Anticonvulsant activity of methanolic extract of *Withania cogulans* in mice

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Research Article

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Abstract

Mental and neurological diseases including depression, Parkinson's disease, dementia, epilepsy, anxiety disorders and bipolar disorders account for a considerable amount of the world's disease burden. Unfortunately, drugs used in the treatment of neurological diseases are expensive, symptomatic and they produce undesirable side effects. People from different cultures prefer to use medicinal plants for the treatment of various ailments ranging from plain to perplex disorders because they are most affordable, cost effective and easily accessible source of treatment in the primary healthcare system throughout the world. *Withania coagulans*, an erect grayish under-shrub belongs to family Solanaceae. It is common in Pakistan, East India, Iran and Afghanistan. The objective of this study was to analyze the anti-seizure activity of crude methanolic extract of *Withania coagulans* fruits (MeWc). For screening of this activity, maximal electroshock seizures model (MES) and chemically-induced seizures models were used. In maximal electroshock seizures test MeWc showed significant dose dependent percent protection against hind-limb tonic extension; significant and dose-dependent increase in latency to myoclonic jerks and tonic clonic convulsions and decrease in seizures duration were observed in PTZ-induced seizures. In strychnine-induced convulsions MeWc significantly increased latency to hind-limb tonic extension and percent protection from death in a dose-dependent manner. Thus, it was inferred from the experiments that extract of *Withania coagulans* showed anticonvulsant activity.

Introduction

The term epilepsy was discovered from the Greek word "*Epilepsia*" which means "to take hold of", "seized" or "attacked" (Fong and Fong 2001). Epileptic seizure generates due to abnormal hyperactivity in the brain (Fisher et al. 2005). It is the widespread, chronic, serious neurological disease affecting 65 million people worldwide (Thurman et al. 2011) and its symptoms include disturbance of movements and sensation (hearing, vision and taste), unconsciousness, anxiety, depression, disturbed mood or other cognitive dysfunctions. Idiopathic epilepsy is the most common type affecting about 60% epileptic people. Other type is symptomatic or secondary epilepsy with known causes. The causes of symptomatic epilepsy may be severe brain injury, stroke, brain tumor, certain genetic disorders, brain infections and birth defects (Megidido et al. 2016).

It has been reported that epilepsy affects approximately thirty thousand people every year and in every 20 people, one man develops epilepsy in his life. The ratio of new epilepsy cases is 20-70 cases/lakh population every year globally (Shorvon, 1990). As compared to elder population, incidence rate of epilepsy is more in children and adults in developed countries, while in developing nations, range of active epilepsy in Asia is 1.5–14 per 1000, in South America 17–57 per 1000 and in African countries this ratio is 5.2–43 per 1000 population (Mac et al. 2007).

There are various pathologies involved in the brain that leads to the development of epileptic disorder such as increased hyperexcitability due to abnormal synaptic connectivity, suppressed GABAergic receptor function, dysfunction of potassium channels and sodium channels, increased

neurotransmission of glutamate or decrease level of GABA level etc. (Wojda et al. 2009). Moreover, variety of models have been used for the screening of antiepileptic activity and for the investigating mechanistic pathway of the unknown compounds. These models include MES model (altering sodium influx and enhances glutamate level), PTZ induced seizures model (inhibiting GABA receptor), Kainic acid-induced seizure model, pilocarpine-induced seizure model (enhancing glutamate receptor activity), strychnine-induced seizure model (antagonism of glycine receptor) (Kandratavicius et al. 2014).

Conventionally, there are various classes of drugs available for the management of epilepsy such as Benzodiazpines, barbiturates, GABA analogs, succinimides, hydantoins, and carbamazepine (Goldenberg 2010). These AEDs are linked with various adverse effects, teratogenicity and dose-related chronic toxicity (Stefan et al. 2004). Drugs used in neurological diseases are expensive and they produce severe side effects which make the treatment difficult; so there is a demand for new drugs which are beneficial in neurological diseases and which are cost effective. One of the approaches is to search for naturally-occurring compounds (Vigil et al. 2005).

Withania coagulans belonging to family Solanaceae is mainly distributed in Pakistan, East India, Iran and Afghanistan (Maurya 2010). It is having globose-shaped smooth berries having diameter up to 6–8 mm and the seeds are somewhat ear-shaped having 2.5–3 mm diameter (Kirtikar 1933). Several phytochemicals like alkaloids, flavonoids, tannins have been extracted, characterized and isolated from aerial parts, roots and berries (Kapoor 2000). The antiepileptic activities of fruits of *Withania coagulans* has not been investigated adequately which influenced us to design this study which would help in exploring a novel agent from the plant source.

The objective of the study was to screen anticonvulsant activity of MeWc, for this maximal electroshock test (MES) and chemical-induced seizure models like pentylenetetrazole (PTZ) and strychnine (STN) models were used. The safety profile of MeWc was investigated by acute toxicity and neurotoxicity tests.

Material And Methods

Animals grouping

Male Balb/c mice weighing 20-30g were used in each model. Animals were obtained from animal house of Department of Pharmaceutical Sciences, Abbottabad University of Science & Technology, Abbottabad. Animals were kept under standard conditions; 25±2°C and 12/12 hr light/dark cycle and were fed with easily accessible food pellets and water.

Chemicals and plant material

Pentylenetetrazole and strychnine were purchased from Sigma Aldrich. Methanol was obtained from Musaji & sons, Rawalpindi while Sodium valproate and Phenytoin were received as gift samples from PolyFine Chem Pharma (Pvt) Ltd Peshawar and Adamjee Pharmaceuticals (Pvt) Ltd. Karachi. All the

drugs and extracts were dissolved in the normal saline. *Withania coagulans* fruits were purchased from local herb market in Karak, Khyber Pakhtoonkhwa, Pakistan. Taxonomical identification of plant was performed by Dr. Syed Mujtaba Shah, Botany Department, Hazara University, Mansehra. A voucher specimen number HUP/S02 for *Withania coagulans* was submitted in the herbarium of said Department. Dried fruits of *Withania coagulans* were ground to fine powder with the help of electric herb grinder and then extracted by maceration with methanol for 15 days. The process was carried out at room temperature with occasional shaking and left the mixture so that the plant material was maximally dissolved in organic solvent. After 15 days, it was filtered with white thin cloth. By repeating the same process thrice methanol soluble filtrate was obtained which was then concentrated with rotary evaporator at 40°C and in this way crude methanolic extract of fruits was obtained.

Toxicity Profile

LD₅₀

LD₅₀ is the dose at which plant extract caused mortality in 50% of the total number of animals in 24 hrs. The LD₅₀ of the MeWc was calculated through the method of Lorke's (Lorke 1983). Intraperitoneal (i.p.) administration of MeWc at 10, 100 and 1000 mg/Kg dose was done to the 3 different groups (n=3). Mortality was checked after 24 hours in each group. For calculating the least and most toxic values four more animals were injected with different doses of MeWc and by calculating geometric mean of these values LD₅₀ was obtained which was 3800 mg/Kg.

Acute Neurotoxicity (TD₅₀)

Acute neurotoxicity of MeWc was determined by method developed by Coughenour and colleagues in 1977 (Coughenour and Parker 1977). It consisted of metal bars to which square platform was attached. The square platform was made of 0.6 cm wire mesh. One day prior to the experiment mice were pretrained on the apparatus. Those failing to climb the inverted screen were excluded. Testing was carried out 30 minutes after intraperitoneal administration of different doses of MeWc. Mice which were unable to climb inverted screen within sixty seconds were rated as failures.

Anticonvulsant activity of extract of *Withania coagulans*

Anticonvulsant screening of the extract was determined by maximal electroshock seizure test. Antiepileptic activity of the material was further validated by chemically induced antiepileptic models in mice. In each set of experiments, animals were divided into five groups (n=8) i.e. control group, reference standard group and 3 test groups.

Maximal electroshock seizure (MES) test

In MES test the electrical stimulus 72 mA for 0.2 s was applied to produce hind-limb tonic extension (HLTE) in control animals which served as group-I. The electrical stimulus was applied through transauricular electrodes (Kupferberg 2008)[119]. In group II phenytoin (20 mg/Kg) was intraperitoneally injected to mice and it served as reference standard group. (100-300 mg/Kg) MeWc were administered to group III-V and it served as test groups. All drugs and extract doses were injected intraperitoneally 30 minutes prior to the stimulus. Mice were considered protected if they were not displaying hind limb tonic extension.

Percent protection was calculated by using the following formula:

$$\% \text{ Protection} = 100 - \left[\left(\frac{\text{Number of Mice exhibited HLTE}}{\text{Total Number of animals}} \right) \times 100 \right]$$

Chemical induced seizure models

Pentylentetrazole (PTZ) seizure test

In PTZ seizure test mice were divided randomly into five groups consisted of 8 mice each. Group-I received NS (10 ml/kg). Group-II was injected with 450 mg/Kg sodium valproate while group III-V were administered with (100 mg/Kg, 200 mg/Kg, 300 mg/Kg) MeWc. After 30 min of the drugs and plant extract treatment, PTZ (85 mg/Kg) was administered subcutaneously to all groups. Each animal was observed for a duration of 30 minutes for onset of myoclonic jerks and onset of tonic-clonic seizures. Total duration of seizures were also noted in 30 minutes observation. (Bum et al. 2008). All doses were administered intraperitoneally except PTZ which was administered subcutaneously.

Strychnine (STN) seizure test

In STN seizure test 1.2 mg/Kg of STN was administered subcutaneously to group-I (Krall et al. 1978). Sodium valproate (550 mg/Kg) was injected to group II (positive control group). Three doses of MeWc (100 mg/Kg, 200 mg/Kg, and 300 mg/Kg) were injected to groups III-V. After administration of doses, STN was administered 30 minutes later to all groups and they were examined for protection against hind limb tonic extension (HLTE) and death.

Statistical Analysis

All experimental data was analyzed statistically for significance by One-Way ANOVA using Origin 6 software. Values were expressed as mean $\pm$ SEM. *P* value of < 0.05 was considered significant. Note that

significant differences from control group were indicated as *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ by one-way ANOVA test.

Results

Toxicity Profile

LD₅₀

LD₅₀ of methanolic extract of fruits of *Withania coagulans* (MeWc) was calculated to be 3800 mg/Kg when administered i.p. Animals were carefully observed for gross behavioral effects during first hour of drug administration. Few animals showed mild decrease in locomotor activity and sedation at higher doses (greater than 2900 mg/Kg).

Acute Neurotoxicity (TD₅₀)

For assessment of acute neurotoxicity, inverted screen test was carried out after 30 minutes of administration of different doses to animals (600, 800, 1000, 1200 mg/Kg; n = 8; i.p.) of *Withania coagulans* extract. At 1000 mg/Kg dose all tested mice climbed the screen illustrating that MeWc didn't show any neurotoxic effects on the said dose.

Anticonvulsant activity

MeWc enhanced the percent protection in MES and increased the duration of seizures in PTZ induced model

Results shown in figure 1a and 1b that MeWc prevented percent protection against hind limb tonic extensor jerks in comparison to PTZ-treated animals. Fig 1a depicted that MeWc at 100, 200 and 300 mg/Kg protected animals from HLTE to 50%, 62.5% and 75% respectively compared to the control group ($P < 0.05$, n = 8, Fig. 1a), while 20 mg/Kg phenytoin used as standard drug showed 100% protection in MES model of seizures.

Similarly, MeWc extract has dose dependent effects on seizures duration. It has been observed that MeWc 100 mg/Kg reduced the duration of seizures to 43 ± 3.35 s from 67.285 ± 3.840 s (PTZ treated group), while in 200 and 300 mg/Kg tested groups the duration of tonic-clonic seizures was further reduced to 29.571 ± 1.75 s and 21.428 ± 1.68 s respectively as compared to the PTZ treated group ($P < 0.001$, n = 8, Fig. 1b). Similarly, Sodium valproate (450mg/Kg) which was used as standard completely prevented the seizures in PTZ induced seizures (Fig 1b).

MeWc increased the onset of myoclonic and tonic-clonic jerks in PTZ-induced seizures test

MeWc delayed the onset of myoclonic and tonic-clonic jerks PTZ-induced seizure model in a dose dependent manner. It has been observed that PTZ (85mg/Kg) caused the onset of myoclonic jerks in PTZ-treated group in 195.57 ± 7.32 s. MeWc in lowest tested dose (100 mg/Kg) significantly delayed the initiation of myoclonic jerks from 195.57 ± 7.32 s (control group) to 215.14 ± 5.16 s (Fig. 2a, $n = 8$, $P < 0.05$). At 200 mg/Kg dose latency to myoclonic jerks was increased to 232.71 ± 9.37 s as compared to control group (195.57 ± 7.32 s). Similarly, at 300 mg/Kg dose, latency to myoclonic jerks was further increased from 195.57 ± 7.32 s (control group) to 255.7 ± 9.60 s. With increasing doses, an increase in latency was observed (Fig 2a). Furthermore, sodium valproate at a dose of 450 mg/Kg prevented the onset of myoclonic jerks in all the animals.

Moreover, our results shown that MeWc delayed significantly the initiation of tonic-clonic seizures in mice in PTZ-induced seizure model. Increase in latency was observed with the incremental doses of plant extract in comparison to PTZ treated group (298 ± 7.93 s). MeWc at different doses (100, 200, 300 mg/Kg) significantly and dose-dependently raised the onset of tonic-clonic seizures in mice in PTZ-induced seizure model. It was noted that MeWc at 100 mg/Kg, latency to tonic-clonic seizures was increased to 354 ± 8.643 s. At 200 mg/Kg, the latency to tonic-clonic seizures was increased to 386 ± 5.773 s and at 300 mg/Kg the latency was further increased to 430 ± 13.42 s as compared to the PTZ-treated group (Fig. 2b, $n = 8$, $P < 0.001$).

MeWc increased the percent protection and latency to HLTE and death in strychnine induced seizures

Hind limb tonic extension is considered as the last stage of the seizure scores that leads to the generalized seizures and ultimately cause death of the animals. It has been observed that MeWc significantly enhanced the % protection from HLTE and also delayed the onset of HLTE and death. It has been noted that HLTE was produced in all the animals by administration of strychnine (1.2 mg/Kg). MeWc at different doses (100, 200 and 300 mg/Kg) produced dose dependent and significant (Fig. 3a, $n = 8$, $P < 0.05$) percent protection from HLTE. Protection against strychnine induced seizures was observed 100% in sodium valproate 550 mg/Kg (standard group).

Similarly, MeWc delayed the time of onset of both HLTE and death in a dose dependent manner (Fig. 3b, $n=8$, $P < 0.05$). In case of strychnine-treated group, latency to HLTE and death was 286.57 ± 11.70 s and 302.28 ± 14.95 s respectively (Fig. 3b, $n = 8$). MeWc at 100 mg/Kg delayed latencies to HLTE and death to 346.85 ± 12.88 s and 352.85 ± 12.46 s respectively as compared to the control group. Similarly, MeWc 200 mg/Kg delayed the latencies to HLTE and death to 376.42 ± 10.12 and 382.85 ± 9.62 respectively. Further, MeWc 300 mg/Kg delayed the latency to hind limb tonic extensions and latency to death further to $395 \pm$

10.05 and 402.42 ± 10.06 respectively. Moreover, sodium valproate prevented all the animals from HLTE and death as compared to the strychnine-treated group.

Discussion

Epilepsy being the second most common neurological disorder which affects approximately 80% people in developing countries (Yemedje et al. 2011) Synthetic drugs that are currently available for treatment of these disorders exhibited undesirable side effects. Therefore, to overcome this situation, search for new drugs from medicinal plants has progressed in the past decade. In the present study, anticonvulsant activity of MeWc was investigated through pentylenetetrazole (PTZ) seizure model, strychnine-induced seizure model and maximal electroshock seizure (MES) (Nicoll, 1995).

It has been known that MES model potentiate influx of sodium and also raise the level of glutamate at the synaptic terminals. Glutamate then binds to the NMDA receptor that ultimately induced symptoms that mimics epileptic seizures in animals. Phenytoin and sodium valproate works by inhibiting NMDA-mediated opening of sodium influx and prevented the spread of the seizures and showed their anticonvulsant effects. The extract, MeWc, was not able to completely prevent HLTE in all mice at any dose but it increased the percent protection against MES induced HLTE with the increasing dose. This protection may be due to presence of chemical constituents in the extract that prevent the spread of seizures by inhibiting Na^+ channels (Porter et al., 1984). Thus, it may be suggested that MeWc exerts its anticonvulsant effects through inhibiting sodium influx and prevented neuronal hyperexcitation.

PTZ may exert its convulsant effects by potentiating calcium currents or by inhibiting GABA (inhibitory neurotransmitter) particularly at GABA_A receptors (Huang et al., 2001). Anticonvulsant activity of an agent may be indicated when it completely stops or delays the onset of tonic-clonic convulsions induced by PTZ (Swiader et al., 2006). As we evaluated in the current experiments that MeWc delayed the occurrence of high frequency seizures in mice and also decreased the duration of convulsions produced by the administration of GABA-A receptor antagonist (PTZ), it is inferred that MeWc may acts through GABA receptor in the prevention of seizures.

Furthermore, strychnine antagonizes glycine receptors in the brain leads to the inhibition of hyperexcitation of the neurons that ultimately produces seizures (McGaraughty & Henry, 1998). Our results demonstrated that administration of MeWc significantly increased the latency of seizures in strychnine-induced convulsions as compared to control group suggesting the possible involvement on glycinergic neurotransmission.

Conclusion

Extract of *Withania coagulans* have shown promising results in increasing percent protection in MES and STN induced models of seizures, delayed the occurrence of myoclonic and tonic-clonic seizures in PTZ-induced seizures and also delayed onset to HLTE and death in strychnine-induced seizure model in mice.

Thus it was concluded from this experiments that *Withania coagulans* have a promising antiseizure activity. Further studies could provide the deep insight into the mechanistic pathway(s) involved in the prevention of epileptic seizures by *Withania coagulans*.

Declarations

Acknowledgement

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Compliance with Ethical Standards

i. Conflict of interest

Authors declare that they have not any scientific or financial conflict of interest to any person or organization. RUH received research grant from Higher Education Commission of Pakistan HEC/NRPU# 3842 and declare no conflict no interest.

ii. Research involving animals

Study has been conducted on laboratory animals according to the approved ethical guidelines of the institution for care and use of laboratory animals.

iii. Consent of authors

All authors are willing to submit this manuscript in Metabolic Brain Disease.

Provision of Data

Data will be provided upon a reasonable request.

Contribution of authors

ZFK, MAS and RUH designed the study. ZFK, BA and MJ performed experiments presented in this study. ZFK and AAA analyzed and interpreted the data and ZFK, MAS and RUH prepared manuscript.

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Figures

Figure 1

MeWc enhanced the percent protection of animals from HLTE in MES model (Fig. 1A) and reduced the duration of seizures in PTZ-induced seizure model (Fig. 1B). $P^{***} < 0.001$ vs. control group by applying one-way ANOVA

Figure 2

MeWc delayed the onset of myoclonic jerks and tonic clonic seizures in mice in PTZ-induced seizure Model. Fig. 2A shown that MeWc delayed the occurrence of myoclonic seizures significantly. Fig. 2B shown the delayed effects of MeWc on the onset of tonic clonic seizures caused by the administration of

PTZ. Data obtained were represented as Mean $\pm$ SEM. P value was taken as significant $P < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$ vs. control group by applying one-way ANOVA.

Figure 3

MeWc enhanced the percent protection from HLTE (3A) in Strychnine-induced seizure model and delayed the onset of HLTE and death (3B) reduced the duration. Data were represented as Mean $\pm$ SEM and analyzed through one-way ANOVA in Origin statistical software. P value < 0.05 was taken as significant.