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Protective Effect of *Cucurbita maxima* against Maximal Electroshock Induced Convulsions

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In the present investigation, an indigenous plant, *Cucurbita maxima* was studied for its protective effect against maximal electroshock (MES) induced convulsions in Wistar albino rats. The rats were pretreated with different doses (100, 200, 400 mg/kg) of hydroalcoholic extract of seeds of *Cucurbita maxima* for 14 days and then, they were subjected to maximal electroshock seizures (40 mA for 0.2 sec) treatment. Hydroalcoholic extract of *Cucurbita maxima* seeds at 200 and 400 mg/kg doses significantly reduced the duration of hind limb extension along with the protection of rats against maximal electroshock induced seizures. The reference standard i.e., phenytoin (20 mg/kg) provided complete protection. Thus, present study revealed anticonvulsant effect of *Cucurbita maxima* against maximal electroshock-induced convulsions in rats.

Keywords: Seizures; phytochemical; chromatography; extract; cucurbita maxima.

1. INTRODUCTION

Epilepsy is a frequent and chronic neurological disorder characterized by recurrent episodes of seizures and other complications [1]. It is usually caused as a result of abnormal electrical discharge of neurons in the brain due to several etiological factors such as cerebral damage, neurocysticercosis, traumas, congenital cortical abnormality, cerebral tumor or infections and an

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imbalance between excitatory and inhibitory neurotransmitters of brain [2]. The conventional therapy includes the treatment with several of antiepileptic drugs such as barbiturates, benzodiazepines, phenytoin, ethosuximide, carbamazepine, gabapentin, levetiracetam etc. acting through different mechanisms [3]. These antiepileptic drugs are associated with wide range of dose dependent side effects, chronic toxicity and teratogenicity and more than 35% of the patients continue to suffer from epileptic seizures in spite of being on antiepileptic drugs which emphasizes on the need of alternative medicines devoid of such adverse effect [4].

Medicinal plants have been frequently used in the treatment of epilepsy in traditional Medicine (TM) wherein they have exhibited their efficacy as promising anticonvulsant medicines and have been put forward as invaluable sources of new antiepileptic drugs [5]. Hence a need arises for novel drugs with improved efficacy and reduced adverse effects and drug interactions unlike the conventional antiepileptic drugs available [6]. *Cucurbita maxima* (*C. maxima*) is one of such herbal medicines can be effective against epilepsy [7]. Pumpkin (*C. maxima*; Family-Cucurbitaceae) is one of the most popular vegetables worldwide possessing various medicinal properties. Several pharmacological activities of *Cucurbita maxima* have been documented including antidiabetic, anti-inflammatory, antihypertensive, antioxidant and antimicrobial activities [8]. *C. maxima* has been traditionally claimed to possess anticonvulsant and nervine tonic properties but hasn't yet been explored scientifically till now to best of our knowledge [7, 9]. In light of this, the investigation of protective effect of *C. maxima* against maximal electroshock (MES) induced convulsions was carried out.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

Fresh seeds of *C. maxima* were collected from area of Marathwada region of Maharashtra, India. The plant materials were taxonomically identified and authenticated by Botanical survey of India. Pune, India. (Voucher specimen no. BSI/WC/Tech./2020/225). The plant materials were shade dried until all the water molecules evaporated, and plants became well dried for grinding. After drying, the plant materials were ground well using mechanical blender into fine powder and transferred into airtight containers with proper labeling for future use.

2.2 Preparation of Extract

Cucurbita maxima seed powder was subjected to extraction by using different types of solvents namely pet. ether, chloroform, ethanol and methanol by continuous Soxhlet extraction method. The solvents were removed by rotary vacuum evaporator; the remaining mass of extract was concentrated and dried [8,9]. The extracts were stored in desiccator for further phytochemical screening. Hydroalcoholic extract of seeds of *C. maxima* (HCM) were used for the further study.

2.3 Preliminary Phytochemical Screening

The hydroalcoholic extract of seeds of *C. maxima* were analyzed for the presence of phytochemical constituents such as terpenoids, alkaloids, quinones, flavonoids, saponins, steroids and phenolic compounds using the standard qualitative phytochemical methods [10,11].

2.4 High Performance Thin Layer Chromatography (HPTLC) Studies of Extract

2.4.1 Instrumentation

HPTLC system of CAMAG, Muttenz, Switzerland, Anchrom Enterprises (I) Pvt. Ltd, Mumbai, consisting of sample applicator (Linomat 5), Twin trough chamber with lid {10×10 cm, CAMAG, Muttenz, Switzerland), UV cabinet (Aetron, Mumbai) with dual wavelength (254/366 nm) and the HPTLC photodocumentation (Aetron, Mumbai) was used for study. FT-IR, NMR were recorded at Department of Chemistry, North Maharashtra University, Jalgaon, LC-MS was carried out at Venture Centre, Pune, IR spectra was recorded using KBr on "JASCO FT-IR 460 plus" by DRIFT method. ^{1}H-NMR spectrum was recorded in $CDCl_3$ solution on "FTNMR VARIAN MERCURY YH-300" using tetramethyl silane (TMS) as internal standard.

2.4.2 Chromatographic conditions

The sample of extract was spotted in the form of bands of width of 6 mm with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminum plate 60 F_{254} (5 cm ×10 cm) with 250 µm thickness (E. MERCK,

Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The slit dimensions 5 mm × 0.45 mm and scanning speed of 20 mm/sec was employed. The linear ascending development was carried out in 10 cm×10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using n-hexane: ethyl acetate: glacial acetic acid (7.5: 2: 0.5 v/v) as mobile phase. The optimized chamber saturation time for mobile phase was 20 min. The length of chromatogram run was 8 cm and development time was approximately 20 min. TLC plates were dried in a current of air with the help of a hair drier [12].

2.4.3 Sample preparation

10 mg of hydroalcoholic extract of seeds of *C. maxima* was extracted with 100 ml of methanol by multiple extractions with smaller volumes method. 10 µl volume of clear supernatant sample was applied on the TLC plate [13].

2.4.4 Calculation of Rf values

Plate was observed in the daylight, under UV light (254 and 366 nm). Retention factor (Rf) was calculated by following formula [14].

$$Rf = A/B$$

A = distance between point of application and central point of spot of material being examined.

B = distance between the point of application and the mobile phase front.

2.5 Drugs and Chemicals

All the chemicals and drugs were purchased from standard vendors from the local market.

2.6 Animals

Swiss male albino mice (18-22g) and Wistar albino rats of either sex (150-200g) were used. They were maintained at 25±2°C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle). The animals had free access to food. All the experiments were carried out between 12:00-16:00 hour. The animals were shifted from animal house to the laboratory one hour prior to the start of the experiment. The respective apparatuses were cleaned with damp cloth wherever necessary to avoid possible bias due to odor trials left by previous animal.

2.7 Preliminary Acute Toxicity Test

Healthy adult male Swiss albino mice (18-22 g) were subjected to acute toxicity studies as per OECD guidelines (AOT 425) suggested by the Organization for Economic Cooperation and Development (OECD-2000). The mice treated with HCM extract at various doses i.e., 175, 550, 1750, 2000 and 5000 were observed continuously for 2 h for behavioral and autonomic profiles and for any sign of toxicity or mortality up to a period of seven days [15].

2.8 General Pharmacological Observation

Behavioral effects of HCM (100, 200 and 400 mg/kg) were assessed by the method described by Irwin et al. (1968). The mice were divided into six groups (n = 6) and treated with HCM at the doses of 100, 200 and 400 mg/kg. The animals were then placed in an observation cage and observed after 30 min of administration up to 2 h for behavioral changes. The observation parameters consisted of body position, locomotion, rearing, respiration, righting reflex and lacrimation. The observation parameters consisted of body position, alertness, reactivity to touch stimuli, righting reflex and lacrimation [16].

2.9 Evaluation of Anticonvulsant Activity Using Maximum Electroshock Induced Convulsions

48 rats (220-250 gm) of either sex were randomly divided in 05 groups each consisting of 06 rats and were treated with vehicle, different doses of HCM (100, 200 and 400 mg/kg) for the period of 14 days. On 14th day one hour after the administration of respective treatment, all rats were subjected to maximal electroshock shock (40 mA for 0.2 sec) using an electroconvulsiometer. The duration of hind limb extension and percentage of rats protected (i.e., abolishment of hind limb extension before 10 seconds) was recorded and compared with control rats. Phenytoin (20 mg/kg p.o.) was used as reference standard [17, 18].

2.10 Statistical Analysis

The results were reported as mean ± SEM. Differences between group means were

assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test to assess the significance of differences between individual groups. P > 0.05 were considered insignificant.

3. Results

3.1 Preliminary Phytochemical Screening

The results of preliminary phytochemical screening hydroalcoholic extract of seeds of *Cucurbita maxima* revealed the presence of alkaloids, flavonoids, sterols, terpenoids, proteins, tannins.

3.2 High Performance Thin Layer Chromatography (HPTLC) Studies of Extract

The Band 3 at Rf Value 0.68 was scratched and subjected to structure elucidation.

Table 1. Preliminary phytochemical evaluation of extracts

Plant constituents	Tests performed	CM Hydrate	CM chloroform
Test for Steroids	1. Salkowski Test	-	-
	2. Liebermann- Burchard Test	-	-
Test for Triterpenoids	1. Salkowski Test	+	+
	2. Liebermann- Burchard Test	+	-
Test for Glycosides	1. Balget's test	-	-
	2. Keller-Kiliani test	-	-
	3. Legal's test	-	-
	4. Borntrager's test	-	-
Tests for Saponins	1. Foam Test	++	+
Tests for Carbohydrates	1. Molisch's test	++	+
	2. Barfoed's test	++	-
	3. Fehling's test	++	+
	4. Benedict's test	++	-
Test for Alkaloids	1. Mayer's Reagent	++	-
	2. Hager's Reagent	++	-
	3. Dragendorff's reagent	++	+
Tests for Flavonoids	1. Ferric-chloride test	-	-
	2. Shinoda test	-	-
Test for Tannins	1. $FeCl_3$ Solution	++	+
	2. Gelatin test	++	-

(-) Absent, (+) Present, (++) Higher concentration

Table 2. Interpretation of FT-IR Spectrum of compound at Rf – 0.68

Sr. No.	Part of molecule	Vibration	General Range (Cm^{-1})	PI 36
1	Ar Rings	a) C=C stretch	1500-1650	1629
		b) C-H stretch	3000-3100	3047
		c) C-H bend	740-762	762
		d) Overtone	1700-2000	1700-2000
2	-O-H	O-H stretch	3200-3600	3391
3	C=O	C=O stretch	1650-1750	1686

Table 3. Interpretation of NMR spectrum

Sr No.	δ	No of Protons	Multiplicity	Type
1	3.662, 4.164, 4.318, 4.464	4 H	S	04 OH Protons of the 02 Ring
2	6.395 and 6.868	2 H	S	02 Protons on the chromen-4-one ring
3	6.712 and 7.134	4 H	D	04 Aromatic protons

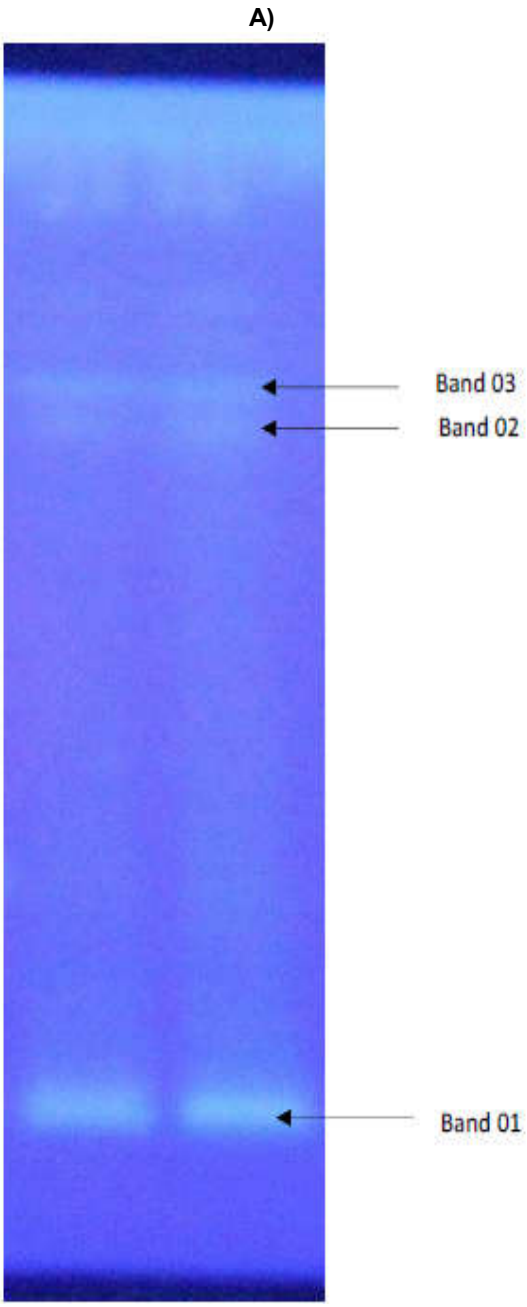

Fig. 1. Hydroalcoholic extract of seeds of *Cucurbita maxima* at 366 nm, Volume applied 10 µl

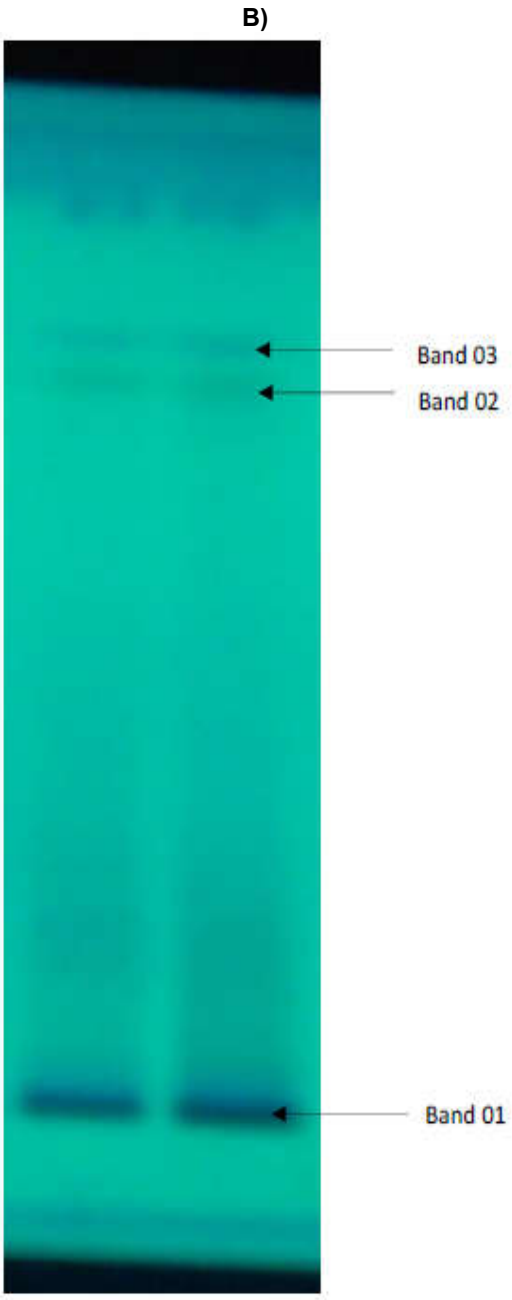

Fig. 2. Hydroalcoholic extract of seeds of *Cucurbita maxima* at 254 nm, Volume applied 10 µl

C)

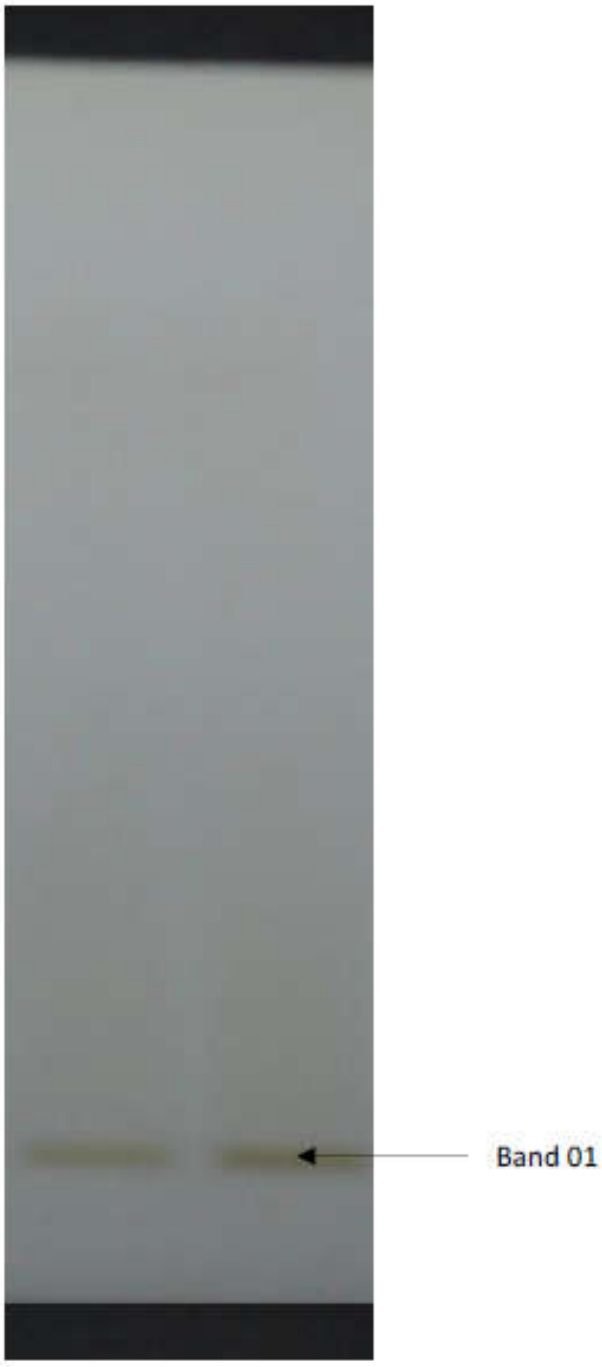

Fig. 3. Hydroalcoholic extract of seeds of *Cucurbita maxima* at visible light, volume applied 10 µl

The spot at Rf Value 0.68 was scratched, extracted with methanol and evaporated to dryness (The process required semipreparative TLC to achieve sufficient amount) for further analysis by IR, NMR and Mass Spectrometry.

A) Spot at Rf Value – 0.68

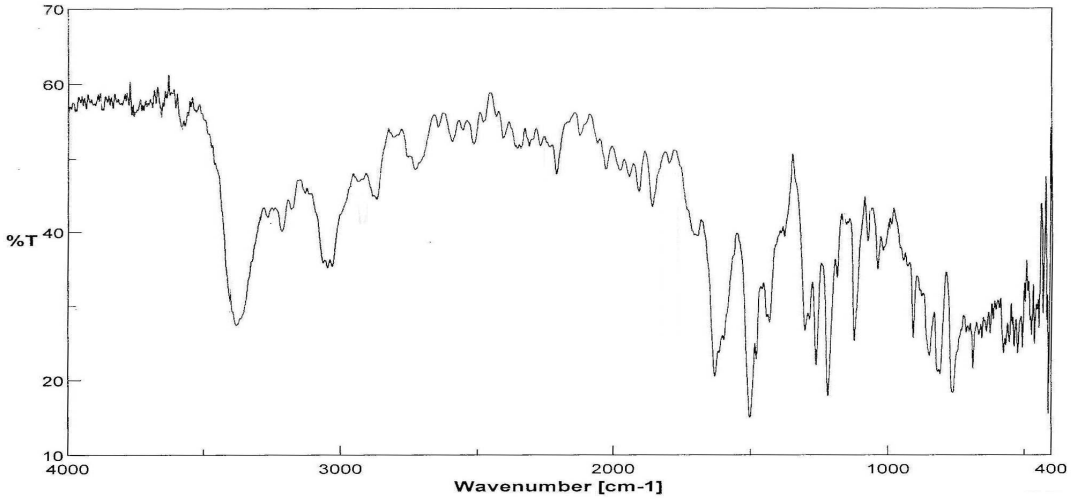

Fig. 4. FT-IR Spectrum of compound at Rf – 0.68

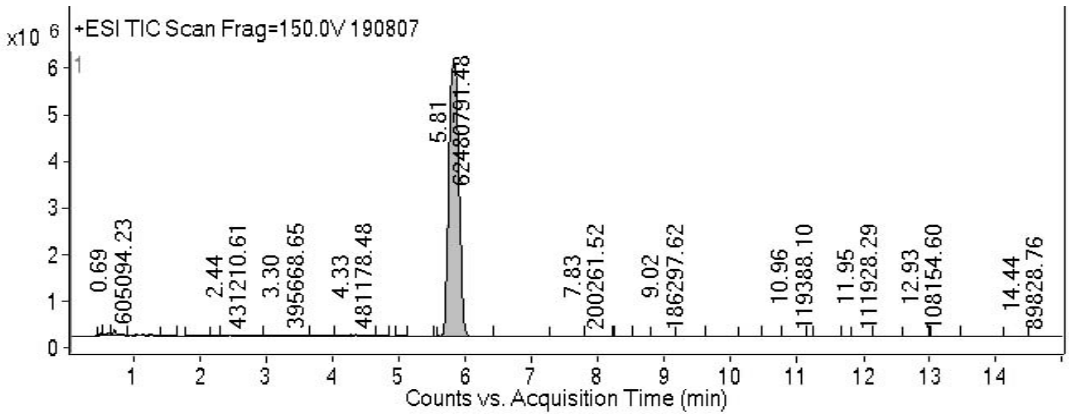

Fig. 5. LC-MS chromatograph of extract

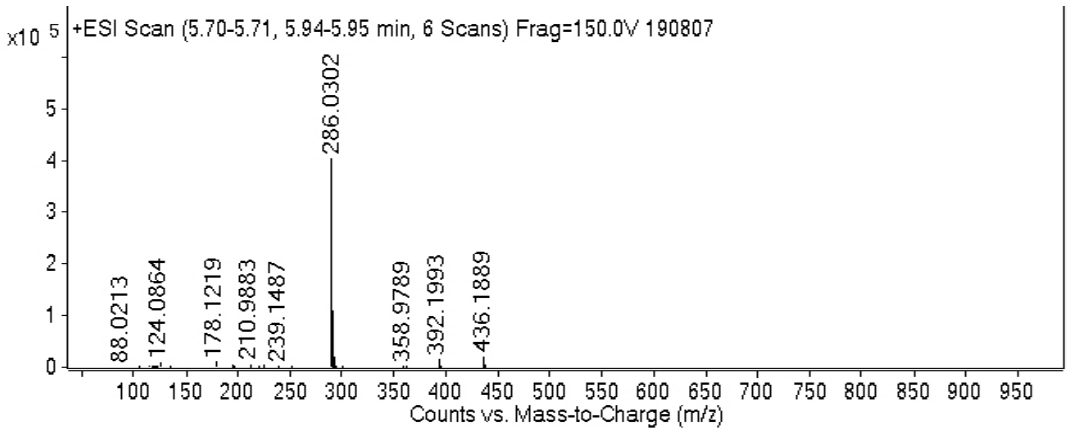

Fig. 6. LC-MS Spectrum of compound at Rf – 0.68

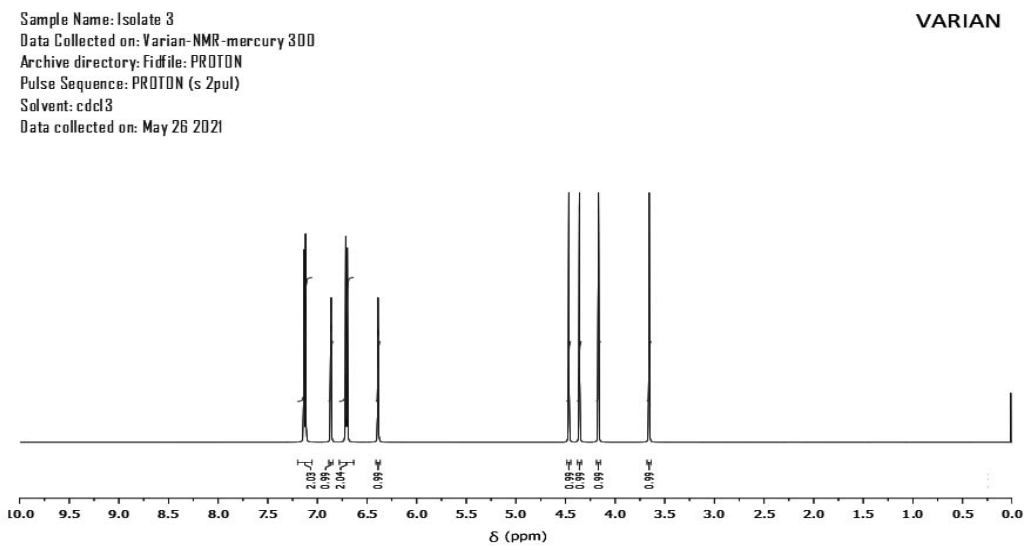

Fig. 7. NMR Spectrum of compound at Rf – 0.68

Probable structure of the isolated compound is as below

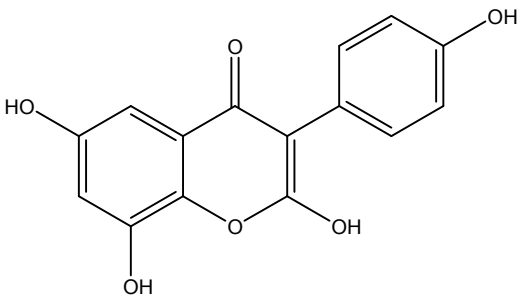

Fig. 8. 2, 6, 8-trihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one

Table 4. Effect of hydroalcoholic extract of *Cucurbita maxima* against maximal electroshock (MES) induced convulsions in rats

Treatment (Dose as per body weight)	Duration of hind limb extension (Mean ± SEM)	Rats protected/ Rats used	% Protection
Vehicle (1 ml/kg)	20.59 ±0.65	0/6	0
HCM (100 mg/kg)	20.78 ± 0.71	0/6	0
HCM (200 mg/kg)	17.25 ± 0.28*	2/6	33.33
HCM (400 mg/kg)	13.85 ± 1.46**	6/6	100
Phenytoin (20 mg/kg)	8.64 ± 0.40**	6/6	100

3.3 Preliminary Acute Toxicity Test

Oral administration of hydroalcoholic extract of seeds of *Cucurbita maxima* did not produce any toxic effect in mice and no mortality was observed up to 2000 mg/kg thus was found to be safe.

3.4 General Pharmacological Observation

Mice orally treated with the HCM extract (100, 200 and 400 mg/kg) and subjected to the general observations did not show any difference in their behavior and other parameters determined during the observation periods. They were alert with normal grooming, touch response and pain response. Alertness, limb tone and grip strength were normal, and the animals did not show staggering gait or contractions.

3.5 Evaluation of Anticonvulsant Activity Using Maximum Electroshock Induced Convulsions

The HCM 200 and 400 mg/kg showed significant reduction in the duration of hind limb extension as compared to vehicle treated control rats. The reference standard phenytoin was most effective in this regard. However as far as prescribed duration of hind limb extension phase to label as protection is concerned then HCM 400 mg/kg and phenytoin 20 mg/kg were found to be equally effective.

4. DISCUSSION

Epilepsy is characterized by recurrent occurrence of seizures, defined as the manifestation of disordered and paroxysmal neuronal discharges in the brain. There are a several conventional anticonvulsant drugs available for the control and treatment of epilepsy in epileptic patients [19,20]. However, most of these synthetic drugs have been documented to possess many toxic effects [21]. It is therefore very essential to approach for the development of safe, effective and cheap anticonvulsant agents from plants and other natural resources. Research in context of application of traditional and herbal medicines for the treatment of several ailments including epilepsy has been increasing in past few decades [22,23]. In light of this, the preclinical screening of anticonvulsant activity of HCM extract was carried out in Wistar albino rats against maximum electroshock induced seizures.

The phytoconstituents in the herbal medicines play an important role in execution of their pharmacological effects [11]. Hence the HCM extract was subjected to preliminary phytochemical analysis and the results revealed the presence of the various phytoconstituents such as alkaloids, flavonoids, sterols, terpenoids, proteins and tannins. Preliminary photochemical analysis does not give us idea about the specific constituent for the anticonvulsant activity. Hence taking into consideration the revealed preliminary phytoconstituents, the extract was further subjected to HPTLC analysis [12-14]. The results of the HPTLC analysis revealed the presence of a phytoconstituent namely 2,6,8-trihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one.

Before evaluating the pharmacological activity of any drug, it must be screened for its toxicity. In this context, the extract was subjected to acute oral toxicity testing [15, 16]. The results reveled that the extract was found to be safe up to the dose 2000 mg/kg without any difference in their behavior and other parameters. The anticonvulsant activity of the extract was determined by using maximum electroshock induced seizure model.

The maximum electroshock seizure test is a widely applicable preclinical model of epilepsy because drugs that are effective against tonic hind limb extension induced by electroshock generally have proven to be effective against partial and tonic clonic seizures in human beings [24]. MES induced convulsions can be blocked either by drugs that block voltage gated sodium channel such as phenytoin. Hence it was used as a reference standard in this study. In the present study, HCM extract reduced the duration of hind limb extension and at doses of 200 and 400 mg/kg in a significant ($P<0.05$ and $P<0.01$ respectively) and dose dependent manner showing potential anticonvulsant activity. HCM showed equipotent activity as that of phenytoin at the dose of 400 mg/kg. It has often been stated that antiepileptic drug that blocks maximal electroshock-induced tonic extension acts by blocking the spread of seizure, [25] which may be possible mechanism of its action and is attributed to the presence.

5. CONCLUSION

In the present investigation, HCM extract showed significant prevention of maximal electroshock-induced seizures which is attributed to the presence of 2,6,8-trihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one. The anticonvulsant activity of *C. maxima* may involve GABAergic transmission as it showed similar activity to that of phenytoin. Further studies are however needed to determine the underlying mechanism of isolated phytoconstituent for its anticonvulsant effect.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Institutional Animal Ethical of Dr. D. Y. Patil College of Pharmacy, Akurdi, Pune approved the protocol (Approval No.-DYPCOP/IAEC/2021/05).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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