

Research Article



Effects of the Aqueous Seed Extract of *Withania somnifera* (Ashwagandha) against Pilocarpine-induced Convulsions in Rats

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ABSTRACT

The present study is designed to investigate effect of Ashwagandha aqueous seed extract against pilocarpine-induced convulsions in rats. Convulsions was induced by pilocarpine injection (300mg/kg, i.p.). After 15 days, the animals were received carbamazepine (100 mg/kg) and Ashwagandha (25 & 50 mg/kg, orally), for 14 days. Death, latencies to the first convulsion and cholinergic signs were observed. Ca level, total antioxidant capacity (TAC) and Na⁺/K⁺-ATPase activity in serum were assessed. Reduced glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO) contents, serotonin and dopamine levels were measured in hippocampus. Finally, brains were isolated for histopathological study. Ashwagandha produced rise in latencies to the first convulsion and showed decrease in serum Ca level and increase in TAC and Na⁺/K⁺-ATPase activity as well as elevation in GSH content and decrease in MDA and NO contents in hippocampus; in addition, Ashwagandha modulates brain serotonin and dopamine in hippocampus as compared to the untreated pilocarpine group. Ashawagandha possess anti-convulsant property against the neurophysiological disorders induced by pilocarpine in rats.

Keywords: Convulsions; Pilocarpine; Ashawagandha; Serotonin; Dopamine; Rats.

INTRODUCTION

Epilepsy is a chronic brain condition with epileptiform neuronal discharges, characterized by recurrent seizures¹. The brain is composed of a neurons network that transmits signals by propagating nerve impulses. The impulse propagation from one neuron's synapse to another is typically controlled by neurotransmitters. When the normal balance between inhibition and excitation by neurotransmitters is disrupted in brain parts, a seizure can take place².

At any age, the first seizure occurrence does not necessary mean epilepsy and a need for antiepileptic drug. Diagnostic procedure should be done to confirm or exclude an etiological reason^{3,4}. The difference between seizures and epilepsy is baffled. Epilepsy is a state of recurrent seizures. If one seizure happens, it may not necessarily mean that it is epilepsy, because the seizure may have been occurred and that individuals may never have a seizure again⁵.

Several anticonvulsant drugs, including lamotrigine, valproate and carbamazepine are used in the acute treatment of bipolar disorders. They have equal efficacy against generalized and partial seizures⁶.

Ashwagandha, *Withania somnifera*, is known as "Indian Winter cherry" or "Indian Ginseng". It belongs to fam. Solanaceae. Ashwagandha is found as a churna or powder which mixed with water, butter or honey. In traditional medicine and remedies its popular herb is used⁷.

The major constituents of Ashwagandha extracts from various parts are steroidal alkaloids and steroidal lactones with ergaostane skeleton (withanolides)⁸. Many toxicological studies stated that Ashwagandha is a safe and edible herb⁹.

Ashwagandha is memory enhancer and improves the brain and nervous system functions. It enhances resilience of the body to stress as it has adaptive power. Ashwagandha improves the cell-mediated immunity increasing the body's defense against disease. Moreover, its extract elicits antioxidant capacity that protect against cellular damage caused by free radicals¹⁰. In addition it displays anti-inflammatory activity¹¹.

Among the herbs, Ashwagandha is the most important plant as brain tonic in the traditional medicine. Although Ashwagandha extracts have possessed neuroregenerative, and anticancer potentials using brain-derived cells in vitro studies¹², Its Pharmacological effects have not been understood to large extent, therefore; the present study was investigated to evaluate the anticonvulsant activity of Ashwagandha

MATERIALS AND METHODS

Animals

Adult male Wister albino rats weighing 120 – 140g purchased from the animal house colony of the National Research Centre (Dokki, Giza, Egypt) and were kept in the animal house under conventional laboratory conditions.



Experiments were performed according to the National Regulations of Animal Welfare and Institutional Animal Ethical Committee (IAEC).

Plant materials

The seeds of *Withenia somnifera* were obtained from Technology of Horticulture Crops Department. The plant was identified by Prof. Dr. Aboelfetoh Mohamed Abdalla, National Research Centre; Giza, Egypt.

Drugs

• Carbamazepine (Tegretol[®]) was purchased from NOVARTIS PHARMA S.A.E. Cairo. Under licence from Novartis Pharma AG., Basil, Switzerland.

• Pilocarpine was purchased from Sigma-Aldrich, Germany

Herb extraction

According to the method of Maheswari and Manisha¹³, Ashwagandha aqueous extract was prepared by weighing accurately 10 g of the seed powder then placed in a 100 ml distilled water (1/10 w/v). Water fractions were combined and filtered through qualitative No.1 Whatman filter paper (Whatman International Ltd, Maidstone, England). In Aroma and Flavoring Department, National Research Center, the filtrate was subjected to lyophilization process through freeze drier (Snijders Scientific-tilburg, Holland) under pressure, 0.1 to 0.5 mbar and temperature -35 to -41°C conditions. The dry extract was stored at -20°C until used. We selected an aqueous extract because most of the Ashwagandha antioxidant components are extracted in it.

Experimental design

Status epilepsy was induced by intraperitoneal injection of a single dose of pilocarpine (300 mg/kg b.w.) dissolved in saline¹⁴. Atropine sulphate was dissolved in saline and injected subcutaneously at a dose of (5 mg/kg b.w.), 30 min before the administration of pilocarpine to prevent peripheral muscarinic stimulation¹⁵. Animals were left for 15 days to establish the chronic phase of induced spontaneous recurrent seizures¹⁶. Thereafter they were enrolled beside the normal groups in the study protocol.

Animals were divided into 5 groups (6 rats each) as follows: Group 1: received saline and served as normal control; group 2: considered as epileptic control without treatment; group 3-5: epileptic rats treated orally for 14 days with carbamazepine (100 mg/kg)¹⁷ and Ashwagandha (25 & 50 mg/kg) after 15 days of induction of epilepsy.

Blood Sample preparation

At the end of the experimental period, blood samples were collected from the retro-orbital venous plexus by heparinized capillary tubes under diethyl ether anesthesia. Blood samples were centrifuged at 3000 rpm for 10 min. The separated sera were stored at -20 °C till examined. After blood collection, the rats were killed, and the whole brains were rapidly removed and hippocampi

were dissected out, weighed and thoroughly washed with isotonic saline. The individual hippocampus of each animal was homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50mM tris-HCl and 300mM sucrose (pH 7.4). The homogenate was centrifuged at 3000 rpm for 10 min in a cooling centrifuge at 0 °C.

Biochemical analysis

Serum Na⁺/K⁺-ATPase was determined according to Tsakiris et al.¹⁸. Serum Ca and TAC levels were measured according to Koracevic et al.¹⁹, Gindler and King²⁰. GSH, MDA, and NO contents in hippocampus were determined according to BEUTLER et al.²¹, Ohkawa et al.²² and Montgomery and Dymock²³ using commercially available kits (Biodiagnostic, Egypt). Serotonin and dopamine levels in hippocampus were also determined according to Kema²⁴ and Miagkova et al.²⁵ using ELIZA kit Biosource EIA kit, Belgium.

Histopathological examination

At the end of the experiment, animals were dissected and the brains were removed carefully and were fixed in buffer formalin for 24 hours. The samples were washed under tap water, dehydrated in ascending grades of ethanol (50, 70, 80, 90, and 100 %) cleared in xylene, embedded in paraffin wax (melting point 55-60 °C). Brain sections of 6µm thickness were prepared and stained with Haematoxylin and eosin according to²⁶. The paraffin sections were stained in Harris's haematoxylin for 5 minutes. Sections were washed in running water for blueing and then stained in 1% watery eosin for 2 minutes, washed in water, dehydrated, cleared and mounted in Canada balsam.

Data analysis

All the values are presented as means ± standard error of the means (SE). Comparisons between different groups were carried out using one way analysis of variance (ANOVA) followed by Tukey HSD. Difference was considered significant when p < 0.05. Graph Pad prism[®] software (version 5) was used to carry out these statistical tests.

RESULTS

Effects of Ashwagandha on serum Ca level and total antioxidant capacity

Induction of epilepsy by Pilocarpine significantly increased serum Ca level and decreased total antioxidant capacity by 19.4% and 4.69%, respectively, as compared with normal control group. Animals received Ashwagandha (25 & 50 mg/kg) significantly decreased serum level of Ca by 12.5% and 15.3% and Ashwagandha (50 mg/kg) only significantly increased serum total antioxidant capacity by 3.3%, carbamazepine, also, significantly decreased serum level of Ca by 13.2% and elevated serum total antioxidant capacity by 4.7%, as compared with epileptic group (Table 1).



Table 1: Effects of treatment with Ashwagandha on serum Ca level, total antioxidant activity and Na/K ATPase

Groups → Parameters↓	Normal control	Epileptic control	Carbamazepine (100 mg/kg)	Ashwagandha (25 mg/kg)	Ashwagandha (50 mg/kg)
Ca (mg/dL)	2.13±0.05	2.55±0.08 ^a	2.21±0.02 ^b	2.23±0.02 ^b	2.16±0.03 ^b
Total antioxidant capacity (mM/L)	1.84±0.00	1.76±0.01 ^a	1.84±0.00 ^b	1.78±0.00 ^a	1.81±0.01 ^b
Na/K ATPase (μmol Pi/h/g)	623.38±13.03	590.68±9.63 ^a	617.12±18.33 ^b	621.12±11.33 ^b	627.35±7.14 ^b

Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. a significantly different from normal control at P<0.05. b Significantly different from epileptic control P<0.05.

Effects of Ashwagandha on hippocampus contents of Na⁺/K⁺-ATPase

The activity of Na⁺/K⁺-ATPase in the epileptic group was significantly decreased by 5.25% comparable to the control. Ashwagandha (25 & 50mg/kg) elevated Na⁺/K⁺-ATPase activity by 5.15% and 6.21%, respectively, carbamazepine also showed rise in it by 4.48 % as compared with epileptic group (Table 1).

Effects of Ashwagandha on hippocampus contents of GSH, MDA, and NO

Induction of epilepsy by Pilocarpine significantly decreased hippocampus GSH content by 53.2% and increased hippocampus MDA and NO contents by 53.7% and 90.4%, respectively, as compared with normal control group. Animals received Ashwagandha (25 & 50 mg/kg) significantly increased hippocampus content of GSH by 54.2% and 60.3%, respectively, decreased hippocampus MDA content by 16.2% and 22.9%, respectively, and decreased the content of NO by 31.8% and 48.6%, respectively, while carbamazepine significantly decreased NO content only by 42.6%, as compared with epileptic group (Fig 1).

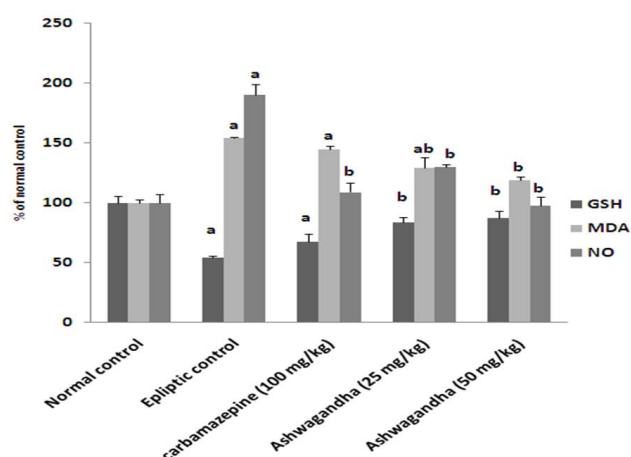


Figure 1: Effects of treatment with Ashwagandha on brain GSH, MDA, and NO contents

Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. a significantly different from normal control at P<0.05. b Significantly different from epileptic control P<0.05.

Effects of Ashwagandha on hippocampus serotonin and dopamine contents

Induction of epilepsy by Pilocarpine significantly increased hippocampus serotonin and dopamine contents by 64.1% and 51%, respectively, as compared with normal control group. Animals received Ashwagandha (50 mg/kg) only significantly decreased hippocampus serotonin and dopamine contents by 19.6% and 22.3%, respectively, also carbamazepine significantly decreased hippocampus serotonin and dopamine contents by 17.2% and 17.7%, respectively, as compared with epileptic group (Fig 2).

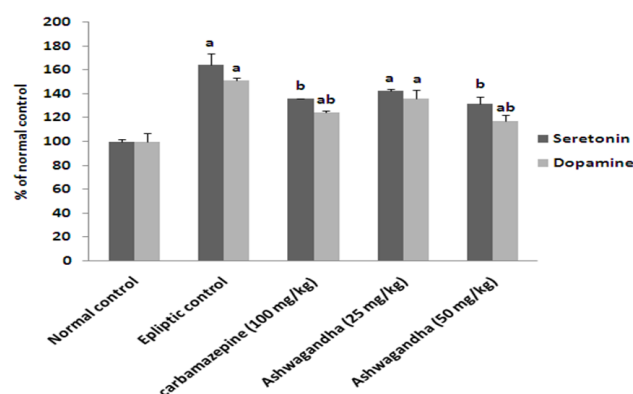


Figure 2: Effects of treatment with Ashwagandha on brain serotonin and dopamine contents

Data were expressed as mean ± SE (n=6). Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. a significantly different from normal control at P<0.05. b Significantly different from epileptic control P<0.05.

Histopathological Results

Histopathological investigation of control rats brain sections shows the highly active neurons that having pale-stained huge nuclei, the nuclear chromatin and prominent nuclei disappeared, glial cells (the surrounding support cells) having densely stained small nuclei, condensed chromatin with no visible nucleoli and background substance (neuropil) are shown in the cortex (Fig 3A).

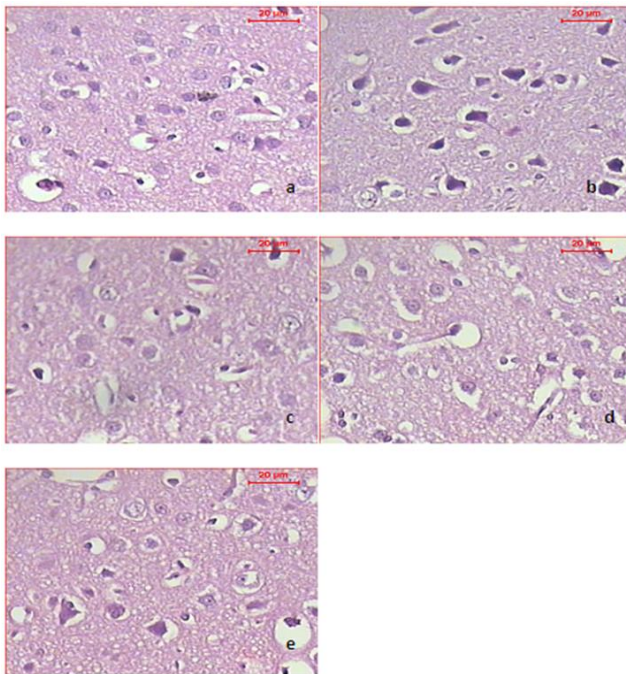


Figure 3: Micrographs of sections in brain cerebrum of a) control rat shows the highly active neurons having huge nuclei with relatively pale-stained, the nuclear chromatin and prominent nucleoli disappeared, the surrounding support cells (glial cells) having small nuclei with densely stained, condensed chromatin with no visible nucleoli and background substance (neuropil) are shown in the cortex, b) epileptic rat shows cluster of injured neurons revealing variation in sizes, vacuolization, shrinking, apoptosis, and lysis, c) epileptic rat treated with low dose of Ashwagandha shows normal structure of the neurons. Notice few cell damage and shrinkage of neurons, d) epileptic rat treated with high dose of Ashwagandha shows normal structure of the neurons. Notice few cell damage and shrinkage of neurons, and e) epileptic rat treated with carbamazepine shows few normal structure of the neurons. Notice numerous damage and shrinkage or vacuolization of neurons (H and E, Scale bar 20 μ m).

Histopathological examination of brain sections from epileptic rats showed cluster of injured neurons revealing variation in sizes, shrinking, vacuolization, apoptosis, and lysis, (Fig 3B). While, in case of epileptic rat treated with low dose of Ashwagandha normal structure of the neurons was noticed. Few shrinkage and damaged neurons were found (Fig 3C). In epileptic rat treated with Ashwagandha high dose, normal structure of the neurons was observed (Fig 3D). Examination of brains of epileptic rats treated with carbamazepine showed few normal neurons. Shrinkage or vacuolization of neurons were present (Fig 3E).

DISCUSSION

In current work, pilocarpine administration to rats initiated cholinergic symptoms as diarrhea, miosis, piloerection, mild tremors then seizures. Epilepsy developed between 20 and 30 min after administration of pilocarpine which included intermittent forelimb and hind limb clonus, head bobbing, hyperextension of tail and hind limb along with loss of posture. These findings are in

a line with those of Ahmad²⁷. On the other hand, Ashwagandha showed the anticonvulsive activity against pilocarpine induced seizure, as revealed by increase in the latency to epilepsy, decrease in the frequency of epileptic episodes, and no mortality.

The significant inhibition in the activity of hippocampal Na⁺, K⁺-ATPase in pilocarpine rats, in the present study, is an evidence of hyperexcitability state arising from Na⁺ influx into the cell as Na⁺, K⁺-ATPase acts to maintain the ionic gradient for neuronal excitability. During a prolonged seizure, the reduced Na⁺, K⁺-ATPase activity could be due to a temporary drop in ATP production²⁸. While Ashwagandha increased serum activity of Na⁺/K⁺-ATPase as compared with epileptic group.

The present investigation showed serum massive influx of Ca²⁺ in rats injected with pilocarpine that is correlated with a state of hyperexcitability arising from the massive release of glutamate which stimulates N-methyl-D-aspartate (NMDA) receptor²⁹. This massive Ca²⁺ influx in serum decreased when animals treated with Ashwagandha as compared with epileptic group.

Other biochemical studies, in our work, indicated a significant decrease in serum TAC and hippocampal GSH levels and increase in MDA and NO contents in the hippocampus of the rats injected with pilocarpine suggesting excessive release of free radicals. Excitatory amino acid receptors overactivation is an important pathogenic factor that leads to seizure genesis and increased oxidative stress has been implicated in the mechanisms of excitotoxicity-induced neurodegeneration³⁰. Therefore, antioxidant therapies have received attention in the treatment of epilepsy via reducing oxidative stress. In current work, animals treated with Ashwagandha showed rise in serum TAC and brain GSH content as well as decrease in brain MDA and NO contents as compared with epileptic group.

Numerous studies over the past two decades indicate that Ashwagandha provides potent antioxidant protection³¹ and stimulates the activation of immune system cells, such as lymphocytes and phagocytes³². Recently, Khan et al.³³ showed the effect of Ashwagandha root extract on amelioration of oxidative stress and autoantibodies production in collagen-induced arthritic rats.

Our results also show that administration of pilocarpine focally evoked seizures, this was reflected by the elicited alterations in the extracellular levels of the neurotransmitters³⁴. Elevation of extracellular dopamine level during seizure is due to an increased efflux mediated by presynaptic muscarinic stimulation. The initiation and spread of seizures in the hippocampus is assisted by endogenous dopamine released onto D1 receptors³⁵. Dopamine D1 and D2 receptors have been implicated in the mediation of excitatory and inhibitory effects³⁶. Moreover the increase in serotonin (5-HT) level in epileptic rats is consistent with the hypothesis of an

augmented rate of synthesis of 5-HT in hippocampus of epileptic rats because pilocarpine administration induces hippocampal 5-HT synthesis³⁷.

The inhibition of pilocarpine-induced seizures may be mediated by stimulation of 5-HT_{1A} and by 5-HT_{1B} receptors blockade that located on the cholinergic terminals³⁸. We have shown significant attenuation of pilocarpine-induced increases in dopamine and serotonin levels in the hippocampus following Ashwagandha administration. In previous study, Ashwagandha has anti-stress and anxiolytic activities³⁹. Ashwagandha, in this study, possesses antiepileptic properties via their major bioactive chemical principles, the glycol-withanolides (WSG). WSG in other studies, inhibit ibotenic acid-induced cognitive deficits in rats by the neurotoxin⁴⁰ and also exert significant antioxidant effect in various rat brain areas⁴¹.

CONCLUSION

The use of Ashwagandha can be a potential approach in arresting or inhibiting the seizure genesis caused by excitotoxic agents via inhibition of oxidative stress and modulation of the hippocampal monoamines release.

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