



# Effect of Aqueous Extract *Withania somnifera* on Tolerance to Antiepileptic Effect of Phenobarbitone in Mice

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## ABSTRACT

**Objective:** To evaluate neuroprotective and antioxidant properties in Phenobarbitone (PHB) induced tolerant effect of aqueous extract of *withania somnifera* (WS) in mice. **Materials & Methods:** Male Swiss albino mice were screened for hind limb tonic extension (HLTE). Animals were divided into 6 groups, Group 1 and 2 served as controls, which received vehicle and PHB (25 mg/kg, i.p.) respectively. Group 3 received PHB (25 mg/kg, i.p.) and after two hr induced to maximal electric shock (MES) at alternate day for 18 days, incidence of HLTE on two consecutive days taken as endpoint. Group 4, 5 and 6 MES was induced 2 hr following co-administration of PHB (25 mg/kg, i.p.) and WS (500, 750 and 1000 mg/kg p.o. respectively). At the end of 18<sup>th</sup> day, mice behaviour tests were performed, after those animals were sacrificed by overdosing of anaesthesia and brain were collected for estimation of malondialdehyde (MDA) and reduced glutathione (GSH). **Results:** After WS treated (500 mg/kg) mice were showed 80% of tolerance effect which has similar to phenobarbitone treated mice. The higher dose treated of WS (750 and 1000 mg/kg) mice were showed maximum at 20% of the tolerance effect in MES induced epilepsy. MDA levels were decreased and GSH levels increased significantly. Higher dose of WS treated mice were showed significant improvement in cognitive functions. **Conclusion:** These findings suggests that aqueous extract of WS (750, 1000 mg/kg) adjuvant to PHB delay in development of tolerance and added advantage of prevention of cognitive impairment and oxidative stress.

**Key words:** Cognition, Epilepsy, Oxidative stress, Phenobarbitone, Tolerance, *Withania somnifera*.

## INTRODUCTION

Epilepsy is a common neurological disorder affecting more than 50 million people worldwide. Approximately 90% of them are suffer from developing countries.<sup>1</sup> In spite of availability of many antiepileptic drugs (AED),

epilepsy cannot be cured, but it can be controlled with proper medications. Surgery may be considered in difficult cases. However up to 30% of patients with epilepsy do not have seizure control even with the best available medications.<sup>2</sup> Phenobarbitone (PHB) is a barbiturate group of drug and still widely used antiepileptic drug (AEDs) for neonatal and childhood seizures and for drug resistant convulsive and nonconvulsive epilepticus. It is a cheap and also cost effective for some industrialized countries. PHB potential to produce sedative, behavioural and mood effects especially in children. Compare to other AEDs greater like hood for PHB to be withdrawn compared with other AEDs possibly

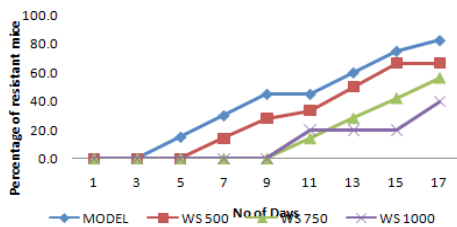
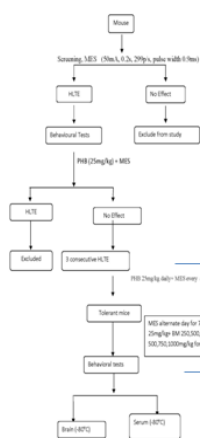
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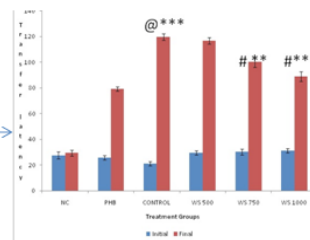
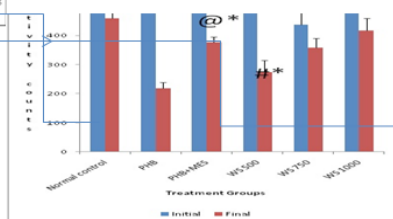
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Groups	GSH (nmol/g wet tissue)	MDA (μg/g wet tissue)
Normal control	109.2±11.01	43.9±4.84
PMB+MES	68.2±9.38**	222.5±16.07****
PMB+MES+ <i>Withania somnifera</i> 500mg/kg	78.3±12.36**	156±16.73**
PMB+MES+ <i>Withania somnifera</i> 750 mg/kg	84.0±16.01**	137±11.26**
PMB+MES+ <i>Withania somnifera</i> 1000mg/kg	88.2±8.04**	115.9±11.47**



Graphical Abstract

due to higher doses and concerns about its toxicity.<sup>3</sup> It is as effective in monotherapy to control seizures as phenytoin and carbamazepine.<sup>4</sup> So therefore need of the drugs that delay in development tolerance, improve cognitive function and reduce epilepsy induced oxidative stress.

The use of complementary and alternative medicine along with allopathic drugs is very common and is increasing.<sup>5</sup> Dried root of *Withania somnifera* Linn called Winter cherry in English and Ashwagandha in Hindi has been used in Ayurvedic medicine for many centuries. Experimental studies show that the plant extract is effective in neuroprotection in cerebral ischemia<sup>6</sup> The extract of ashwagandha is effective against oxidative stress and prevents the cyclophosphamide induced toxicity in rats.<sup>7</sup> *Withania somnifera* root has been studied for CNS effects. It was reported that the anticonvulsant effect of extract *Withania somnifera* against PTZ seizure threshold paradigm involved the gabaergic modulation in experimental model of mice.<sup>8</sup> The antioxidant effects of active principle of *Withania somnifera* demonstrated anti-stress, immunomodulatory, cognition-facilitating, anti-inflammatory and anti aging effects produced by them in experimental animals.<sup>9-12</sup> it has a mild tranquilizing action. It modulates acetylcholinesterase activity and thus enhances cognition and improves memory.<sup>10</sup> Therefore in this study, the effects of aqueous extracts of *Withania somnifera* (WS), were evaluated with co-administration of WS extracts

on development of tolerance to antiepileptic effects of phenobarbitone as well as effect of cognitive improvement in phenobarbitone tolerant mice.

## MATERIALS AND METHODS

### Plant materials

The selected plant extracts namely *Withania somnifera* (root) was obtained from Natural Remedies Pvt Ltd, Bangalore, and Karnataka, India. Plant extracts were prepared by using aqueous extraction method and were concentrated using rotary evaporation followed by freeze drying. The profiling of all selected plant extracts was done and a certificate of analysis was provided by Natural Remedies Pvt Ltd.

### Chemicals

Phenobarbitone was obtained from Nichlos Pirmal Pvt Ltd, Mumbai.

### Experimental animals

Adult Swiss Albino mice of male sex weighing 25-30 g were used in the pharmacological study and kept in the animal house of AIIMS, Department of Pharmacology, Ansari Nagar, and New Delhi, India. The animals were maintained in well-ventilated room, temperature was

maintained at  $22 \pm 1^\circ\text{C}$  with humidity at  $55 \pm 5\%$ . They were fed balanced rodent pelleted diet, and tap water *ad libitum* throughout the experimental period. The animals were housed for one week, prior to the experiments to acclimatize to laboratory condition. All the experimental protocol was followed according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi, India. All the experimental procedures were approved by the standing Institutional Animal Ethics Committee (IAEC) of AIIMS, New Delhi, India. (667/IAEC/12).

## Experimental design

Albino mice were divided into six groups each group consist of six animals. Group 1 considered as a normal control; group 2 received PHB (25 mg/kg, i.p.) for 18 days; group 3 received PHB (25 mg/kg, i.p.) with MES (50 mA, 299 pulse/sec, 0.2 sec); Group 4, 5 and 6 received WS (500, 750 and 1000 mg/kg, p.o) respectively for 18 days along PHB and MES treated.

## Maximal Electric Shock

The MES models as described.<sup>13</sup> Ear clip electrodes were used. Before applying the electrodes, the ears of mouse were wiped with cotton soaked in normal saline (to ensure good conductivity of the electroshock current). The shock was delivered using ECT unit (57800-001, UGO Basile, Italy). The frequency (299 p/s), current duration (0.2 s) and pulse width (0.9 ms) were kept constant whereas the current intensity varied. The mice were observed for incidence latency and duration of hind limb tonic extension (HLTE).

## Development of PHB induced tolerance

For induction of phenobarbitone induced tolerance, Animals were pretreated with phenobarbitone (PHB) 100% protective anticonvulsant dose i.e., 25 mg/kg i.p. And 2 hours later electroshock were given.<sup>14</sup> Electroshock were repeated after every alternate day while phenobarbitone were administrated daily till animals become tolerant and respond with HLTE on two consecutive tests or 18 days whichever is earlier. After this animals were separated for further experimentation.

## Behavioural test

In order to assess the effect of different treatment protocols on cognition in mice, assessment was carried out using three different tests i.e., Closed field activity using actophotometer, Grip strength scores and Elevated plus maze.<sup>15,16</sup>

## Elevated Plus Maze

The apparatus consists of two open arms and two enclosed arms perpendicular to each other and, elevated from the ground. The mice were placed on the open arm facing outwards and the transfer latency (TL) (the time in which the rat moves from the open arm to the closed arms) was noted. On the next day, the mice were placed similarly on the open arm and the TL noted again. This procedure was repeated on day 18 i.e. after development of tolerance.

## Closed field activity test

Spontaneous loco motor activity was assessed before initiation of protocol for development of tolerance, at day 18. Each mouse was observed for a period of 10 minutes in square closed arena equipped with infrared light sensitive photocells using a digital photoactometer (Techno, India Ltd.). The apparatus was housed in a darkened light and sound attenuated ventilated testing room.

## Grip test

The apparatus was a string of 50 cm length, pulled taut between two vertical supports and elevated 40 cm from a flat surface. The mice were placed on the string at a point midway between supports and evaluated according to the following scale: 0 – fall off, 1- hangs onto string by two forepaws, 2- as for 1 but attempts to climb on string, 3- hangs onto string by two forepaws plus one or both hind paws, 4- hangs onto string by all fore paws plus tail wrapped around string, 5- escape.

## Biochemical test

### Oxidative Stress

The oxidative stress markers, malondialdehyde (MDA) and reduced glutathione levels (GSH) were estimated in mouse whole brain tissue homogenate after 18 days in all the 6 groups. The mice were decapitated under ether anesthesia and the brain was quickly removed, cleaned by rinsing with chilled normal saline and stored at  $-20^\circ\text{C}$  until analysis. The biochemical analysis was performed within 1 week. Malondialdehyde (indicator of lipid peroxidation) and reduced glutathione were estimated as described<sup>17,18</sup> respectively.

## Statistical analysis

The results were presented as Mean  $\pm$  SEM. Results were analyzed by one way ANOVA followed by non-parametric Kruskal-Wallis test of using SPSS version 11.5.  $P < 0.05$  as considered being statistically significant.

## RESULTS

### Effect of WS (500, 750 and 1000 mg/kg) on development of tolerance to PHB

In MES + PHB treated group, repeated administration of PHB (25 mg/kg) daily, and MES on alternate day for 18 days in 80% mice development tolerance. Administration of WS (500 mg/kg) along with PHB + MES also showed tolerance 80% as MES + PHB. However WS at higher doses (750 and 1000 mg/kg) preventing development of tolerance to 40%, 25% respectively. (Figure 1)

### Effect of WS (500, 750 and 1000 mg/kg) on spontaneous loco motor activity

Spontaneous locomotors activity significantly reduced in

PHB + MES, PHB + MES + WS 500 group compared day 1 and day 19 (  $P < 0.05$ ). However this reduction was not significant in PHB + MES + WS 750 and PHB + MES + WS 1000 group. (Figure 2)

### Effect of WS (500, 750 and 1000 mg/kg) on Elevated Plus maze activity

Significant increase in latency time was noted in tolerant mice & WS 500 group in comparison to normal control indicating impairment of memory .while in WS 750 & 1000 mg/kg groups significantly reduced the transfer in latency time. (Figure 3)

### Effect of WS (500, 750 and 1000 mg/kg) on Muscle Grip strength activity

Statistically significant decline in grip test score was seen

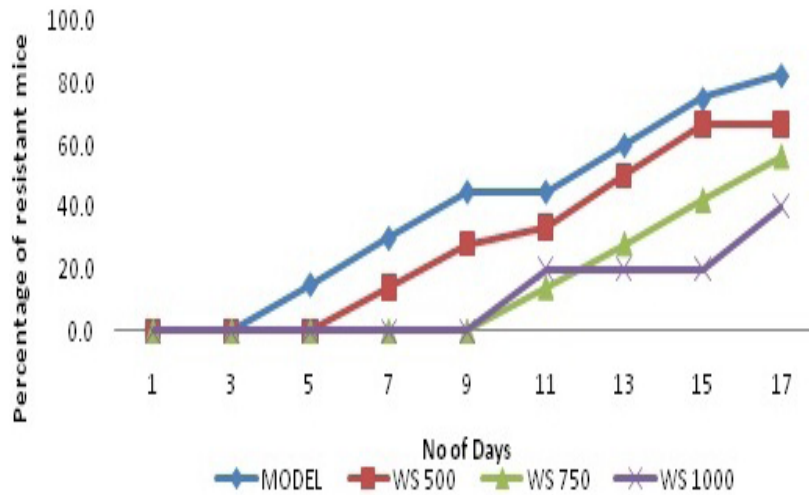


Figure 1: Effect of different doses of *Withania somnifera* on development of tolerance to phenobarbitone in mice

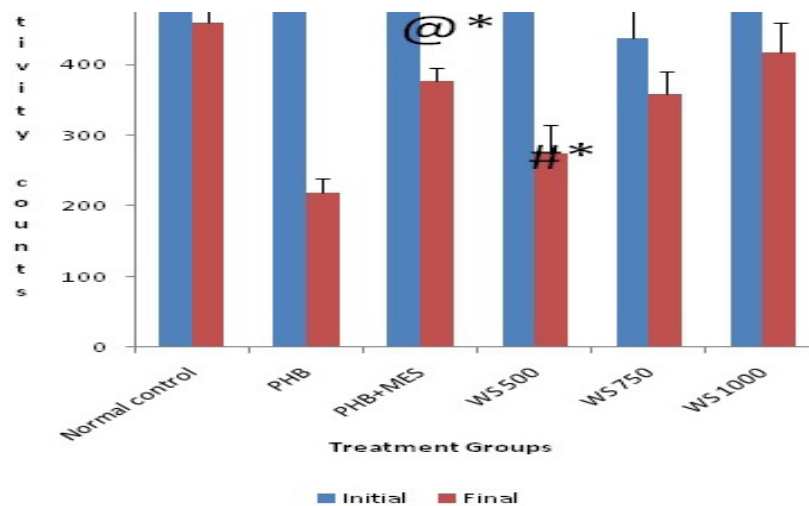
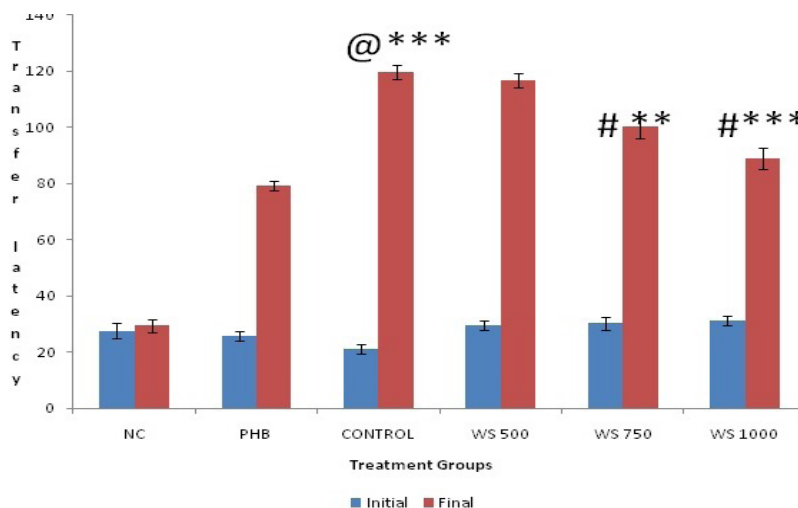


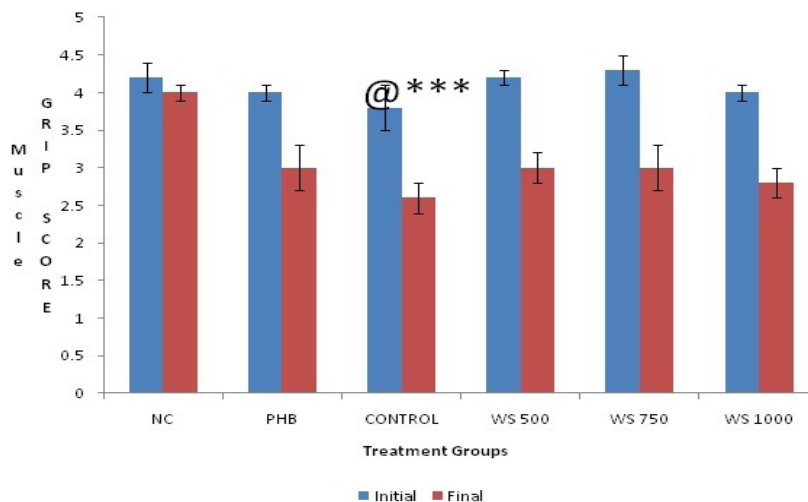
Figure 2: Effect of different doses of *Withania somnifera* on spontaneous locomotor activity using actophotometer in mice (10 min)

Each value represents mean  $\pm$  SEM for 6 mice PHB represents Phenobarbitone; MES represents maximal electroshock @ p value for comparison with normal control group # p value for comparison with PHB+MES group \*  $p < 0.05$ ; \$  $p > 0.05$



**Figure 3: Effect of different doses of *Withania somnifera* on spontaneous locomotor activity using actophotometer in mice (10 min)**

Each value represents mean ± SEM for 6 mice PHB represents Phenobarbitone; MES represents maximal electroshock @ p value for comparison with normal control group # p value for comparison with PHB+MES group \* p < 0.05; \$ p > 0.05



**Figure 4: Effect of different doses of *Withania somnifera* on grip strength in mice**

Each value represents mean ± SEM for 6 mice PHB represents Phenobarbitone; MES represents maximal electroshock @ p value for comparison with normal control group # p value for comparison with PHB+MES group \* p < 0.05; \$ p > 0.05

in tolerant mice however all WS groups showed no effect on grip scores when compared to tolerant mice. (Figure 4)

### Level of reduced GSH in resistant mice brain

A significant change was observed in the reduced GSH level between normal, control and tolerant group. WS at dose 500 mg/kg did not significantly alter the brain reduced GSH level in comparison to normal control and tolerant mice. However at the dose of 750 mg/kg and 1000 mg/kg significant increase in brain reduced GSH levels were observed compared to tolerant mice (Table 1).

### Effect of drug resistance on level of MDA in mice brain

The level of MDA was significantly increased in mice developing tolerance to PHB as compared to normal control group. Interestingly treatment with WS at all dose levels significantly decreased the brain MDA levels as compared to tolerant mice. (Table 1).

## DISCUSSION

Pharmacoresistance is encountered in nearly one third of epilepsy patients. Pharmacoresistance leads to shortened life spans due to a higher incidence of sudden deaths,

**Table 1: Effect of different doses of *Withania somnifera* administered during development of tolerance to phenobarbitone on GSH & MDA levels in mice brain**

Groups	GSH(nmol/g wet tissue)	MDA( $\mu$ g/g wet tissue)
Normal Control	109.2 $\pm$ 21.01	43.9 $\pm$ 4.84
RHB + MES	68.5 $\pm$ 9.58***	222.5 $\pm$ 16.01***
RHB + MES + <i>Withania somnifera</i> 500 mg/kg	78.3 $\pm$ 12.56#*	156 $\pm$ 26.23#*
RHB + MES + <i>Withania somnifera</i> 750 mg/kg	84.0 $\pm$ 16.01#*	137 $\pm$ 11.26#*
RHB + MES + <i>Withania somnifera</i> 1000 mg/kg	88.2 $\pm$ 8.04#*	115.9 $\pm$ 11.47**

Each value represents mean  $\pm$  SEM for 6 mice. PHB represents Phenobarbitone; MES represents maximal electroshock @ p value for comparison with normal control group # p value for comparison with PHB + MES group \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \$p>0.05

psychiatric issues and other antiepileptic drugs (AEDs) related toxicities.<sup>19</sup> Pharmacoresistance is complex with even controlled patients relapsing and developing resistant epilepsy. While previously resistant patients would experience remission (about 4% of adults per year), of which a sizable proportion goes on to eventually relapse.<sup>20</sup> Resistant epilepsy patients adopted for various treatment strategies are curative or palliative surgery and procedures such as vagal nerve stimulation, ketogenic diet etc. But, even these have limited utility and search is on for newer more effective AEDs.<sup>21</sup> Pharmacoresistance is explained intrinsic severity hypothesis, the target hypothesis and the transporter hypothesis.<sup>22</sup> Drug related factors like tolerance have also been involved in development of pharmacoresistance. Genetics, alterations in drug targets, structural brain alterations, progression of disease, ineffective mechanism of drug action etc are the other factors.<sup>23</sup> Tolerance is reported to all AEDs i.e. conventional as well as the newer agents both in experimental and clinical studies.<sup>24</sup> Tolerance may lead to drug resistance. Thus we decided that strategies ameliorating tolerance development and would prevent development of drug resistance.

The effect of aqueous extracts of herb *WS* on development of tolerance was studied. The reason for selecting herbals and particularly *WS* was herbals have emerged as viable options to conventional synthetic drugs owing to the rich repository of their use in ancient literature. *WS* is not only of prominence in ancient literature but also many workers have attempted to scientifically validate the different facets of their action namely, anticonvulsant action against PTZ induced seizures,<sup>8</sup> effect on learning and memory<sup>10</sup> stress etc. Here it is also worthwhile to mention that this herbal has been in use, mostly as mental tonics, for a long period and shown to be safe.

An alteration in oxidative stress may lead to pharmacoresistance by multiple mechanisms. Increased production of ROS may modify ion channel properties and lead to pharmacoresistance. Drug resistant temporal lobe epilepsy is associated with an oxidative stress condition that was favourably modified by surgical resection of the epileptic foci.<sup>25</sup> An over expression of MDR1 or P-gp in epileptogenic brain tissue of patients with different types of multidrug-resistant epilepsy, has been demonstrated leading to the multidrug-transporter hypothesis of medically intractable epilepsy.<sup>22</sup> Consistent with their profile, *WS* had a beneficial effect on oxidative stress parameters i.e MDA and GSH. On treating mice with the *WS* along with phenobarbitone, there was a significant decrease in the incidence of tolerance to phenobarbitone. The mechanism by which *WS* prevent development of tolerance is unclear. It could be related to anti-oxidant activity directly or indirectly via P-gp or any other mechanism. Apart from this, since cognitive and behavioural problems are a major concern in patients with intractable epilepsy, mice were evaluated using a battery of behavioural tests.<sup>26</sup> Behavioural changes in rats can be taken as a predictor for pharmacoresistant epilepsy.<sup>27</sup> *WS* has demonstrated a favourable effect in behavioural studies previously. In this study, also the effect on all the behavioural parameters assessed excepting grip strength was consistent with a favourable effect on cognition and memory.

We conclude that aqueous extract of *WS* at higher doses prevented development of tolerance to antiepileptic effect PHB as well as improvement in cognitive function.

Major limitation of study was to find out mechanism of action of tolerance development.

## CONCLUSION

These findings suggests that aqueous extract of *Withania somnifera* adjuvant to PHB delay in development of tolerance and added advantage of prevention of cognitive impairment and oxidative stress.

## CONFLICT OF INTEREST

Authors declared there is no conflict of interest.

## ACKNOWLEDGEMENT

Natural remedies Bangalore for providing extract.

## Highlights of Paper

- Prolong use of Phenobarbitone in Epilepsy leads to development of tolerance (antiepileptic effect) and cognitive impairment.
- Aqueous extract of *Withania somnifera* add on therapy with Phenobarbitone reduces the tolerance and improves cognitive impairment.
- *Withania somnifera* add on therapy with Phenobarbitone beneficial effect in epilepsy patients.

## Author Profile

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