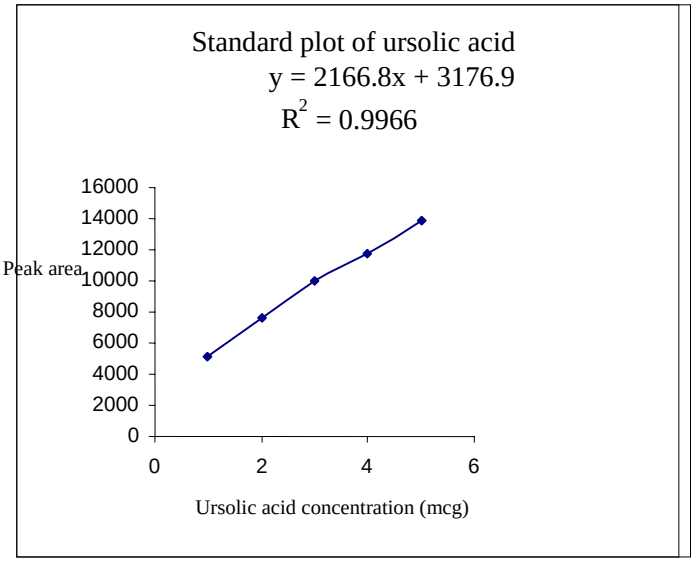

Figure 1 Standard plot of ursolic acid.

Ursolic acid →

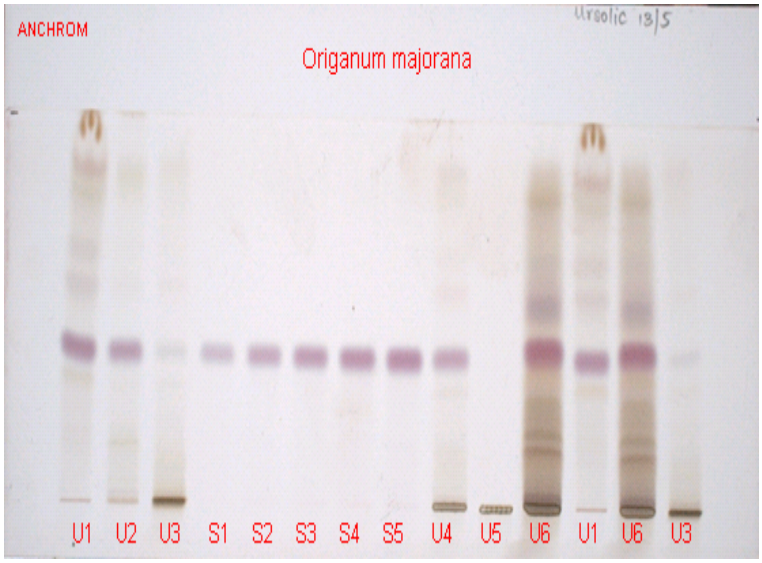

Figure 2: Quantification of ursolic acid in the extracts of *O. majorana*. (λ_{max}- 366 nm.)

Presence of ursolic acid in the extracts is shown in the figure by arrow.

U1: PEOM, U2: CEOM, U3: ACEOM, S1 to S5 (Concentration grading from 1 to 5 mcg): Ursolic acid (standard), U4: MEOM, U5: AQEOM, U6: TAF

Table 1: The % w/w of ursolic acid present in *O. majorana* extracts.

Extracts	**(%w/w) Ursolic acid**
PEOM	0.9
CEOM	1.13
ACEOM	0.11
MEOM	0.34
AQEOM	-
TAF	1.18

Effect of *O. majorana* extracts in PTZ test

The results of the PTZ test are presented in Table 2. In animals treated with vehicle, clonic convulsions appeared at 78.50±2.69 s after PTZ injection (70 mg/kg, i.p.) and all animals died after seizures. The PEOM, CEOM, MEOM and TAF delayed the onset of seizures and decreased the duration of seizures significantly ($p<0.05$) as compared to the control group. No extract had complete seizure and mortality protective effect but the CEOM and TAF showed maximum % reduction in the duration of seizures. Diazepam (10 mg/kg i.p.) inhibited seizures completely.

Table 2: Effect of *O. majorana* extracts in PTZ-induced seizure in mice.

Treatmet	Dose (mg/kg i.p.)	Onset of seizure (s)	Duration of seizure (s)	(%) Reduction in duration of seizure	(%) Mortality protection
Control	-	78.50 ±2.69	52.17±2.18	0	0
Diazepam	10	-	-	100	100
PEOM	250	193.2±2.30**	32.17±2.35**	38.33	33.34
PEOM	500	216.2±2.50**	26.50±3.08**	49.20	50
CEOM	250	231.2±2.82**	26.83±2.05**	48.56	70
CEOM	500	262.5±2.15**	21.67±1.80**	58.47	66.67
ACEOM	250	97.83±2.76**	46.17±1.83*	11.50	16.67
ACEOM	500	104.2±2.38**	43.17±0.94*	17.25	33.34
MEOM	250	139.2±2.52**	35.67±2.24**	31.63	50
MEOM	500	153.5±2.34**	29.17±2.53**	44.09	50
AQEOM	250	111.8±2.38**	42.83±1.79*	17.89	33.34
AQEOM	500	124.2±2.41**	39.50±1.52**	24.28	33.34
TAF	250	270.3±2.64**	23.17±2.00**	55.59	66.67
TAF	500	294.2±3.20**	18.50±1.87**	64.54	66.67

Values are expressed as mean ± S.E.M. (n=6), *$p<0.05$, **$P< 0.01$ compared to control.

Effect of O. majorana extracts in MES test

The results of the electroconvulsive threshold and maximal electroshock (50mA, 0.3 s) are showen in Table 3 and 4 respectively. The PEOM, CEOM, MEOM and TAF showed significant change in the electroconvulsive threshold compared to the control group.

In the MES test the duration of hind limb tonic extension in mice treated with vehicle was 24.17±1.10 s. The PEOM. CEOM, MEOM and TAF at the dose of 500 mg/kg, i.p. exhibited significant reduction in the duration of seizures compared to control group. Also, no mortality was observed in case of all the tested extracts of *O. majorana* with MES (50 mA, 0.3 s).

Discussion

The present study indicated that all the test extracts of *O. majorana* possess significant anticonvulsant activity in PTZ and MES induced seizures at 500 mg/kg dose. Maximum reduction in seizure duration was shown by TAF (64.54 and 59.31 % in PTZ and MES tests respectively). The most popular and widely used animal seizure models are the traditional MES and PTZ test. The MES test is considered to be a predictor of likely therapeutic efficacy against generalized tonic-clonic seizures. By contrast PTZ test represents a valid model for human generalized myoclonic seizures and also generalized seizures of petit mal (Loscher et al,1998). Thus, our results suggest the *O. majorana* extracts may be effective against human generalized myoclonic seizures and also absence ones. PTZ a chemoconvulsant is reported to induce seizures by depressing chloride channel function by binding to picrotoxin site on the Gamma amino butyric acid (GABA) receptor complex (Kulkarni et al, 1993). It has been indicated that PTZ induced seizures can be prevented by drugs that enhance $GABA_A$ receptor-mediated inhibitory neurotransmission (Rogawski et al, 1995; Macdonald et al, 1995). Preliminary phytochemical analysis performed in this study showed that terpenoids, steriods and flavonoids are the major components of *O. majorana* extracts.

Table 3: Effect of *O. majorana* extracts on the threshold of electroshock induced seizure in mice.

Treatment	Dose (mg/kg, i.p.)	Electroconvulsive threshold (mA)
Control	-	9.50±0.50
Phenytoin	20	21.50±0.61**
PEOM	250	13.00±1.00*
PEOM	500	13.50±0.67**
CEOM	250	13.50±0.71**
CEOM	500	13.50±1.02**
ACEOM	250	10.00±0.63
ACEOM	500	12.50±0.92
MEOM	250	12.00±0.77
MEOM	500	13.00±0.63*
AQEOM	250	11.50±1.20
AQEOM	500	12.00±1.09
TAF	250	13.00±0.57*
TAF	500	14.50±0.50**

Values are expressed as mean ± S.E.M. (n=6), *p<0.05, **P< 0.01 compared to control

Table 4: Effect of *O. majorana* extracts in MES-induced seizure in mice.

Treatment	Dose (mg/kg, i.p.)	Duration of seizure (s)	(%) Reduction in duration of seizure
Control	-	24.17±1.10	0
Phynetoin	20	7.16±1.18**	70.37
PEOM	250	17.33±1.43*	28.28
PEOM	500	15.67±1.54**	35.17
CEOM	250	15.83±1.16**	34.48
CEOM	500	13.33±0.71**	44.83
ACEOM	250	18.83±1.40*	22.07
ACEOM	500	18.17±1.22*	24.83
MEOM	250	18.17±1.57*	26.21
MEOM	500	15.50±1.28**	35.86
AQEOM	250	18.67±1.54*	22.76
AQEOM	500	17.37±1.36*	28.14
TAF	250	12.83±1.30**	46.90
TAF	500	9.83±0.94**	59.31

Values are expressed as mean ± S.E.M. (n=6), *p<0.05, **P< 0.01 compared to control

It is reported that anticonvulsant activity of terpene SL-1, a synthetic monoterpene homologue of GABA, demonstrated anticonvulsant activity in PTZ induced seizures (Librowski et al,2000). Linnalool is another terpene compound which has protective effect against PTZ induced seizures (Silva Brum et al, 2001). Moreover pinene, eugenol and methyleugenol exhibited anticonvulsant profile in some experimental seizures such as MES and PTZ tests (Consroe et al,1981; Dallmeier et al, 1981).

Modulation of glutaminergic and gabaminergic transmission is one of the possible mechanisms indicated for anticonvulsant action of the terpenes like linalool and eugenol (Wie et al, 1981). Hence, it is possible that the anticonvulsant effects of *O. majorana* may be related in part to terpenes and terpenoid compounds present in the extracts.

The mechanism of anticonvulsant activity of the extracts is not clear. The quantity of ursolic acid present in all extracts is revealed by HPTLC analysis. TAF showed good anticonvulsant activity as it contains more amount of ursolic acid (1.18 %w/w of TAF) compared to the test extracts. Ursolic acid has been reported to have some effect on CNS. In a study, it was demonstrated that the ursolic acid of *O. majorana* appeared to be a potent acetyl cholinesterase inhibitor in Alzeimer's Disease (Yo-Kyung Chung et al, 2001). As Alzeimer's disease is related to CNS, it is possible that the ursolic acid may be responsible for the anticonvulsant activity of *O. majorana* extracts.

The results of the study revealed that *O. majorana* has potential anticonvulsant activity. However detailed experimental studies should be performed to elucidate the mechanism of action and to support this suggestion. The constituents like terpenoids and ursolic acid can be further explored to be the probable active constituents.

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LIST OF NON-STANDARD ABBREVIATIONS

PEOM: Pet ether extract of *O. majorana*

CEOM: Chloroform extract *O. majorana*

ACEOM: Acetone extract *O. majorana*

MEOM: Methanol extract *O. majorana*

AQEOM: Aqueous extract *O. majorana*

TAF: Triterpenoic acid fraction