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Original Research Article

# A comparative study of the efficiency of *Withania somnifera* and carbamazepine on lifespan, reproduction and epileptic phenotype – A study in *Drosophila* paralytic mutant

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ABSTRACT

*Background:* Seizure disorders are considered a serious health issue because of the vast number of people affected globally and the limited treatment options. Approximately 15 million epileptic patients worldwide do not respond to any of the currently available medications. Carbamazepine (CBZ) is one of the most widely used antiepileptic drugs (AEDs) for the treatment of epilepsy, which is discontinued in less than 5% of epileptic patients due to its side effects. In traditional medicine, to establish the foundation of health care, plant extracts are utilized to a great extent to treat different pathologies. *Withania somnifera (W. somnifera)* is an herbal component with anticonvulsant properties.
*Objectives:* To compare the medicinal effects of *W. somnifera* on lifespan, fecundity, fertility and epileptic phenotype in *Drosophila* paralytic mutant ($para^{bss1}$) model system with CBZ, a commonly used AED.
*Material and methods:* Flies were exposed to three different doses of *W. somnifera* or CBZ in standard wheat flour-agar media for six days. *Drosophila* Oregon-R strain was used as a control.
*Results:* Results indicate that a high dose of *W. somnifera* increased the lifespan in *Drosophila* $para^{bss1}$ while remaining safe for fecundity and fertility. CBZ decreased the lifespan of $para^{bss1}$ mutant at higher dose (40 μg/ml), as expected, and also reduced the fecundity and fertility of the flies. Our findings indicate that *W. somnifera* was more effective than CBZ to control epileptic phenotype.
*Conclusion: W. somnifera* is an effective medication with no side effects for treating epilepsy in *Drosophila* paralytic mutant.


## 1. Introduction

One of the most common neurological disorders in humans is epilepsy, and it is caused by a persistent disruption of normal brain electrical activity, which results in unpredictable and frequent seizure attacks [1,2]. Epilepsy is caused by the imbalance between stimulatory and inhibitory neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA), which play a critical role [3].

Model organisms are extremely useful for tracking the long-term effects of antiepileptic drugs (AEDs) on functional biological traits like average or maximum lifespan, in addition to their impact on reproductive ability [4]. The main base for epilepsy treatment is pharmacotherapy to control seizures with no side effects. Even though, AEDs do not cure epilepsy, they suppress seizures [5].

Carbamazepine (CBZ) is one of the most commonly used AEDs as a first-line treatment for various neuropathic pain and epilepsy [6], but CBZ has no therapeutic impact in 30–40% of epileptic patients [7]. In epileptic patients, there is a high risk of premature death [8]. Despite the fact that more than 70% of newly diagnosed epileptic patients were cured of their seizures, the mortality rate remains substantially higher compared to the age matched healthy individuals [9].

Reproductive physiology may be affected by epilepsy [10]. Epilepsy can have a significant impact on reproduction and fertility [11] in both sexes throughout their fertile years [12], as well as disrupt the regulation of reproductive hormone secretion [13]. Fertility is lower in men and women with epilepsy than in the general population [14]. In addition AEDs are also associated with adverse effects on reproductive endocrine functions in patients

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[15], which has been linked to decreased fertility in both men and women [16]. However, it's unknown if these problems are caused by epilepsy, AED treatment, or both [17]. CBZ has been discontinued in less than 5% of patients due to the side effects such as altered endocrine activity, e.g., changes in circulating pituitary and sex hormone level, which finally leads to the change of the medication [18]. However, AEDs like CBZ have been linked to the higher circulating levels of sex hormone-binding globulin, which reduces testosterone and estradiol bioactivity [19,20]. According to clinical studies, men who take AEDs, especially CBZ, Oxcarbazepine, and Valproic acid, have more sperm abnormalities and lower sperm motility than men who do not have epilepsy [21,22]. It is well understood in biology that improvements in sperm quality have a direct impact on male fertility [14].

Approximately 3–4 out of 1000 pregnancies occur in women with epilepsy (WWE) [23]. A change in reproductive endocrine system has been observed in female epileptic patients who take CBZ. There is a connection between CBZ treatment and menstrual disorders, which could be triggered by decreased Sex hormone-binding globulin (SHBG) concentrations, and this may be a sign of polycystic ovary syndrome (PCOS), a common hormone disorder in women of reproductive age that can lead to female infertility [24].

In the treatment of epilepsy disorder, despite the availability of various AEDs on the market, there are certain drawbacks that make allopathic medications less appealing, such as higher costs and unavailability of physician's access [25], and their side effects [26].

Herbal therapy is relatively less expensive and culturally acceptable, and it could be an effective cure for epileptic patients across the world. *Withania somnifera* (*Ashwagandha*), also known as Indian ginseng, is a complementary and alternative medicine (CAM) used to treat epilepsy [27]. It has an antioxidative mechanism that increases the levels of gamma-aminobutyric acid (GABA) and cortical muscarinic acetylcholine (Ach), as well as enhancing neurite regeneration throughout the brain [28]. The screening of active compounds from *W. somnifera* root extract will help treat epilepsy. The pharmacological effect of the roots of *W. somnifera* is assigned to its active ingredients, withanolides, which have a wide variety of medicinal uses [29], and are a derivative of steroids that is rich in iron and has anticonvulsant properties [30]. *W. somnifera* extends lifespan in both flies with Alzheimer's disease and wild-type flies. By targeting the Insulin/IGF-1 signaling pathway, *W. somnifera* was able to extend the lifespan of human (epidermal growth factor receptor) EGFR-driven cancerous *Caenorhabditis elegans* by about 20% [31]. *W. somnifera's* beneficial activity may be attributed to its antioxidant and free radical scavenging effects, and *W. somnifera* may have a therapeutic role in preventing glycation-induced pathogenesis in diabetes mellitus and aging [32]. It has been approved that when *W. somnifera* is used alone or in combination with other medications, it cures impotency and enhances sex appeal and fertility [33]. *W. somnifera*, as a novel drug, has antioxidant and rejuvenating properties, as well as the ability to maintain the cellular probity of testicular cells, which is essential for the normal functioning of the testis [34]. The sexual function index improved, and sexual distress index was declined substantially with *W. somnifera* in healthy married women [35].

To our knowledge, this is the first research study that assessed *W. somnifera's* antiepileptic effect on paralytic mutant model organisms. Multiple steps of the screening procedure can be automated based on the biological characteristics of the *Drosophila* paralytic strain in order to detect and discover a new antiepileptic compound with the best efficacy and fewest side effects in flies. Furthermore, we tested and contrasted the effects of *W. somnifera* and the commercially available antiepileptic drug CBZ on lifespan, reproductive traits in *Drosophila* paralytic mutant strain in terms of fecundity and fertility, and epileptic phenotype.

*Drosophila paralytic bang-senseless*$^{1}$ mutant strain, known as, *para*$^{bss1}$ flies, carry a gain of function mutation in an allele of the voltage-gated sodium (Na+) channel gene (*para gene*) located on chromosome X, and it is semi-dominant in phenotype [36]. The structure and function of all voltage-gated Na+ channels in humans and flies are identical [37].

The *para gene* has been identified as a structural gene for Na+ channels [38,39]. Structural genes on the X chromosome of *Drosophila* are expressed equally in both sexes, despite being present in two doses in somatic cells of females (XX) and a single dose (XY) in males. This phenomenon was named “dosage compensation” by Muller [40]. The X chromosome in male *Drosophila* promotes dose compensation, resulting in a hypertranscription mechanism with more transcribe copies of its genes [41]. As a result, the gene dosage in *para*$^{bss1}$ is equal in homozygous females (*para*$^{bss1}$/*para*$^{bss1}$) and hemizygous males (*para*$^{bss1}$/*Y*). The phenotypic severity for *para*$^{bss1}$ is: *para*$^{bss1}$/*Y* = *para*$^{bss1}$/*para*$^{bss1}$ [42].

This mutant is commonly utilized as a model for intractable epilepsy because of its high seizure sensitivity and resistance to treatment with AEDs. The *para*$^{bss1}$ mutant appears to be the best model for epilepsies that exhibit alternating tonic-clonic responses that is not seen in other *Drosophila* paralytic mutant genotypes [36].

The current study examined homozygous females and hemizygous males of *Drosophila para*$^{bss1}$ mutant strains.

## 2. Material and methods

### 2.1. Chemicals

Carbamazepine (CAS 298-46-4; EC number 206-062-7; Synonym: 5H-Dibenz[b, f]azepine-5-carboxamide) was procured from Sigma–Aldrich, India.

### 2.2. Standardized W. somnifera extract

*W. somnifera* root extract standard powder (Withanolides, 2.57%; Withaferin A, 2.38%) was procured from M/s Sami Labs Ltd., Bengaluru, India. The biologically active chemical constituents of *W. somnifera* include alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine, etc.) and steroidal lactones (withanolides, withaferins) [43]. Withanolides have been considered a major therapeutic component of *W. somnifera*, naturally occurring C-28 steroidal lactones, because of their antioxidant properties built on an intact ergostane structure, in which C-22 and C-26 are oxidized to form a six-membered lactone ring [44].

### 2.3. Fly stock

Paralytic mutant strains of *Drosophila melanogaster* were raised on standard wheat flour-agar media containing yeast granules and held at a constant temperature of (22 ± 1 °C) and relative humidity of 70–80%. The *D. melanogaster* paralytic mutant strain used in present study, *para*$^{bss1}$ mutant, was obtained from the fly facility department of the National Centre for Biological Sciences (NCBS) in Bangalore, India. As a wild-type control, *Drosophila* Oregon-R strain was obtained from the University of Mysore in Mysore, India.

### 2.4. Standardization of drug dosage

Mohammad's modified protocol has been used for drug dose standardization [45]. The 50 percent lethality, or lethal concentration (LC50), of adult flies after seven days of exposure to various doses of *W. somnifera* or CBZ in the standard wheat flour-agar media was used to assess drug dosage standardization. In the present study, doses of interest for *W. somnifera* and CBZ were at

0.01% w/w and 10 μg/ml, considered mid-doses, respectively. In addition to the mid-dose, the low dose and high dose of both compounds were also examined in this study.

### 2.5. Treatment of flies with W. somnifera and CBZ

*W. somnifera* standardized powder at 0.005, 0.01, and 0.05 percent w/w [29] or CBZ at 5, 10, and 40 μg/ml were added to the *Drosophila para$^{bss1}$* mutant and *Drosophila* Oregon-R strains' standard wheat flour-agar media [46]. Both compounds were dissolved in distilled water. Both *Drosophila* strains' control cultures were raised on the same diet with distilled water added to the media but were not exposed to any compounds [46]. The compounds i.e., *W. somnifera* and CBZ, were added and mixed in the partially cooled media and the doses reflected the final concentration of compounds in the food and were supplemented to adult unmated male and virgin female flies. It's worth noting that, unlike gestational mammals, developmental exposure and food intake in *Drosophila* life cycles are entirely voluntary [47]. All experiments were done in triplicate.

### 2.6. Fecundity

Freshly eclosed virgin female and male *Drosophila para$^{bss1}$* and Oregon-R strains were collected within 8 h of eclosing, in an uncrowded condition after mild anesthesia with diethyl ether for about 1 min, and were mated two days prior to monitoring their fecundity.

Fifty glass vials (25 × 100 mm) containing 50 pairs of mated flies (for each dose) in three replicates were allowed to lay eggs on 5 ml media without (control culture) and with three different doses of *W. somnifera* or CBZ.

Likewise, for the six-day experiment, three consecutive transfers once in two days were rendered into fresh supplemented media without being etherized. To determine the rate of fecundity, the number of eggs laid by females in each vial were counted every two days under a stereo zoom microscope [48].

### 2.7. Fertility

Fertility was assessed using the same set of vials that were used to measure the fecundity. Fertility assay was evaluated by larval hatch rate based on counting the number of first instar larvae hatched out from eggs in each vial during the six-day experiment [48].

### 2.8. Lifespan

For lifespan assay, 50 newly eclosed non-mated pairs of flies were collected from each vial of fertility sets. Collected male and female flies were separately transferred into fresh media vials without (control) and with *W. somnifera* or CBZ at 2-day intervals until all of the flies were dead. The lifespan of every *Drosophila para$^{bss1}$* and Oregon-R strains in each compound set was recorded. Every condition was replicated three times [48].

### 2.9. Epileptic phenotype analysis

*Drosophila* paralytic *bang-senseless$^{1}$* (*para$^{bss1}$*) mutant flies display ordinary behaviors under normal conditions. A mechanical-stressor-induced epileptic behavior, a brief vortex mixing (a "bang") induces epileptic-like behavior in *para$^{bss1}$* flies, resulting in a complex epileptic phenotype with different phases including seizure, paralysis, tonic-clonic activity, and a refractory recovery, in which no more seizures can be triggered in behaviorally normal flies throughout this time, and, finally, complete recovery [42].

In the present study, epileptic phenotype analysis was performed on 50 males and 50 females *Drosophila para$^{bss1}$* mutants seven days post eclosion that were reared in 5 ml media without control culture and with three different doses of *W. somnifera* or CBZ for 24 h at constant temperature of (22 ± 1 °C). Then, each fly was placed in a clean glass vials and vibrated for 10 s with a VWR vortex mixer set to the maximum speed, 10 [49]. Mean recovery times (MRTs) were defined as the average time it took any individual fly that exhibited epileptic behavior due to mechanical stressor to recover and were recorded until the entire population had recovered. Recovery of *para$^{bss1}$* flies was defined as the ability of flies to stand and walk normally [50]. Three replicates were performed for each compound and dosage. The wild-type flies were unaffected by this treatment [49].

### 2.10. Statistical analysis

Statistical analysis was performed using the GraphPad Prism statistical package version 8.4.3 (GraphPad Software, San Diego, CA) for each experiment conducted. For lifespan assay, survival curve analysis and the log-rank (Mantel–Cox) test were used. Analysis of variance (ANOVA) was used to compare the variations in mean, maximum, and minimum lifespans in treatment settings, followed by descriptive statistics.

For fecundity and fertility assay, statistical analysis was done using analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

For Epileptic phenotype analysis, analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used. In the present study, the groups with a $p$ value $\leq 0.05$ were considered statistically significant.

**Table 1**
Mean ± SEM of lifespan (in days) of male and female *Drosophila para$^{bss1}$* mutant on exposure to carbamazepine and *W. somnifera*.

| | CBZ | | | | | | | | *W. somnifera* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (A) Male | | | | (B) Female | | | | (A) Male | | | | (B) Female | | | |
| | Min | Max | Mean ± SEM | $p$ value | Min | Max | Mean ± SEM | $p$ value | Min | Max | Mean ± SEM | $p$ value | Min | Max | Mean ± SEM | $p$ value |
| C[a] | 10 | 65 | 41.52 ± 2.44 | – | 11 | 71 | 42.52 ± 2.48 | – | 10 | 65 | 41.52 ± 2.44 | – | 11 | 71 | 42.52 ± 2.48 | – |
| L[b] | 11 | 64 | 39.62 ± 2.46 | 0.3452 | 13 | 70 | 43.20 ± 2.33 | 0.8103 | 11 | 64 | 41.08 ± 2.47 | 0.6004 | 10 | 70 | 42.28 ± 2.50 | 0.7388 |
| M[c] | 17 | 71 | 43.00 ± 2.62 | 0.0980 | 16 | 75 | 49.44 ± 2.31 | 0.5630 | 10 | 66 | 41.36 ± 2.50 | 0.6213 | 11 | 71 | 42.04 ± 2.48 | 0.7901 |
| H[d] | 8 | 59 | 32.86 ± 2.45 | 0.0019 | 10 | 59 | 33.80 ± 2.00 | 0.0050 | 18 | 75 | 48.14 ± 2.56 | 0.0097 | 16 | 76 | 48.60 ± 2.81 | 0.0080 |

Data were analyzed by survival curve log-rank (Mantel–Cox) test. For minimum and maximum lifespan, one-way ANOVA followed by descriptive test was used. (n = 50 flies per replicate, three such replication used for assay). CBZ: (**$p$ = 0.0019, **$p$ = 0.0050); *W. somnifera*: (**$p$ = 0.0097, **$p$ = 0.0080).

[a] Control.
[b] Low dose (CBZ- 5 μg/ml; *W. somnifera*- 0.005% w/w).
[c] Mid-dose (CBZ- 10 μg/ml; *W. somnifera*- 0.01% w/w).
[d] High dose (CBZ- 40 μg/ml; *W. somnifera*- 0.05% w/w).

## 3. Results

The current study evaluated the impact of three different concentration of *W. somnifera* (0.005%, 0.01%, 0.05% w/w) and CBZ (5, 10, and 40 μg/ml) on lifespan, fecundity, fertility and epileptic phenotype in paralytic *D. melanogaster, bang-senseless*$^{1}$ *(para*$^{bss1}$*)*. *Drosophila* Oregon-R (wild-type) strain was used as a control group.

### 3.1. Lifespan

Male and female *Drosophila para*$^{bss1}$ and Oregon-R strains did not show a difference in lifespan vs. the control flies with low dose, 0.005% ($p = 0.6004$ and $p = 0.7388$ for mean lifespan of male and female *para*$^{bss1}$ strain, $p = 0.9042$ and $p = 0.9680$ for mean lifespan of male and female Oregon-R strain, respectively) and mid-dose,

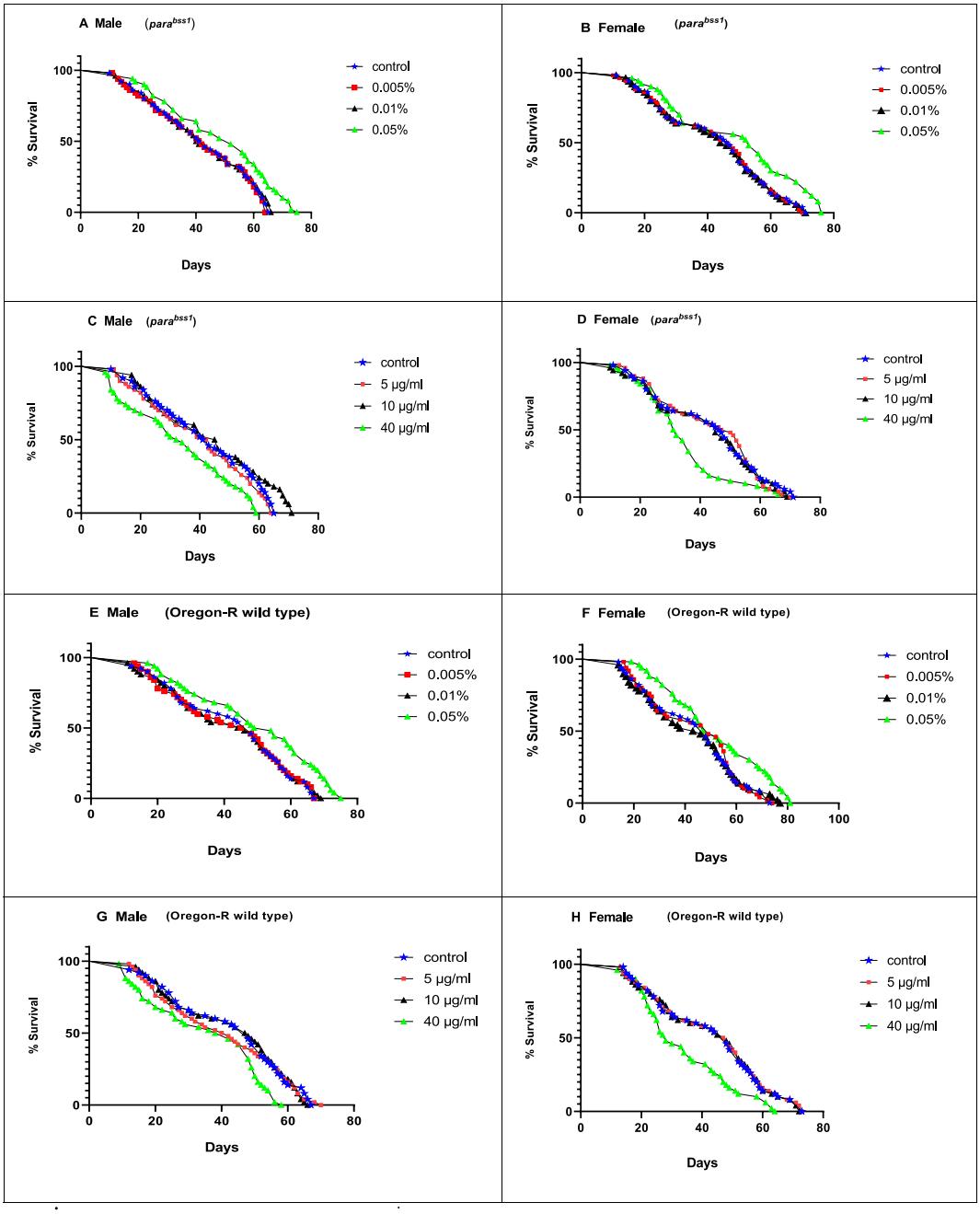


**Fig. 1.** The effect of *Withania somnifera* on the lifespan of male (**A**) and female (**B**) *Drosophila para*$^{bss1}$ mutant. At 0.005% and 0.01% in both sexes, *Withania somnifera* showed no statistically significant differences in mean and maximum lifespan. *W. somnifera* at 0.05% increased mean and maximum lifespan in both sexes significantly (**$p = 0.0097$ for mean lifespan in male and **$p = 0.0080$ for mean lifespan in female). The effect of Carbamazepine on the lifespan of male (**C**) and female (**D**) *Drosophila para*$^{bss1}$ mutant. At 5 and 10 μg/ml in both sexes, CBZ showed no statistically significant differences in mean and maximum lifespan. CBZ at 40 μg/ml decreased mean and maximum lifespan in both sexes significantly (**$p = 0.0019$ for mean lifespan in male and **$p = 0.0050$ for mean lifespan in female). The effect of *Withania somnifera* on the lifespan of male (**E**) and female (**F**) *Drosophila* Oregon-R wild type. At 0.005% and 0.01% in both sexes, *Withania somnifera* showed no statistically significant differences in mean and maximum lifespan. *W. somnifera* at 0.05% increased mean and maximum lifespan in both sexes significantly (**$p = 0.0044$ for mean lifespan in male and **$p = 0.0085$ for mean lifespan in female). The effect of Carbamazepine on the lifespan of male (**G**) and female (**H**) *Drosophila* Oregon-R wild type. At 5 and 10 μg/ml in both sexes, CBZ showed no statistically significant differences in mean and maximum lifespan. CBZ at 40 μg/ml decreased mean and maximum lifespan in both sexes significantly (**$p = 0.0017$ for mean lifespan in male and **$p = 0.0028$ for mean lifespan in female). Values are means ± SEM and units are days. (n =50 flies per replicate, three such replication used for assay).

**Table 2**
Mean ± SEM of lifespan (in days) of male and female *Drosophila* Oregon-R wild type on exposure to carbamazepine and *W. somnifera*.

| | CBZ | | | | | | | | *W. somnifera* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (A) Male | | | | (B) Female | | | | (A) Male | | | | (B) Female | | | |
| | Min | Max | Mean ± SEM | *p* value | Min | Max | Mean ± SEM | *p* value | Min | Max | Mean ± SEM | *p* value | Min | Max | Mean ± SEM | *p* value |
| C[a] | 12 | 67 | 42.36 ± 2.46 | – | 14 | 73 | 43.14 ± 2.55 | – | 12 | 67 | 42.36 ± 2.46 | – | 14 | 73 | 43.14 ± 2.55 | – |
| L[b] | 12 | 70 | 39.72 ± 2.57 | 0.7350 | 13 | 73 | 42.92 ± 2.57 | 0.7827 | 13 | 67 | 41.40 ± 2.54 | 0.9042 | 16 | 74 | 43.56 ± 2.50 | 0.9680 |
| M[c] | 14 | 66 | 42.24 ± 2.46 | 0.5716 | 13 | 72 | 43.02 ± 2.58 | 0.7242 | 11 | 69 | 41.14 ± 2.57 | 0.8244 | 14 | 77 | 42.02 ± 2.68 | 0.8467 |
| H[d] | 9 | 58 | 34.82 ± 2.38 | 0.0017 | 12 | 64 | 33.70 ± 2.11 | 0.0028 | 17 | 75 | 49.00 ± 2.64 | 0.0044 | 19 | 81 | 51.78 ± 2.64 | 0.0085 |

Data was analyzed by survival curve log-rank (Mantel–Cox) test. For minimum and maximum lifespan, one-way ANOVA followed by descriptive test was used. (n = 50 flies per replicate, three such replication used for assay). CBZ: (**$p = 0.0017$, **$p = 0.0028$); *W. somnifera*: (**$p = 0.0044$, **$p = 0.0085$).
[a] Control.
[b] Low dose (CBZ- 5 μg/ml; *W. somnifera*- 0.005% w/w).
[c] Mid-dose (CBZ- 10 μg/ml; *W. somnifera*- 0.01% w/w).
[d] High dose (CBZ- 40 μg/ml; *W. somnifera*- 0.05% w/w).

0.01% *W. somnifera* treatment ($p = 0.6213$ and $p = 0.7901$ for mean lifespan of male and female *para*$^{bss1}$ strain, $p = 0.8244$ and $p = 0.8467$ for mean lifespan of male and female Oregon-R strain, respectively) (Table 1, Fig. 1A and 1B ; Table 2, Fig. 1E and 1F).

The lifespan extensions were observed in both male and female *para*$^{bss1}$ and Oregon-R strain with high dose, 0.05% *W. somnifera* treatment compared to the control groups. In male *para*$^{bss1}$ flies, mean and maximum lifespan was increased by 6.62 days (15.94%) and 10 days (15.38%), respectively (**$p = 0.0097$ for mean lifespan). In female *para*$^{bss1}$ flies, mean and maximum lifespan was increased by 6.08 days (14.29%) and 5 days (7.04%), respectively (**$p = 0.0080$ for mean lifespan) (Table 1, Fig. 1A and 1B). In male Oregon-R flies also, the mean and maximum lifespan was increased by 6.64 days (15.67%) and 8 days (11.94%), respectively (**$p = 0.0044$ for mean lifespan). In female Oregon-R flies, the mean and maximum lifespan was increased by 8.64 days (20.02%) and 8 days (10.95%), respectively (**$p = 0.0085$ for mean lifespan) (Table 2, Fig. 1E and 1F).

Male and female *para*$^{bss1}$ and Oregon-R strains, fed on media supplemented with 5 μg/ml CBZ ($p = 0.3452$ and $p = 0.8103$ for mean lifespan of male and female *para*$^{bss1}$ strain, $p = 0.7350$ and $p = 0.7827$ for mean lifespan of male and female Oregon-R strain, respectively) and 10 μg/ml CBZ ($p = 0.0980$ and $p = 0.5630$ for mean lifespan of male and female *para*$^{bss1}$ strain, $p = 0.5716$ and $p = 0.7242$ for mean lifespan of male and female Oregon-R strain, respectively), did not show any significant changes in lifespan (Table 1, Fig. 1C and 1D; Table 2, Fig. 1G and 1H).

In contrast to the above results, high doses of CBZ (40 μg/ml) resulted in a significant decline in *Drosophila para*$^{bss1}$ and Oregon-R strains' lifespan. Mean and maximum lifespan in male and female *Drosophila para*$^{bss1}$ mutant were decreased significantly by 8.66 days (20.85%) and 6 days (9.23%) (for male flies) and 8.72 days (20.50%) and 12 days (16.90%) (for female flies), respectively (**$p = 0.0019$ for male mean lifespan and **$p = 0.0050$ for female mean lifespan) (Table 1, Fig. 1C and 1D). Mean and maximum lifespan in male and female *Drosophila* Oregon-R were decreased significantly by 7.54 days (17.79%) and 9 days (13.43%) (for male flies) and 9.44 days (21.88%) and 9 days (12.32%) (for female flies), respectively (**$p = 0.0017$ for mean lifespan in male and **$p = 0.0028$ for mean lifespan in female) (Table 2, Fig. 1G and 1H).

### 3.2. Fecundity and fertility

The effect of *W. somnifera* or CBZ on adult flies' fecundity was measured by counting their eggs during six days of exposure to different doses of supplemented media. There were no differences in fecundity between control and treated *Drosophila para*$^{bss1}$ and Oregon-R after exposure to three doses of *W. somnifera* (Table 3, Fig. 2A; Table 4, Fig. 2C).

CBZ exposure had no effect on fecundity in *Drosophila para*$^{bss1}$ and Oregon-R at 5 μg/ml (*p value* for *para*$^{bss1}$ = 0.6928 and *p value* for Oregon-R = 0.8766) and 10 μg/ml (*p value* for *para*$^{bss1}$ = 0.9961 and *p value* for Oregon-R = 0.1590) compared to the control groups. For both *Drosophila* strains, fecundity was found to be significantly reduced at 40 μg/ml CBZ (****$p < 0.0001$) (Table 3, Fig. 2B; Table 4, Fig. 2D).

There were no significant variations in fertility (the percentage of eggs hatched out as first instar larvae) in both *Drosophila* strains on exposure to three doses of *W. somnifera* compared to the control flies (Fig. 3A and 3C).

5 μg/ml (*p value* for *para*$^{bss1}$ = 0.9769 and *p value* for Oregon-R = 0.8647) and 10 μg/ml CBZ (*p value* for *para*$^{bss1}$ = 0.9400 and *p value* for Oregon-R = 0.7540), did not affect the fertility in both *Drosophila* strains compared to control flies. However, a significant decrease in fertility of both *Drosophila* strains at 40 μg/ml CBZ were observed, as predicted (****$p < 0.0001$) (Fig. 3B and 3D).

**Table 3**
Mean ± SEM of fecundity in *Drosophila para*$^{bss1}$ mutant strain on exposure to carbamazepine and *W. somnifera*.

| | CBZ | | | | *W. somnifera* | | | |
|---|---|---|---|---|---|---|---|---|
| | Min | Max | Mean ± SEM | *p* value | Min | Max | Mean ± SEM | *p* value |
| C[a] | 35.50 | 75.05 | 48.32 ± 0.90 | – | 33.50 | 75.05 | 48.32 ± 0.90 | – |
| L[b] | 33.20 | 74.30 | 47.52 ± 0.91 | 0.6928 | 32.80 | 64.30 | 49.20 ± 0.92 | 0.7656 |
| M[c] | 37.80 | 56.65 | 48.16 ± 0.68 | 0.9961 | 40.20 | 68.90 | 50.71 ± 0.97 | 0.0634 |
| H[d] | 4.30 | 19.15 | 10.53 ± 0.53 | <0.0001 | 38.80 | 58.65 | 49.23 ± 0.67 | 0.7470 |

Data was analyzed by Two-way ANOVA followed by Tukey's test. For minimum and maximum fecundity, descriptive test was used. (n = 50 flies per replicate, three such replication used for assay). CBZ: (****$p < 0.0001$).
[a] Control.
[b] Low dose (CBZ- 5 μg/ml; *W. somnifera*- 0.005% w/w).
[c] Mid-dose (CBZ- 10 μg/ml; *W. somnifera*- 0.01% w/w).
[d] High dose (CBZ- 40 μg/ml; *W. somnifera*- 0.05% w/w).

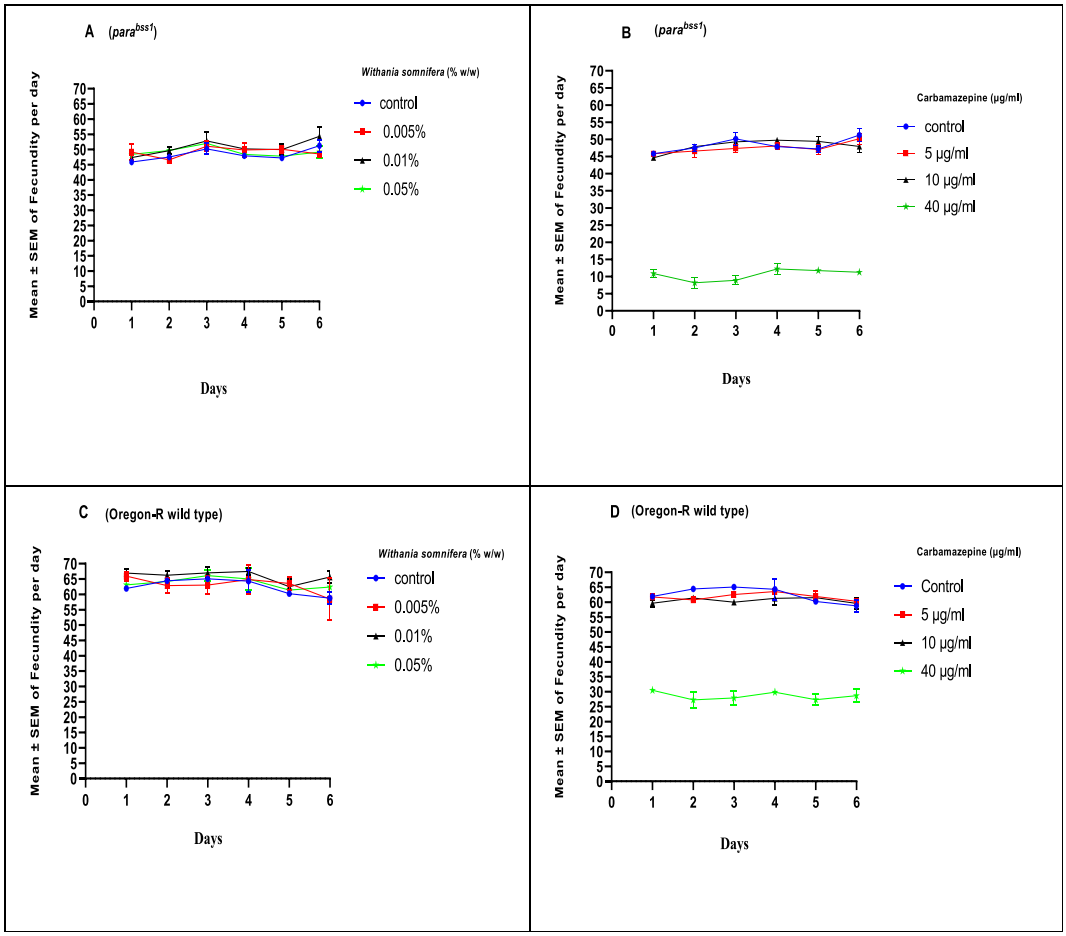


**Fig. 2.** The effect of *W. somnifera* on fecundity in *Drosophila para*$^{bss1}$ mutant at 0.005 %, 0.01 %, 0.05 %. Values are means ± SEM (**A**), The effect of carbamazepine on fecundity in *Drosophila para*$^{bss1}$ mutant at 5, 10 and 40 µg/ml (****$p < 0.0001$ for 40 µg/ml). Values are means ± SEM (**B**). The effect of *W. somnifera* on fecundity in *Drosophila* Oregon-R wild type at 0.005 %, 0.01 %, 0.05 %. Values are means ± SEM (**C**), The effect of carbamazepine on fecundity in *Drosophila* Oregon-R wild type at 5, 10 and 40 µg/ml (****$p < 0.0001$ for 40 µg/ml). Values are means ± SEM (**D**). (n= 50 flies per replicate, three such replication used for assay); Data was analyzed by Two-way ANOVA followed by Tukey's test.

### 3.3. Epileptic phenotype analysis

This study compared the antiepileptic effects of *W. somnifera* with CBZ on the MRTs from epileptic phenotypes induced in *Drosophila para*$^{bss1}$ mutant by mechanical-stressor (vortex).

There was no significant effect on the MRTs in male and female *Drosophila para*$^{bss1}$ mutants fed on media supplemented with three doses of CBZ compared to the control *para*$^{bss1}$ flies (Fig. 4C and 4D).

The high dose of *W. somnifera* (0.05%) showed a significant reduction in the MRTs in both male and female *para*$^{bss1}$ compared to the control *para*$^{bss1}$. In male *para*$^{bss1}$ flies, the MRT was reduced by 47 s (20.25%) (**$p = 0.0043$). In female *para*$^{bss1}$ flies, the MRT was reduced by 45 s (18.29%) (**$p = 0.0018$). Low dose (0.005%) and mid-dose (0.01%) had no significant effects on the MRTs of *para*$^{bss1}$ flies (Fig. 4A and 4B).

## 4. Discussion

*Drosophila* is an excellent model for quickly identifying potential therapeutic agents [51]. *D. melanogaster* is used in high-throughput

**Table 4**
Mean ± SEM of fecundity in *Drosophila* Oregon-R wild-type strain on exposure to carbamazepine and *W. somnifera*.

| | CBZ | | | | *W. somnifera* | | | |
|---|---|---|---|---|---|---|---|---|
| | Min | Max | Mean ± SEM | *p* value | Min | Max | Mean ± SEM | *p* value |
| C[a] | 56.50 | 69.33 | 62.46 ± 0.51 | – | 56.50 | 69.33 | 62.46 ± 0.51 | – |
| L[b] | 45.16 | 79.16 | 61.81 ± 0.79 | 0.8766 | 54.50 | 71.16 | 62.89 ± 0.47 | 0.9556 |
| M[c] | 45.66 | 69.33 | 60.57 ± 0.65 | 0.1590 | 42.66 | 68.83 | 63.04 ± 0.59 | 0.0870 |
| H[d] | 22.50 | 37.66 | 28.58 ± 0.37 | <0.0001 | 51.33 | 73.33 | 63.69 ± 0.74 | 0.8171 |

Data was analyzed by Two-way ANOVA followed by Tukey's test. For minimum and maximum fecundity, descriptive test was used. (n = 50 flies per replicate, three such replication used for assay). CBZ: (****$p < 0.0001$).

[a] Control.
[b] Low dose (CBZ- 5 µg/ml; *W. somnifera*- 0.005% w/w).
[c] Mid-dose (CBZ- 10 µg/ml; *W. somnifera*- 0.01% w/w).
[d] High dose (CBZ- 40 µg/ml; *W. somnifera*- 0.05% w/w).

pharmacological screens and behavioral assays, as well as genetic analysis of genes related to human diseases [52]. The main goal of this study was to compare the effects of *W. somnifera*, an herbal ingredient, and carbamazepine, a common antiepileptic drug, on the lifespan, fecundity, fertility and epileptic phenotype of *Drosophila para*$^{bss1}$ mutant strain.

Since *Drosophila para*$^{bss1}$ mutant strain is a paralytic fly, to rule out the epilepsy effects on biological parameters in *para*$^{bss1}$ mutant, we used *Drosophila* Oregon-R, a wild-type fly, as a control group. In present study, both compounds had the same effects on lifespan, fecundity, and fertility in both *Drosophila* strains. Furthermore, based on the results, all the significant changes in lifespan and reproductive parameters in the *Drosophila para*$^{bss1}$ mutant strain could be attributed to the effects of the desired compounds alone.

In recent years, using traditional and complementary medicines to treat diseases has become more popular. As a result, introducing a new medicinal combination with less side effects have become a potential goal for treating diseases [53].

According to the literature, anti-aging properties are one of the therapeutic benefits of *W. somnifera* root extract [32]. *W. somnifera* appears to play a significant role in extending lifespan in *para*$^{bss1}$ mutants, based on present study data. The lifespan of male and female *Drosophila para*$^{bss1}$ mutant and Oregon-R strains were significantly increased with administration of high dose (0.05%) of *W. somnifera.* While the lifespan of both *Drosophila* strains was unaffected by the low (0.005%) and mid-doses (0.01%) of *W. somnifera* (Table 1, Fig. 1A and 1B; Table 2, Fig. 1E and 1F). Based on several studies, the antioxidant property of *W. somnifera* is effective against cellular damage prompted by free radicals [54]. The presence of a constant telomere at the ends of chromosomes results in a longer lifespan. It is well documented that with each DNA replication, the telomeres are progressively shortened. The telomerase enzyme synthesizes telomeric DNA sequences [55]. Enhancing telomerase activity is one way to delay aging for a health lifespan [56]. *W. somnifera* enhanced telomerase activity by ~45% in human HeLa cell lines [57]. The antioxidant property of *W. somnifera* and its effect on telomerase activity enhancement may explain the lifespan extension in both *Drosophila para*$^{bss1}$ mutant and Oregon-R strains.

In present study, low dose (5 μg/ml) and mid-dose (10 μg/ml) CBZ concentrations had no effect on the lifespan of male and female flies in both *Drosophila* strains. High dose (40 μg/ml) CBZ decreased the lifespan of both male and female *Drosophila para*$^{bss1}$ and Oregon-R strains (Table 1, Fig. 1C and 1D; Table 2, Fig. 1G and 1H). According to Gagne's study, CBZ had less potential for inducing lipid peroxidation in vitro. Rainbow trout hepatocytes exposed to CBZ were studied for oxidative metabolism and cytotoxicity. CBZ had a concentration threshold of 20 M. Cell viability was significantly decreased at a higher concentration (90 M) [58]. According to Sarikaya and Yüksels' findings, the higher concentration of CBZ, 20 and 40 μg/ml, were toxic in *Drosophila*, and the results suggest that CBZ could have genotoxic effects [46]. Furthermore, the present data suggest that lifespan reductions in flies after exposure to a high dose of CBZ (40 μg/ml) may be due to genotoxicity.

One of the most important factors to consider when evaluating a drug's perinatal toxicity is its effect on reproductive performance

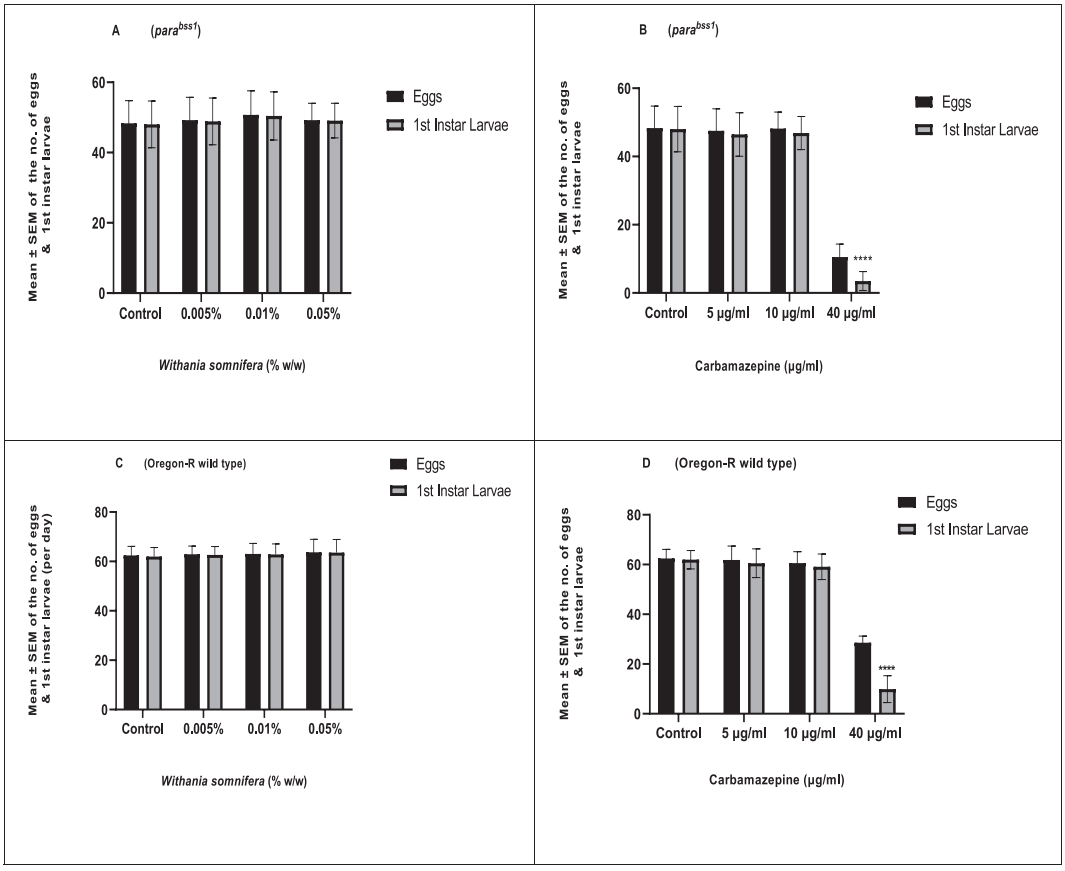


**Fig. 3.** The effect of *W. somnifera* on fertility in *Drosophila para*$^{bss1}$ mutant at 0.005 %, 0.01 %, 0.05 %. The bars display mean ± SEM (**A**), The effect of CBZ on fertility in *Drosophila para*$^{bss1}$ mutant at 5, 10 and 40 μg/ml (****$p < 0.0001$ for 40 μg/ml). The bars display mean ± SEM (**B**). The effect of *W. somnifera* on fertility in *Drosophila* Oregon-R wild type at 0.005 %, 0.01 %, 0.05 %. The bars display mean ± SEM (**C**), The effect of CBZ on fertility in *Drosophila* Oregon-R wild type at 5, 10 and 40 μg/ml (****$p < 0.0001$ for 40 μg/ml). The bars display mean ± SEM (**D**). (n= 50 flies per replicate, three such replication used for assay); Data was analyzed by Two-way ANOVA followed by Tukey's test.

[59]. Based on present study, there were no significant changes in fecundity and fertility in either *Drosophila para$^{bss1}$* mutant or Oregon-R strains in all the experimental trials on exposure to three different doses of *W. somnifera* (Table 3, Fig. 2A; Table 4, Fig. 2C; Fig. 3A and 3C).

After exposure to the high dose of CBZ, 40 μg/ml, extreme reductions in fecundity and fertility were observed in both *Drosophila* strains, as predicted. Low (5 μg/ml) and mid-dose (10 μg/ml) exposure of CBZ did not show any significant differences in fecundity and fertility of both *Drosophila* strains (Table 3, Fig. 2B; Table 4, Fig. 2D; Fig. 3B and 3D).

According to previous studies, there were no variations in the prevalence of reproductive disorders between women treated with CBZ and control women [60,61]. Based on the reports from mice studies, CBZ has no impact on female reproductive ability [62]. In comparison to the general population, men with epilepsy have a lower rate of births than women on CBZ [14], and men with epilepsy have more reproductive endocrine disorders and sexual dysfunction than the general population [63]. AEDs can have adverse effect on male sexual function, in addition to the fact that epilepsy disorder is often associated with the aforementioned disorders [64].

CBZ reduces total sperm count and fertility in rats [65] and inhibits testosterone formation in an in-vitro Leydig cell model at clinically appropriate doses [66]. As a result of this research, decreased fecundity and fertility in both *Drosophila para$^{bss1}$* mutant and Oregon-R strains after exposure to high dose (40 μg/ml) of CBZ may be due to its negative effect on male flies' reproductive system.

This experiment demonstrates that High dose (0.05%) of *W. somnifera's* pharmacological approach, as an AED, can successfully ameliorate the epileptic behavior, i.e., shortened the MRTs of male and female *para$^{bss1}$ Drosophila* mutants after exposure to mechanical-stressor (Fig. 4A and 4B).

In the absence of mechanical stimulation, *Drosophila para$^{bss1}$* mutants showed no significant behavioral impairments. Following a mechanical-stressor-induced epileptic behavior (vortex), *para$^{bss1}$* mutants lost coordination and went through a set of epileptic-like behaviors. Mainly, initial paralysis in homozygotes female and hemizygote male *para$^{bss1}$* is followed by an extended period of tonic-clonic activity. The fly is usually quiescent (tonic phase) at this phase, but multiple bouts of clonus-like activity break this up. Tonic-clonic activity in *para$^{bss1}$* prolongs the recovery time to 240 s [42,67].

Following mechanical stress, a high dose (0.05%) of *W. somnifera* administration reduced tonic–clonic-like activity in both homozygotes female and hemizygote male *para$^{bss1}$* mutants, resulting in lower MRTs. The traditional and clinical use of *W. somnifera* reveals that the effects of *W. somnifera* are associated to GABAergic activity [30,68]. GABA is the most prominent inhibitory neurotransmitter in the central nervous system, and it plays a vital role in epilepsy [69]. The potential effects of *W. somnifera* on GABA receptors suggest that some of its constituents may be useful to create new GABAergic tools and pharmacological treatments for neurological disorders involving GABAergic pathways such as epilepsy [70]. According to Bhattarai's study, GABA activity is directly affected by *W. somnifera* via GABAA receptors on mice's gonadotropin-releasing hormone (GnRH) neurons [71]. Based on our findings, *W. somnifera* improves the epileptic phenotype in *Drosophila para$^{bss1}$* mutants by reduction in MRT, which may be due to the GABAergic system or inhibition involved in the epileptic behavioral response.

The present study shows that CBZ was ineffective at ameliorating epileptic phenotypes in *para$^{bss1}$* flies (Fig. 4C and 4D), indicating that not all antiepileptic drugs used to treat human epilepsy disorders are effective against *Drosophila* seizures. Even though CBZ blocks sodium channels in an activity-dependent manner, it is thought to have a very low affinity for the sodium

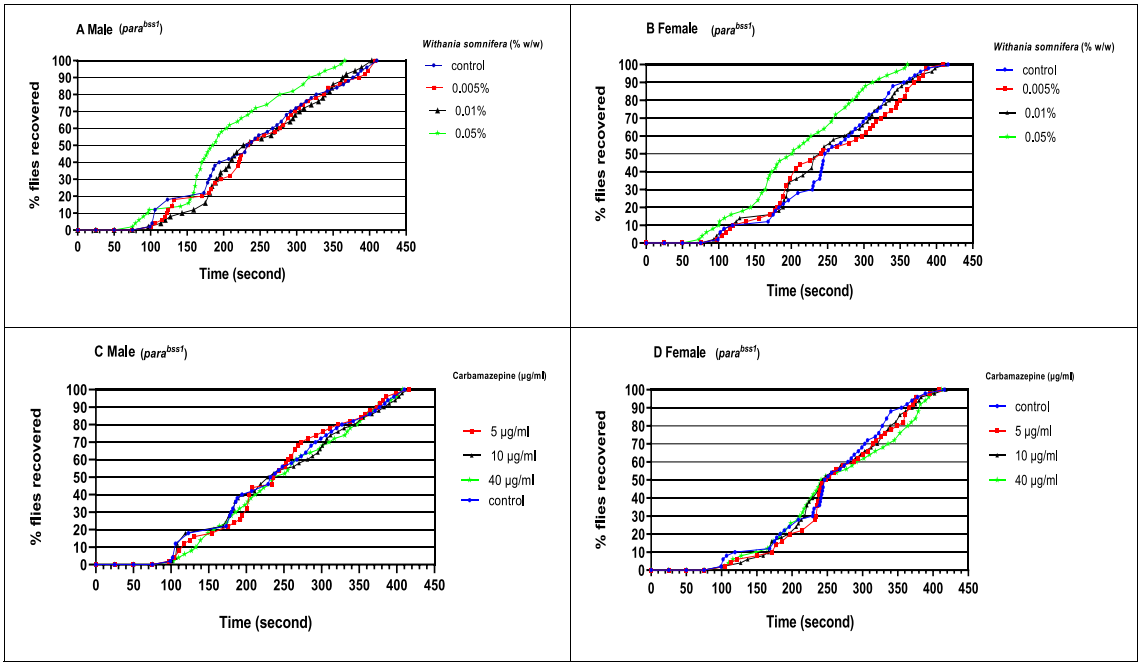


**Fig. 4.** The effect of *Withania somnifera* on Mean Recovery Time (second) from epileptic behavior after mechanical stressor in male (**A**) and female (**B**) *Drosophila para$^{bss1}$* mutant. At 0.005% and 0.01% in both sexes, *Withania somnifera* showed no statistically significant differences in MRT. W. somnifera at 0.05% reduced MRT in both sexes significantly (**$p$ = 0.0037 for male *para$^{bss1}$*, **$p$ = 0.0018 for female *para$^{bss1}$*). Values are MRT ± SEM. The effect of Carbamazepine on Mean Recovery Time (second) from epileptic behavior after mechanical stressor in male (**C**) and female (**D**) *Drosophila para$^{bss1}$* mutant. CBZ showed no statistically significant differences in MRT at any of the three doses. Values are MRT ± SEM. (n =50 flies per replicate, three such replication used for assay); Data was analyzed by One-way ANOVA followed by Tukey's test.

channel in the *Drosophila* system [72]. According to Reynolds's study, treatment with CBZ had no significant effect on the MRT in *Drosophila para*$^{bss1}$ [50]. As a result, the insignificant effect of CBZ on the epileptic phenotype in *Drosophila para*$^{bss1}$ mutants could be explained by its low affinity for the sodium channel in the *Drosophila* system.

## 5. Conclusion

The findings of animal toxicity studies indicate that *W. somnifera*, a medicinal herbal component, has the ability to extend the lifespan of *Drosophila para*$^{bss1}$ mutant strain when given in high dose, while still being safe for fecundity and fertility, and also ameliorates epileptic behavior in *Drosophila para*$^{bss1}$ mutant. Furthermore, the previous reports based on experimental data clearly outline *W. somnifera*'s efficacy as a therapeutic potent in neurodegenerative disorders such as epilepsy. The current findings indicate that high dose (40 μg/ml) of CBZ, a commonly used synthetic AED, significantly decreased the lifespan of *Drosophila para*$^{bss1}$ mutant strain, which may be due to its genotoxicity, and also has negative effects on flies' reproductive traits, fecundity and fertility, which may be due to its negative impact on the male flies' reproductive system. Aside from that, CBZ did not affect the epileptic behavior in *para*$^{bss1}$ flies after mechanical stress.

## Author agreement

All authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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## Conflict of Interest

None.

## Author contributions

**Sara Moghimi**: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition. **B. P. Harini**: Conceptualization, Supervision, Validation, Resources, Project administration.

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