Research Article

Evaluation of Protective Neuro Pharmacological Activity of seeds of *Cucurbita maxima* against Ethidium Bromide Induced Demyelination in Rat Model

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Received: 12-01-2018; **Revised:** 04-02-2018; **Accepted:** 15-02-2018.

ABSTRACT

The main aim and objective of the present study was to evaluate the neuro protective effect of seeds of Cucurbita maxima on ethidium bromide induced demyelination in Wistar rats. The petroleum ether extract of the seed was tested at 50 mg/kg and 100 mg/kg body weight administered daily by oral route and continued for a period of 28 days in demyelinated Wistar rats. The demyelination was effected by giving intracranial injection of Ethidium bromide at a dose of 1mg/0.03ml of PBS/kg body weight. The efficacy of the extract was evaluated in terms of their behaviour in beam walk, rotarod, grip strength, and open field tests on the 1st, 2nd and 4th week. The animals were sacrificed after 28 days brain tissue was separated and subjected to histopathological studies and Biochemical analysis. The results from the behavioral and histopathological studies and bio chemical Analysis revealed that the petroleum ether extract of Cucurbita maxima seeds have potential protective effect in the ethidium bromide induced demyelination on rat by intracranial administration, showing an improvement in muscle strength and muscle coordination in motor neuron diseases when compared with standard oral anti Multiple sclerosis drug (Fingolimod).

Keywords: Multiple sclerosis, Ethidium bromide, Histopathology, Biochemical analysis.

INTRODUCTION

Axonal myelination in the vertebrate central nervous system is pivotal for saltatory nerve impulses, whereas demyelination can construct serious diseases such as multiple sclerosis (MS)[1]. MS is recognized as an autoimmune, inflammatory, demyelinating and neurodegenerative disease of the central nervous system affecting over 2 million people worldwide[2]. It is characterized by a progressive clinical decline due to axonal loss from chronic demyelination[3]. Nowadays it is widely accepted that cognitive dysfunction occurs in 4070% of MS patients[4]. The most common cognitive deficits are memory dysfunction and spatial perception impairment[5, 6]. Recent studies, especially using magnetic resonance imaging (MRI) technique, has indicated that structural derangements of both the cerebral cortex and the hippocampus (a principle locus of memory consolidation) take place in patients with MS[7, 8].

Although different mechanisms may contribute to the demyelination and neurodegeneration in MS, it became clear in recent years that oxidative stress plays the key role in the process[9, 10]. The brain is believed to be particularly vulnerable to free radical damage because of the high content of polyunsaturated fatty acids (PUFA), the high utilization of oxygen account and the limited possession of antioxidant defenses in comparison to other organs. Thus it is clear that oxidative stress is involved in the formation and persistence of MS lesions[11]. Recently, researchers have turned to experimental autoimmune encephalomyelitis in order to explore the relationship between hippocampal dysfunction and neuropathology during MS[12]. Intriguingly, it has been observed that the progressive decline in spatial learning and memory function correlated with oxidative stress in the brain of experimental autoimmune encephalomyelitis models. Toxic demyelination by ethidium bromide (EB) is one of the most commonly used models for analyzing the cellular changes that occur during demyelinating pathologies such as MS. Direct injection of EB induces focal demyelinating lesions at the site of injection by selectively damaging oligodendrocytes and astrocytes, and consequently interfering with the demyelination and remyelination processes[13]. Previous studies have indicated the increase in oxidative stress following EB injection in different areas of the brain[14]. Antioxidants as substances which could delay or inhibit oxidative damage even in small quantities compared to an oxidizable substrate can help in disease prevention by effectively scavenging free radicals or inhibiting damage caused by them.

Cucurbita maxima Duch.ex Lam. (Family: Cucurbitaceae) known as Dadhiphala in Sanskrit, Red Gourd in English and Kashiphala in Hindi, widely available in temperate regions such as North America and Australia[15, 16]. The chemical constituents from seeds contain 30% unsaturated fixed oil (linoleic and oleic fatty acids). triterpenoids flavonoids, coumarins, saponins, cucurbitacins, vitamins, minerals notably zinc, amino acid known as cucurbitin[17] which has anthelmintic effect, high amount of carotenoid content which include lutein and beta-carotene. It has also been suggested that phytosterols present in the seed may play some role in the treatment of enlarged prostate gland and micturation problem related to irritable bladder. Long chain

hydrocarbons and fatty acids in fruits, spinasterol in flowers have been reported as antioxidant, anti-inflammatory, immunological activity.

Pulp is applied to burns, scalds, inflammations, abscesses, boils and is remedy for migraine, neuralgia, haemoptosis & hemorrhages[18, 19]. It is also efficient in enhancing cognitive behaviour has indicated that structural derangement of both the cerebral cortex and hippocampus (a principle locus of memory consolidation) take place in patients with MS. Although different mechanism may contribute to the demyelination and neurodegeneration in MS, it became clear in recent years that oxidative stress plays the role in this process. The brain is believed to be particularly vulnerable to free radical damage because of the high content of polyunsaturated fatty acids (PUFA), the high utilization of oxygen account and limited possession of antioxidant defenses in comparision to other organs. Thus it is clear that oxidative stress is involved in the formation and persistence of MS lesions. Recently researchers have turned to experimental autoimmune encephalomyelitis. Recent work has revealed that the Cucurbita maxima seeds petroleum ether extract protective effects on spatial memory impairment and oxidative stress and inflammation induced in rats. However, previous studies have not investigated the possibilities that saffron extract may improve learning and memory deficits and oxidative stress in clinical or experimental models of MS. The aim of the present study was to investigate the effect of a 7day intra hippocampal administration of Cucurbita maxima seeds petroleum ether extract on the learning and memory of EB demyelinated rats and their oxidative status and inflammation in the hippocampus

MATERIALS AND METHODS

Experimental Pharmacognosy

Reagents and chemicals

All the reagents, chemicals and solvents used for the experiments were of analytical grade and obtained from Sigma Aldrich (USA).

Plant material and extraction

Cucurbita maxima seeds were purchased from local market in The Nilgiris, Ooty. The *Cucurbita maxima* seeds were authenticated at Tamil Nadu Agricultural University, Coimbatore. The voucher specimen number is F-196.

Extraction methodology

Cucurbita maxima seeds were dried at 40 °C for 48 hours in hot air oven. Dried seeds were powdered using an electric mixer (Remi). Soxhlet extraction technique was used to prepare petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts. Extraction was carried for 18 hours at a temperature not exceeding the boiling point of the solvents. The extracts were filtered using Whatmann filter paper (No.1), concentrated in vacuum under reduced pressure using rotary flask evaporator, and dried in a vacuum desiccators. The extracts were stored in amber colored bottles at 2-4 °C until further use. The petroleum ether extract was chilled in refrigerator until the use.

Experimental Pharmacology

Animals

Adult male and female Wistar rats weighing 200 -250 g were housed in standard hygienic plastic cages (four in each) Under a 12 h light/dark cycle (lights on at 07:00 a.m.) in a room with controlled temperature (23 ± 2 °C). Food and water were available *ad libitum*. The experiments were carried out during the light phase of the cycle. All animal procedures were performed according to the National Institutes of Health: Guide for the care and use of laboratory animals. Efforts were made to minimize the number of animals used and their suffering.

Protocol

Wistar rats of both sexes (12 weeks old, weight 200–250 g) will be housed under standard conditions in a temperature-controlled environment (23 °C ± 2 °C) and a 12 h light/dark cycle the rats are then allocated into dietary regimens by feeding with the rat pellet supplied by Hindustan levers Ltd., Bangalore. After the 2 weeks of dietary manipulation, the animals were randomly divided into groups Animals were deeply anasthetised with intraperitoneal injection of Ketamine (100 mg/kg) and xylazine (20 mg /kg) the rats are placed on the rat stereotaxic instrument in the skull- flat position. Hair of the skull surface was shaved and then an incision is made to expose the rat skull two holes are drilled in the skull according to appropriate coordinates. Two guide cannulae are inserted into the holes and fixed using dental cement after surgery dummy inner cannulae are inserted into the guide cannulae and left in the place and till the injections are made. All animals are allowed to recover for one week before starting the microinjections Experimental model of MS is induced bilaterally by direct single injection of 3 µl of 0.01% Ethidium bromide in sterile 0.9% saline. After the demyelination the animals are treated with the extracts and are observed for their behavioral studies after 28 days the animals were sacrificed and subjected for histopathology and biochemical analysis. The proposed experiment was approved by IAEC, JSS University, and Mysore. Proposed No. JSSCP/IAEC/PH.D/PH.COG/03/2016-2017.

Grouping of animals

The experimental design of the study was carried out in five groups with five Albino Wistar rats of both sexes (200-250gms) in each group.

(I) Group G_0 - Served as solvent control, received only 0.3%CMC (2ml) orally and daily.

(II) Group G_1 - Received Ethidium bromide (EB) 1µg/ kg body weight in 0.03ml PBS (sterile) intra cerebrally

(III) Group G_2- Received EB and standard drug for MS (Fingolimod) 100mg/kg body weight in 0.3% CMC orally and daily.

(IV) Group G_3 - Received EB and dried petroleum ether extract of *Cucurbita maxima* seeds 100mg/kg body weight in 0.3% CMC orally and daily.

(V) Group G_4 - Received EB and dried petroleum ether extract of *Cucurbita maxima* seeds 200 mg/kg body weight in 0.3% CMC orally and daily.

Studies of behavioural pharmacoly[20, 21, 22, 23]

Open field exploratory behaviour test

This test has been utilized for behavioral changes in rats by exposing the animals to a novel environment and has been used to detect anxiogenic and anxiolytic activity under identical situation. A typical apparatus suitable for rats, comprises of large black area (96×96cm) with wall height (96cm). The floor was divided into 16 squares by white lines and the apparatus was placed in a dim light room. Mice were placed individually at one corner of the apparatus and were observed, thereafter for the next 10-15 minutes. The parameters noted were:

(i) Ambulation (no. of squares crossed)

(ii) Freeze (periods of immobility)

(iii) Rearing (no. of times animal stand on its rear paws)

(iv) Defecation (no. of fecal pellets).

Grip strength

The test is being used to assess muscular strength or neuromuscular function in rats which can be influenced not only by sedative drug and muscle relaxant compounds but also by toxic agents. The method was discovered as 'test de 1' gripment' by Boissler and Simon (1960). Male or female rats were posed to a horizontal thin thread or metallic wire suspended about 10 cm in the air, which they immediately grasp with the fore limbs. Normal animals were able to catch the wire with hind limb and climb up within 5 seconds. The animals that are not able to touch the wire are considered as impaired.

Rota rod test

The test is used to evaluate the activity of drugs interfering with motor coordination. The apparatus consists of horizontal metal rod of 3 cm diameter attached to a motor with the speed 20-25 revolution per minute. The rod is divided into five sections with wooden compartment, it allows simultaneous testing of 5 rats, the rod is in height of 50 cm above the table top in order to discourage the animal from falling off. The compartment made with wooden cardboard to restrict escape of animals when they fall from the rod, the test animals were tested for the time of falling from the roller.

Beam walks Test

The test is used to evaluate the ability of animals to walk on beam. The apparatus consists of a horizontal metal rod of 1 cm diameter supported by two side stands of 30 cm height and animals are kept in the center of the rod to allow walking on the beam. The parameters evaluated are fecal pellets, distance walk, time of immobility and falling time.

Assessment of biochemical parameters

After completion of behavior study the animals were sacrificed by cervical dislocation for biochemical study. The whole brain was immediately dissected out, washed in normal ice cold saline and immediately cerebral cortex and midbrain was separated and processed for various biochemical estimation

Evaluation of anti oxidant activity

Malondialdehyde (MDA)

Lipid peroxidation was assayed by measuring the level of Malondialdehyde (MDA) in the brain tissues. Malondialdehyde was determined by measuring thio barbituric reactive species using the method of Ruiz-Larrea et al. (1994) in which the thio barbituric acid reactive substances react with thio barbituric acid to produce a red colored complex having peak absorbance at 532 nm.

Superoxide Dismutase (SOD)

SOD activity was assayed according to the method of winter bourn et al. (1975). Enzyme activity is expressed as units per milligram of protein. Bovine serum albumin (BSA) is added to keep the solution from forming precipitates when bathos cuproine disulfonic acid (BCS) is added. BCS (an electron chain-associated free radical production inhibitor) and diethylenetriaminepentaacetic acid (DETAPAC) are both added to inhibit iron-associated redox cycling and free radical production. Various amounts of protein are added into tubes until maximum inhibition of NBT reduction. Colored complex having peak absorbance at 532 nm was observed.

Evaluation of anti inflammatory activity

TNF- α and IL-6 were quantified according to the guidelines using enzyme-linked immunosorbent assay kit. These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra assay precision and small amount of tissue sample required to conduct the assay.

Study of histopathology

Histopathological study was done according to (Bancroft *et al*, 1996) method. Tissue from mid brain and cerebral cortex were separated and fixed in 10 per cent formalin and were processed by conventional method, embedded in paraffin, sectioned at 4-5 µM and stained by heamotoxylin and eosin. Tissues were examined under a light microscope.

RESULTS

Behavioural Pharmacology

The animal tests were carried out to assess the induction and to evaluate the remyelination in animals. The tests are

(i) Open field exploratory behavior test

(ii) Rota rod test

(iii) Grip strength test

(iv) Beam walk test

The animals subjected for the above tests were observed for four weeks. The results obtained are as follows:

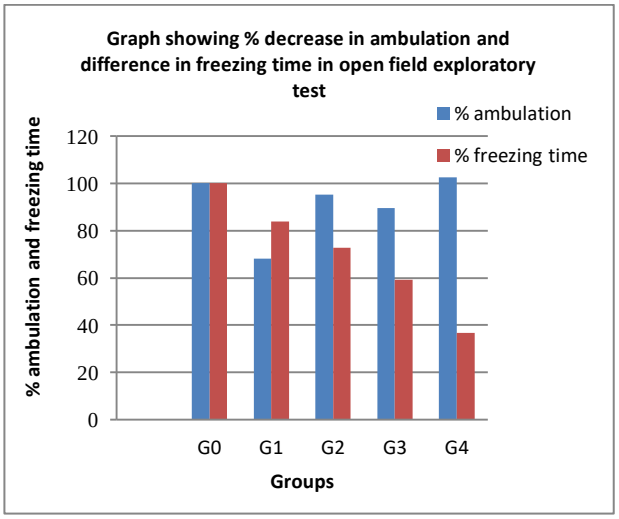


Figure 1: Showing % decrease in ambulation and difference in freezing time in open field test.

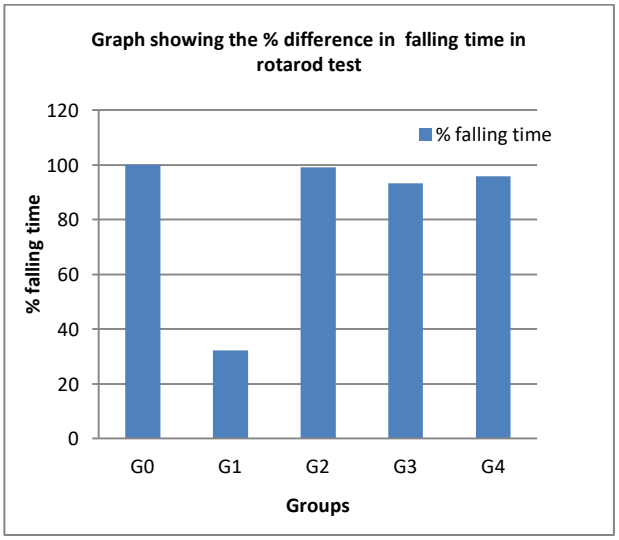


Figure 2: Showing the % difference in falling time in Rota rod test.

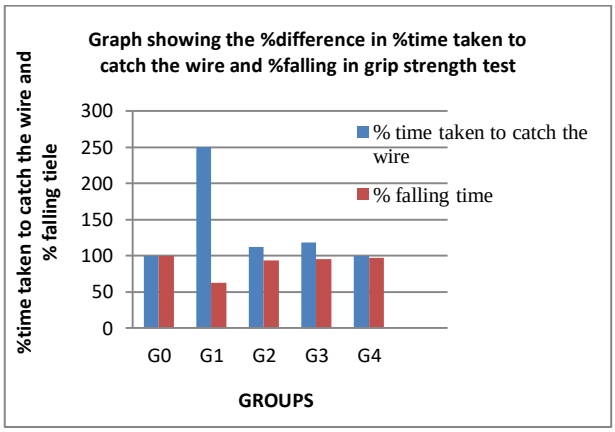


Figure 3: Shwoing % difference in time taken to catch the wire and % falling time in grip strength test.

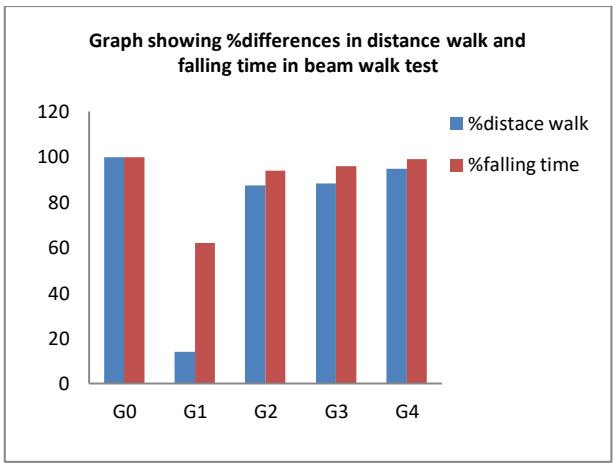


Figure 4: Showing % difference in distance walk and falling time in beam walk test.

Assesment of biochemical parameters

Antioxidant activity

Table 1: Effect of *Cucurbita maxima* seeds petroleum ether extract on the concentration of malondialdehyde (MDA)

S.No	Sample Name	Concentration	nmol/gm/tissue
1	*Cucurbita maxima* seeds petroleum ether extract	Saline	25±1.06
		Ethidium Bromide	40±1.53
		Extract 100 mg	36±1.22
		Extract 200 mg	29±0.98

Table 2: Effect of Fingolimodon the concentration of malondialdehyde (MDA)

S.No	Sample Name	Concentration	nmol/gm/tissue
2	Fingolimod	Saline	25±1.06
		Ethidium Bromide	40±1.53
		Extract 100 mg	25±1.23
		Extract 200 mg	18±1.02

Table 3: Effect of *Cucurbita maxima* seeds petroleum ether extraction of Superoxide Dismutase (SOD)

S.No	Sample Name	Concentration	mg/tissue
2	*Cucurbita maxima* seeds petroleum ether extract	Saline	30±0.85
		Ethidium Bromide	48±1.13
		Extract 100mg	39±0.94
		Extract 200mg	24±1.26

Table 4: Effect of Fingolimodon the concentration of malondialdehyde (MDA)

S.No	Sample Name	Concentration	nmol/gm/tissue
3	Fingolimod	Saline	25±1.06
		Ethidium Bromide	40±1.53
		Extract 100 mg	25±1.23
		Extract 200 mg	18±1.02

Anti inflammatory activity:

Table 5: Effect of *Cucurbita maxima* seeds petroleum ether on TNF - α

S.No	Sample Name	Concentration	Pg/ml
1	*Cucurbita maxima* seeds petroleum ether extract	Saline	14.5±0.098
		Ethidium Bromide	19.9±0.126
		Extract 100 mg	12.6±0.118
		Extract 200 mg	11.9±0.086

Table 6: Effect of Fingolimodon on TNF - α

S.No	Sample Name	Concentration	Pg/ml
4	Fingolimod	Saline	14.5±0.098
		Ethidium Bromide	19.9±0.126
		Extract 100 mg	12.5±0.102
		Extract 200 mg	10.6±0.114

Table 7: Effect of *Cucurbita maxima* seeds petroleum ether on IL-6

S.No	Sample Name	Concentration	Pg/ml
1	*Cucurbita maxima* seeds petroleum ether extract	Saline	23.7±0.145
		Ethidium Bromide	36.9±0.106
		Extract 100 mg	35.8±0.954
		Extract 200 mg	28.4±0.859

Table 8: Effect of Fingolimodon on IL-6

S.No	Sample Name	Concentration	Pg/ml
8	Fingolimod	Saline	23.7±0.145
		Ethidium Bromide	36.9±0.106
		Extract 100 mg	20.4±0.114
		Extract 200 mg	15.2±0.956

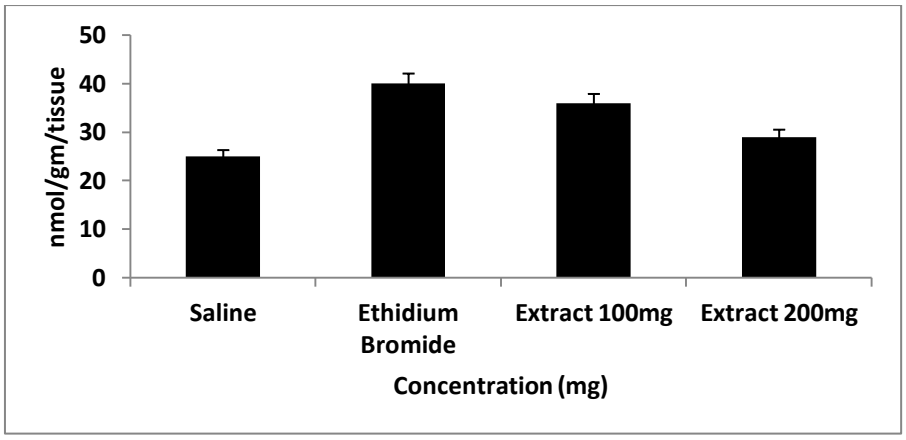


Figure 5: Effect of *Cucurbita maxima* seeds petroleum ether extract on the concentration of Malondialdehyde (MDA) in the cortex of rats subjected to in-tracerebral injection of ethidium bromide

Table 9: Histopathology:

BRAIN						
Microscopic features		**Group 0**	**Group I**	**Group II**	**Group III**	**Group IV**
HIPPOCAMPAL AREA						
1.	NEURONS :-					
	(a)Vacuolar degeneration	-	+	-	-	-
	(b) Necrosis	-	++	-	mild	-
	c) Prominence in Substantia nigra	Well seen	Less prominent	+	+	+ +
2.	GLIOSIS :-					
	(a) Astrocytes	+	++	+	+	+
	(b) Oligodentroglial cells	-	+	_	+	_
3.	CONGESTION :-					
	(a)Brain Parenchymal vessels	+	_	+	+	+
	(b) Choroid plexus	-	+	_	_	_

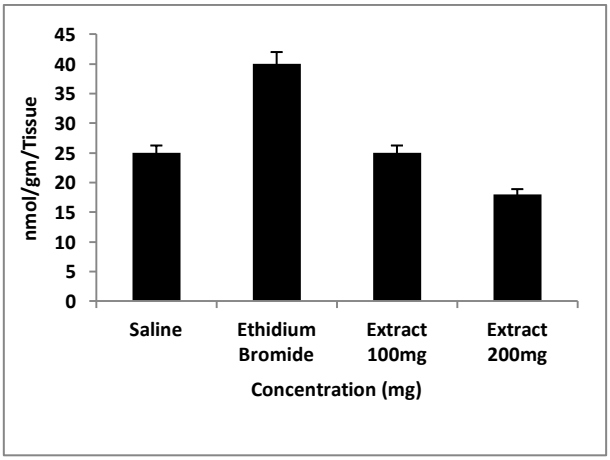


Figure 6: Effect of Fingolimodon the concentration of malondialde-hyde (MDA) in the cortex of rats subjected to in-tracerebral injection of ethidium bromide.

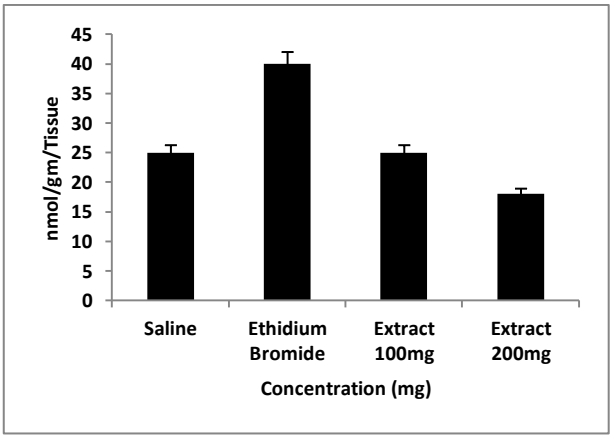


Figure 8: Effect of Fingolimodon the concentration of malondialde-hyde (MDA) in the cortex of rats subjected to in-tracerebral injection of ethidium bromide.

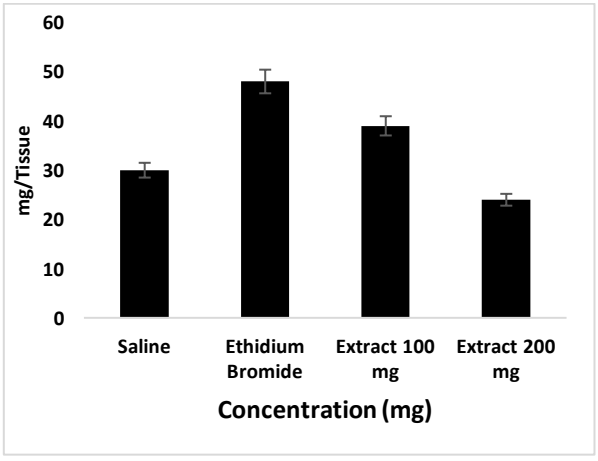


Figure 7: Effect of *Cucurbita maxima* seeds petroleum ether extraction of Superoxide Dismutase (SOD).

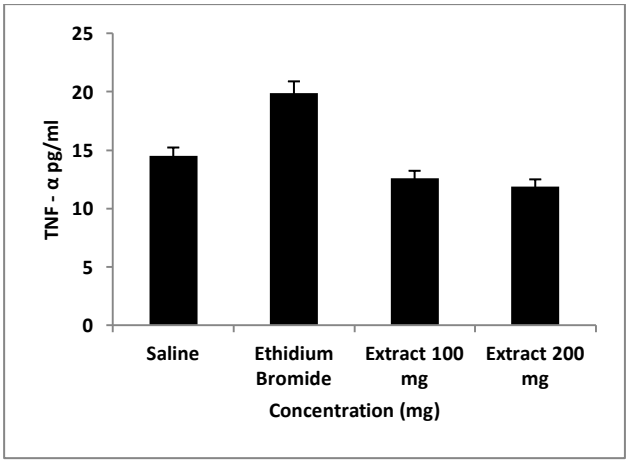


Figure 9: Effect of *Cucurbita maxima* seeds petroleum ether on TNF - α

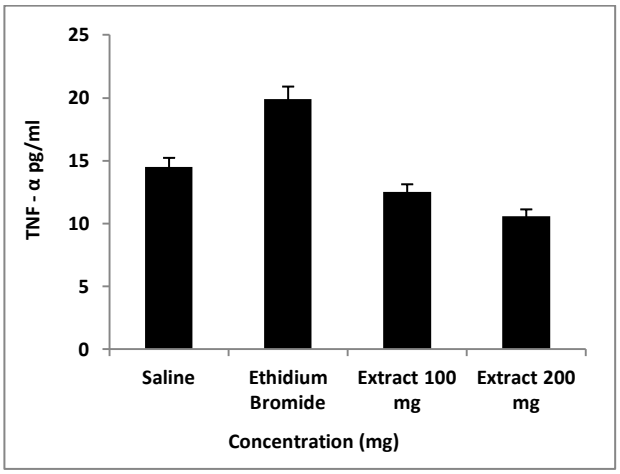


Figure 10: Effect of Fingolimodon the concentration on TNF - α

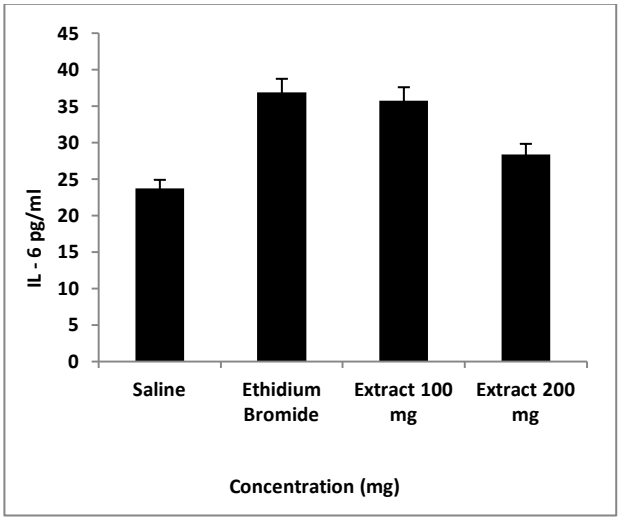


Figure 11: Effect of *Cucurbita maxima* seeds petroleum ether on IL-6.

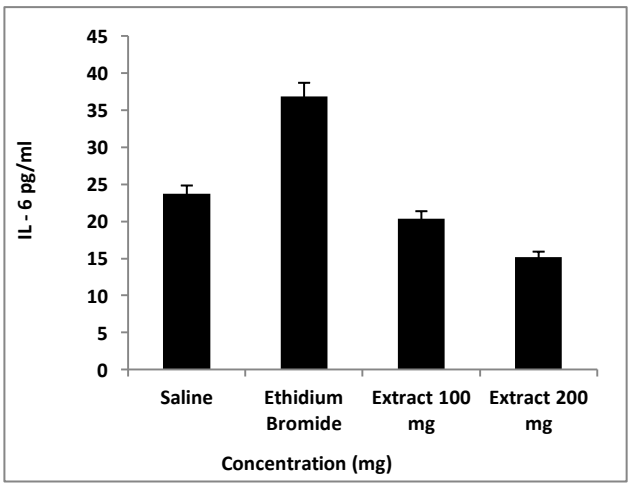
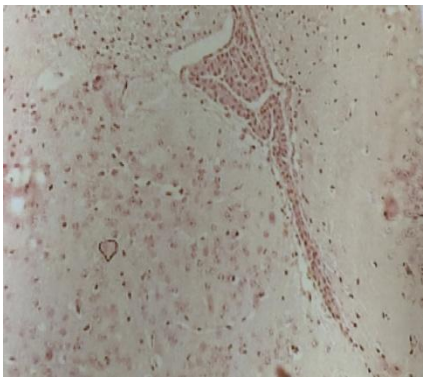
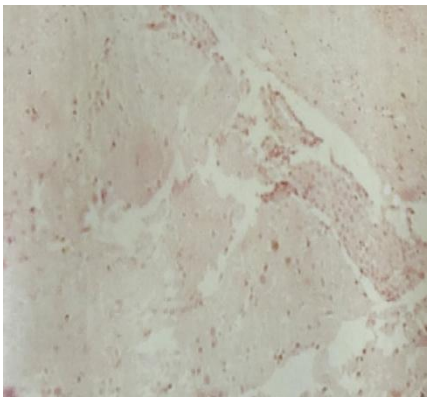


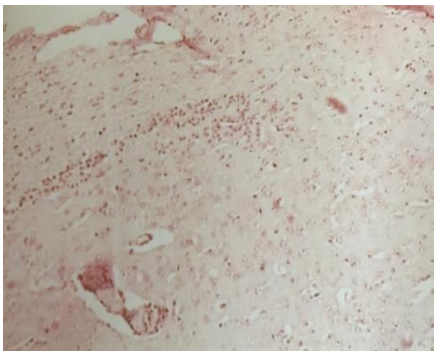
Figure 12: Effect of Fingolimod on the concentration on IL-6



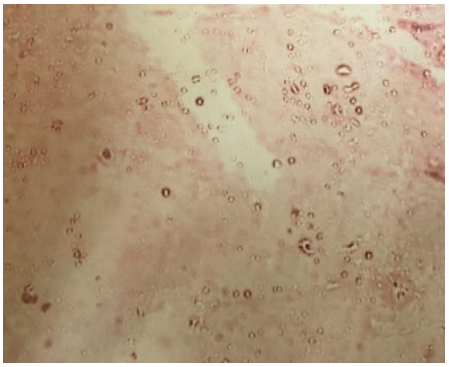
A) Solvent control



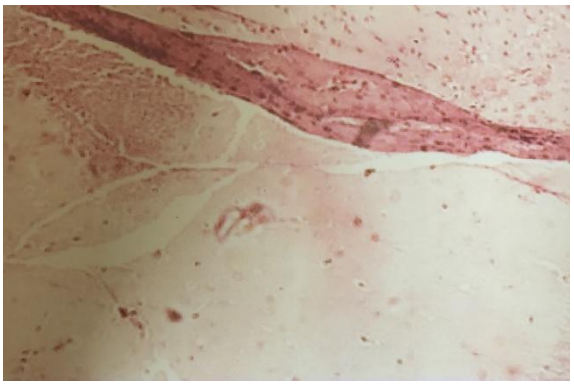
B) Induction group



C) Standard group



D) *Cucurbita maxima* seed extract (100mg/kg)



D*) Cucurbita maxima* seed extract (200 mg/kg)

Figure 13: Histopathology

DISCUSSION

Our present study has been carried out with a view to determine the pharmacological evaluation of their activity the *Cucurbita maxima* seeds have been used traditionally for various nervous disorders and brain tonic. Because of this reason this plant has been selected to evaluate scientifically the protective effect of the seeds on ethidium bromide induced demyelination. Here the petroleum ether extract of *Cucurbita maxima* seeds used to evaluation of the protective effect on ethidium bromide induced demyelination in wistar rats by compared with standard.

Mostly demyelination of CNS produces deficit in locomotor and behavioral pattern. Symptoms are confusion, muscle weakness, numbness, muscle disco ordination and hind limb paralysis. Hence the following animal's tests were carried out to assess the induction and to evaluate the remyelination in animals. The tests are

-Open field exploratory behavior test

-Rota rod test

-Beam walk test

-Grip strength test.

The animals subjected for above tests were observed for four weeks except the open field exploratory behavior test, which was carried out for only one week. The brain tissue of two animals in each group was subjected for biochemical and histopathological study.

In the present study histopathological lesions were observed in mice brain by exposing to the petroleum ether seeds extract of *Cucurbita maxima* (100 & 200 mg/kg) at repeated dose during 28days exposure. Various pathological changes like necrosis and cellular degenerative with massive diffuse cells and changes have been observed in experimental mice brain in plant extract treated rats compared to control. The present investigation with severity of histopathological lesions indicates that the repeated exposure to plant extracts cause deleterious effects and making less fit for better survival. Our results are in consistent with similar to necrotic lesion observed following exposure to certain toxic substance such as ethidium bromide. Similar study on histopathological changes in animals by heavy metals has been reported earlier by several workers (Kumar and Pant, 1981; Akhilender Naidu, 1982; Usha Rani, 1986). The present results are in agreement with the results of (Savory and Garruto 1998: Vogelbruch et al, 2000) with neuronal degeneration and neurodegenerative diseases associated with aluminum. The cerebral cortexes are the key structures of memory formation. In the present study the brains tissue of experimental animal showed changes in histo architecture and necrosis in cerebral cortex and in mid brain compared to control which are form of neuro-degeneration. Similar result was reported by (Buraimoh, et al, 2011) with accumulation of Aluminium in the brain regions. Evidence of mild lesions in parts of the brain indicates the ability of the extract to cross the blood-brain barrier. In this study, pre treatment with plant extract, produced a reversal trend in most of the negative effects of behavior and showing protection in the brain tissue from pathological lesion with absence of necrosis and cellular Lipid peroxidation and SOD are related with cellular injury and is commonly used as an indicator of oxidative damage in cells and tissues and it is regarded as one of the primary key events in cellular damage. With increased LPO and SOD in myocardial homogenate of isoproterenol administered rats.

CONCLUSION

In the pharmacological evaluation of selected petroleum ether extract of *Cucurbita maxima* seeds for their protective effect in ethidium bromide induced demyelination on rats result in improvement in muscle strength and muscle coordination, thus proving the protective effect of this plant in motor neuron diseases by compared with standard oral anti MS drug. The components present in the seeds may be having the neutralization effect on ethidium bromide.

In the biochemical and Histopathological study, it's observed that only 200 mg/kg dose is showing the protective effect, which is not in agreement with the findings of the behavioral study. From the result s, it is found that the petroleum ether extract of *Cucurbita maxima* seeds are useful in nervous diseases (demyelination diseases) by compared with the oral anti MS drug (Fingolimod).

Acknowledgement: I am thankful to the AICTE, grant in aid in the form of AICTE Fellowship for (QIP) making this investigation possible.

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Source of Support: Nil, **Conflict of Interest:** None.