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Attenuation of Seizures, Cognitive Deficits, and Brain Histopathology by Phytochemicals of *Imperata cylindrica (L.) P. Beauv* (Poaceae) in Acute and Chronic Mutant *Drosophila melanogaster* Epilepsy Models

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Abstract

Imperata cylindrica is a globally distributed plant known for its antiepileptic attributes, but there is a scarcity of robust evidence for its efficacy. The study investigated neuroprotective attributes of *Imperata cylindrica* root extract on neuropathological features of epilepsy in a *Drosophila melanogaster* mutant model of epilepsy. It was conducted on 10-day-old (at the initiation of study) male post-eclosion bang-senseless paralytic *Drosophila* ($para^{bss\,1}$) involved acute (1-3 h) and chronic (6-18 days) experiments; n = 50 flies per group (convulsions tests); n = 100 flies per group (learning/memory tests and histological examination). Administrations were done in 1 g standard fly food, per os. The mutant flies of study ($para^{bss\,1}$) showed marked age-dependent progressive brain neurodegeneration and axonal degeneration, significant ($P < 0.05$) bang sensitivity and convulsions, and cognitive deficits due to up-regulation of the *paralytic* gene in our mutants. The neuropathological findings were significantly ($P < 0.05$) alleviated in dose and duration-dependent fashions to near normal/normal after acute and chronic treatment with extract similar to sodium valproate. Therefore, *para* is expressed in neurons of brain tissues in our mutant flies to bring about epilepsy phenotypes and behaviors of the current juvenile and old-adult mutant *D. melanogaster* models of epilepsy. The herb exerts neuroprotection by anticonvulsant and antiepileptogenic mechanisms in mutant *D. melanogaster* due to plant flavonoids, polyphenols, and chromones (**1** and **2**) which exert antioxidative and receptor or voltage-gated sodium ion channels' inhibitory properties, and thus causing reduced inflammation and apoptosis, increased tissue repair, and improved cell biology in the brain of mutant flies. The methanol root extract provides anticonvulsant and antiepileptogenic medicinal values which protect epileptic *D. melanogaster*. Therefore, the herb should be advanced for more experimental and clinical studies to confirm its efficacy in treating epilepsy.

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Keywords
cognition disorder, drosophila melanogaster brain, epilepsy, imperata cylindrica, neuroprotection, seizure characteristics

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Introduction

Epilepsy is a chronic noncommunicable brain (neurological) disorder associated with excessive neuronal activity and unprovoked seizures referred to as 'epileptic seizures' and sensory-motor deficits that cause neurobiological, cognitive, psychological, and social consequences to the patient.[1-4] It is one of the most common neurological disorders worldwide,[1-5] is among the most common chronic brain diseases, and affects people of all ages. Around 50 million people or more worldwide have epilepsy, with almost 80% of them living in low- and middle-income countries.[2-4] The global prevalence of active epilepsy is 0.64%, with a lifetime prevalence of 0.76% and an incidence of 61.4 per 100,000 person-years. The prevalence of the disease increases with age and is higher in people who are socially deprived.[6] Africa is estimated to contribute about 20–23% of the global cases, and the prevalence of epilepsy in Uganda is estimated to be ranging between 0.22 and 13 per 1000 population, with an estimated incidence of 156 cases per 100,000 each year.[7,8]

Genetic epilepsy arises from mutations in one or multiple genes and can occur in non-ion and ion channel genes.[9] Of huge significance are the voltage-gated sodium ion channels (VGSCs) which are transporters of ions facilitating the depolarization of action potentials and transmission of signals in neurons. The most common types of epilepsy occurring in humans are the genetic forms due to genetic disorder (s) in the VGSCs of the brain (brain sodium channelopathies).[9-11] Studies involving animal models with genetic forms of epilepsy due to brain sodium channelopathies are vital for a better understanding of the pathophysiology of neuropathological features of epilepsy associated with these channelopathies. This knowledge can be utilized to develop newer effective therapies for the genetic epilepsies that arise due to channelopathies.[11]

Drosophila melanogaster is a good model for studying neurological disease pathways and for utilization during drug discovery for neurological diseases including epilepsy mainly because the flies and humans have similar basic biology, physiology, and neurological properties, with 75% of disease-causing human genes being conserved in the flies.[12] In *D. melanogaster* flies the VGSCs are mainly coded by a single gene called the *paralytic* (*para*) gene.[13,14] A mutation in the *para* gene (overexpression of *para*) in the neuronal brain tissues of bang-senseless (bss) mutant epileptic *Drosophila* models ($para^{bss}$) gives rise to epileptic neuropathological features in the flies that are similar to those in epileptic mammals. This mutation induces a reduction in seizure stimulation threshold in $para^{bss}$ which makes the flies more susceptible to getting seizures following electrical, mechanical, or physical stimulation of the animals.[15,16] In addition, gene mutations or alterations due to the *para* gene in *D. melanogaster* fly models are associated with defective metabolism and viability in neurons of these flies leading to defective neurophysiology and behavior.[17,18] Again, the gene defects are associated with extensive neurodegeneration in the brain of the flies[17] as well as an impairment of sensory-motor activities, and neurobiological, and cognitive deficits in the animals.[19,20] Because almost 67% of human disease genes are conserved in *Drosophila,*[21] the defects and deficits observed in the epileptic fly models (referred to as '*para* neuropathology') are related to the 'epileptic syndrome' in people with genetic epilepsy that is characterized by brain sclerosis, convulsions, cognitive deficits, motor deficits, etc.[21-25]

Some epileptic patients (almost a third of them) do not get the expected relief from seizures and other neuropathological features of epilepsy after administration with the current anti-epileptic treatments,[10,26] also, the anti-epileptic drugs are associated with numerous severe side-effects causing discomfort, drug non-compliance, and drug resistance in the patients.[16,27,28] Other challenges faced by epileptic patients include the inadequate number of neurologists and the lack of good healthcare facilities for epilepsy patients in developing countries.[29,30] Therefore, herbal extracts are an important source for the development of novel complementary and alternative antiepileptic medicines both in developing and developed countries.[30-33]

Imperata cylindrica (L.) P. Beauv (Poaceae) is native to tropical regions of Africa, tropical and subtropical zones, southwestern Asia, and some parts of the U.S.A,[34,35] is an important neuromedicinal plant in Africa, Asia, and elsewhere, and could be a good source for the development and discovery of novel complementary and alternative antiepileptic medicines globally due to its widespread utilization in treating epilepsy in people with epilepsy.[34-36] Phytochemicals from the herb such as the 2-(2-Phenylethyl) and 5-Hydroxy-2-(2-phenylethyl) chromones have been found to possess neuroprotective potential on glutamate-induced neurotoxicity in rat cerebral cortical cells,[37,38] additionally plant-based nonenzymatic antioxidants mainly phenols, flavonoids, chromones, and glycosides provide neuroprotective attributes against brain cell damage through their strong antioxidant potentials that improve brain cell biology,[37,39] provide anticonvulsant and antiepileptogenic attributes,[40] and alleviate cognitive deficits[41] of animals by the antioxidation mechanism which controls oxidative stress markers in the brain of animals.[39-41]

Although it is widely used, there is a lack of strong evidence for the efficacy and safety of *Imperata cylindrica* or *I. cylindrica* as an anticonvulsant in folk medicine in Africa, Asia etc,[35,36,42] therefore, information on evidence-based scrutiny of antiepileptic/anticonvulsant efficacy and safety of this

commonly used neuromedicinal (antiepileptic) herb is crucial for establishing targeted and effective community-based intervention policies and programs during the management of epilepsy.[32,43,44]

The study aimed to establish the neuroprotective effects of phytochemicals and changes in convulsion, cognitive, and histological parameters in brain tissues caused by *Imperata cylindrica* root extract on the neuropathological features of *Drosophila melanogaster* mutant models of epilepsy.

Materials and Methods

Plant Materials

Fresh plants of *Imperata cylindrica* were harvested in Ishaka-Bushenyi Municipality, Bushenyi District, western Uganda (GPS coordinates; Latitude: −0.53828 S 0° 32′17.79583″; Longitude: 30.14546 E 30°8′43.64405″), and identified and authenticated by a botanist at the Department of botany, Mbarara University of Science and Technology (voucher No. *P. Beauv* /11/2020). The plants were cleaned and washed with distilled water to remove all foreign matter and the roots were collected and made into pieces and dried under shade. The methanol root extract was prepared using standard methods.[45-47] Briefly, the dried roots were ground using a grinding machine into fine powders that were kept in air-tight glass containers. The methanolic extract of the plant was prepared by soaking 100 g of the powdered sample in 1000 mL of 70% methanol in an Erlenmeyer conical flask (Corning® CLS431145, Darmstadt, Germany). This was placed on an orbital shaker incubator (Amerex®, Gyromax 727, NY, USA) to allow complete mixing (24 h, 100 rpm, 30°C). The debris was removed by centrifugation (CYAN CL008N, USA; 4000 rpm, 15 min) to obtain a fine extract that was then filtered using Whatman number 1 filter papers (Whatman® qualitative filter paper, Grade 1, WHA1001325, Darmstadt, Germany). The extract was concentrated to dryness using a rotary evaporator (Büchi® Rotavapor® R-210, Büchi 23011V010, Darmstadt, Germany) at 53°C to evaporate off the methanol, followed by a concentration to dryness at 53° C using an electric oven (GallenKamp BS300, London, England). The remaining dry concentrated extract was weighed and stored in universal bottles put in a freezer at 4°C (Samsung RF22N9781SR, UPC: 887276259277, Brooklyn, NY, USA) till utilization.[45-47]

Animals

The study was conducted on the male adult or post-eclosion *D. melanogaster* flies at 10 days of age at the time of study initiation following previous studies on bang-senseless (bss) *D. melanogaster* models ($para^{bss}$) and novel anti-epileptic treatments.[15,16] The *Drosophila* models of the bang sensitive (BS) family used in the study were the bang senseless or bss1 mutants of the *paralytic* gene ($para^{bss1}$),[48,49] and the wild-type strains used as controls were *Oregon-R* or *OrR* and *Canton-Special* (*CS*).[49-51] Male flies were used as a previous laboratory study has shown differences between female and male bss *Drosophila* because the $para^{bss}$ is located on the X chromosome, and heterozygous $para^{bss/+}$ female *Drosophila* have significantly decreased recovery times.[52] Only animals that underwent seizures were included in the study during behavioral (convulsion and learning/memory tests) and histological assessments.[49]

The bang-sensitive mutant flies ($para^{bss1}$) obtained from Prof. Richard Baines laboratory (University of Manchester, UK)[48,49] were used, and the wild-type flies ie, *OrR* (BDSC stock #25211) and *CS* (BDSC stock #64349), were obtained from Bloomington Drosophila Stock Center, Indiana University, U.S.A, and all the stocks were delivered to the Institute of Biomedical Research for culturing into sufficient stock colonies. The bss paralytic (para) *D. melanogaster* mutant flies used in the study have a gain-of-function mutation in the *para* sodium channel gene located at the *para* locus in neurons of the mutant flies.[52-54]

The flies were cultured on a standard yeast/cornmeal/agar diet (distilled water, 6.65% cornmeal, 7.15% dextrose, 5% yeast, 0.66% industrial agar supplemented with 3.4 mL/L propionic acid, and 2.2% nipagin) for *Drosophila* flies following previous methods,[55,56] (Supplementary file 1). The flies were kept in incubators at 25°C, 65% humidity, and on a 12-h light/dark cycle (Fly incubator with programmable day/night cycle; Powers Scientific Inc., S33SD, USA). Flies were transferred to fresh plastic fly vials (Genesee Scientific, 32-116, USA), every 3 days, and fly density was kept to 10 flies per plastic fly vial (Genesee Scientific, 32-116, USA).

Experimental Design and Flies' Treatment

The study was conducted for 1, 2, 3 h, and 6, 12, and 18 days for acute and chronic experiments respectively based on previous studies.[52,57,58] The preliminary acute toxicity tests on *D. melanogaster* from our laboratory using a previous method,[59] found the lethal concentration 50 (LC_{50}) of *I. cylindrica P. Beauv* methanol root extract (acute oral exposure for 24 h in 20-day-old post-eclosion *Drosophila*) to be 3 g/mL of extract in the medium, and doses below 2 g/mL produced no lethality. Mutant animals treated with the extract solution appeared healthy and showed reduced seizure-like behavior compared to the untreated mutant controls. Therefore, we decided to expand the assessments to lower extract concentrations (0.6, 0.8, and 1 g/mL) and (0.0125, 0.025, and 0.05 g/mL) in our acute and chronic experiments respectively. The doses of sodium valproate or SV (0.3 and 0.15 mg/mL, p.o) that were used in standard control groups of the study were based on previous studies that have shown them to provide significant anticonvulsant, anti-epileptic activities, and alleviative control of bss behaviour in epileptic *D. melanogaster* models eg $para^{bss}$.[15,60,61]

The initial experiments assayed the bang-sensitivity and convulsion characteristics of our mutant flies, and also evaluated the extract doses that provide anticonvulsant potential on

the mutant flies of the study following short-term and prolonged treatments. To do this, juvenile adult flies (10 days) were anesthetized using CO_2 anesthesia (Fly CO_2 anesthesia setup; Genesee Scientific, 59-114/54-104M, USA), sexing was performed on a CO_2 perfused pad to separate flies into females and males (dissecting stereo microscope; Leica Microsystems, Leica M60 CMO), and the males were randomly assigned to plastic fly vials (Genesee Scientific, 32-116, USA), with 10 flies each. Male Animals were subjected to a vortex stimulus to induce seizures,[62,63] seizing males were separated from non-seizing males and the former were then randomly assigned to the different groups (negative/mutant control, standard control, and low and high-dose extract tests groups), and the wild-type flies were used in the normal control groups, Table 1.

Then, during the subsequent learning, memory, and histological tests, juvenile adult flies (10 days) were also anesthetized using CO_2 anesthesia with Fly CO_2 anesthesia setup (Genesee Scientific, 59-114/54-104M, USA), separated on a CO_2 perfused pad into females and males (dissecting stereo microscope; Leica Microsystems, Leica M60 CMO), and the males were randomly assigned to plastic fly vials (Genesee Scientific, 32-116, USA), with 10 flies each. The flies were then tested for bang sensitivity (seizures) and the animals that underwent seizures were then randomly assigned to the different groups (negative/mutant control, standard control, and extract test groups), while wild-type flies were used in the normal control group, Table 2.

Drug Preparation and Administration

I. cylindrica root extract and sodium valproate were administered by mixing them with standard cornmeal fly food (Supplementary file 1), and extract and drug solutions were prepared fresh prior to each experiment. The experiment does are expressed in g of extract per mL of fly food and mg of sodium valproate per mL of fly food. For extract doses (0.6, 0.8, 1, 0.0125, 0.025, and 0.05 g/mL) the dry concentrated root extract was dissolved in distilled water in a beaker at room temperature and warm food (50°C) was added and mixed before aliquoting approximately 1.5 mL per testing vial. For sodium valproate, one uncoated 100 mg tablet (Agog Pharma Ltd, India) was crushed, 0.3 mg or 0.15 mg were suspended in 1 mL distilled water each, and the drug suspension was then mixed with 5 mL of warm food to produce low and high sodium valproate concentrations respectively; SV 0.3 mg/mL and 0.15 mg.

Administration of the extract and standard control drug were done following previous methods.[46,49,64,65] For acute study models, 10-day-old flies in replicates of 10 flies per plastic fly vial (Genesee Scientific, 32-116, USA), were starved for 15 h to induce appetite, after which they were fed 1 g of standard yeast/cornmeal/agar media without extract (negative or mutant controls and wild-type controls for acute study), or 1 g of standard media mixed directly with 0.6 g/mL, 0.8 g/mL, or 1 g/mL extract (extract tests for acute study), or 1 g of standard media mixed with 0.3 mg/mL SV (standard controls for acute study), and for the acute tests, the flies were allowed to feed for 1, 2, or 3 h (each treatment was tested for 1-3 h) before being transferred into individual empty plastic fly vials (Genesee Scientific, 32-116, USA) that were capped with a cotton plug. The flies were allowed to sit undisturbed for 20 min before tests were conducted.[46,49,64,65] For chronic experiments, 10-day-old flies at the start of experiments in replicates of 10 flies per plastic fly vial (Genesee Scientific, 32-116, USA), were fed 1 g of standard yeast/cornmeal/agar media without extract (mutant/negative controls and wild-type controls for chronic study) or 1 g of standard media mixed directly with 0.0125 g/mL, 0.025 g/mL or 0.05 g/mL extract (extract

Table 1. Study Design on Treatment Groups Of Bang-Sensitivity and Convulsion Tests.

Group	Study Group	Treatment	Number of Flies
Acute experiments			
I	Mutant and normal controls	1 g of standard fly food without extract, per os (p.o) for 2 h (hrs.)	50 flies per fly genotype, total = 150 flies per group
II	Low-dose extract test	0.6 g/mL extract mixed directly in 1g of standard fly food p.o for 2 h.	50 per genotype, total = 150
III	Moderate-dose extract test	0.8 g/mL extract mixed directly in 1g of standard fly food p.o for 2 h.	50 per genotype, total = 150
IV	High-dose extract test	1 g/ml extract mixed directly in 1g of standard fly food p.o for 2 h.	50 per fly genotype, total = 150
V	Standard control	0.3 mg/mL sodium valproate (SV) mixed directly in 1g standard fly food p.o for 2 h.	50 per fly genotype, total = 150
Chronic experiments			
VI	Mutant and normal controls	1 g of standard fly food without extract, p.o for 12 days	50 per fly genotype, total = 150
VII	Low-dose extract test	0.0125 g/mL extract mixed directly in 1g of standard fly food p.o for 12 days	50 per fly genotype, total = 150
VIII	Moderate-dose extract test	0.025 g/mL extract mixed directly in 1g of standard fly food p.o for 12 days	50 per fly genotype, total = 150
IX	High-dose extract test	0.05 g/mL extract mixed directly in 1g of standard fly food p.o for 12 days	50 per fly genotype, total = 150
X	Standard control	0.15 mg/mL SV mixed directly in 1g standard fly food p.o for 12 days	50 per fly genotype, total = 150

Table 2. Study Design on Treatment Groups of Learning, Memory, and Histological Tests.

Group	Study Group	Treatment	Number of Flies
Acute experiments			
XI	Mutant and normal controls	1 g of standard fly food without extract, p.o for 1, 2, or 3 h.	100 flies per genotype, total = 100 × 3 = 300 flies per sub-group in this group
XII	Extract test	0.8 g/mL of extract mixed directly in 1g of standard fly food p.o for 1, 2, 3 h.	100 per genotype, total = 300 per sub-group
XIII	Standard control	0.3 mg/mL SV mixed directly in 1g standard fly food p.o for 1, 2, 3 h.	100 per genotype, total = 300 per sub-group
Chronic experiments			
XIV	Mutant and normal controls	1 g of standard fly food without extract, p.o for 6, 12, or 12 days	100 per genotype, total = 300 per sub-group
XV	Extract test	0.025 g/mL extract mixed directly in 1g of standard fly food p.o for 6, 12, and 18 days	100 per genotype, total = 300 per sub-group
XVI	Standard control	0.15 mg/mL SV mixed directly in 1g standard fly food p.o for 6, 12, and 18 days	100 per genotype, total = 300 per sub-group

tests for chronic study), or 1 g of standard media mixed with 0.15 mg/mL SV (standard controls for chronic study), and the flies were transferred to fresh plastic fly vials (Genesee Scientific, 32-116, USA) every 3 days. The flies for chronic studies were allowed to feed for 6, 12, or 18 days (each treatment was tested for 6-18 days) before being transferred into individual empty plastic fly vials (Genesee Scientific, 32-116, USA) which were capped with cotton plug, and the flies were left to sit undisturbed for 20 min after which the tests were conducted.[46,49,64,65]

Analytical Procedures

Phytochemical Analysis of I. cylindrica Root Extract. Phytochemical analysis of the methanol root extract of the plant was performed in triplicates in the Ethnobotany Laboratory, Department of Biological Sciences, Makerere University in Uganda. Qualitative analysis of the main bioactive compounds of the plant (alkaloids, saponins, tannins, cardiac glycosides, flavonoids, phenols, terpenoids, etc)[34,35,66] was carried out using standard qualitative analytical methods described previously.[67,68] Quantitative analyses of the isolated compounds of the crude extract of the herb were carried out following standard spectrophotometric analytical methods (PerkinElmer's Lambda 35 UV/Vis, USA) described previously.[69-75]

Saponin Analysis. The levels of saponins were measured by the vanillin–sulfuric acid method described by Shiau et al,[74] at absorbance (O.D) 535 nm, expressed as milligrams of saponin per gram of dry extract.

Cardiac Glycoside Analysis. Amounts of glycosides in the powdered sample were determined using the Baljet's test described previously,[75] and the O.D of the colored complex was determined spectrophotometrically at 495 nm, expressed as milligrams of glycoside per gram of dry extract.

Flavonoid Analysis. The flavonoid levels were analyzed using the aluminum chloride test using a method described previously,[69,72] at O.D = 420 nm expressed as milligrams of flavonoid per gram of dry extract.

Polyphenol Analysis. The polyphenol contents were assessed using the Folin-Ciocalteu method described previously,[69,71] at a spectrophotometer O.D of 730 nm, and the result was expressed as milligrams of phenol per gram of dry extract.

Tannin analysis. The tannin levels were determined using the vanillin-hydrochloric acid test by employing a method by Morrison et al[73] as suggested by Piana,[69] at O.D = 500 nm. The data were expressed as milligrams of tannin per gram of dry extract.

Alkaloid Analysis. The contents of alkaloids were analyzed using Dragendorff's test described previously,[69,76] at O.D = 435 nm. The data were expressed as milligrams of alkaloids per gram of each fraction.

Chromone Analysis. The amounts of two chromone compounds ie, 5-hydroxy-2-(2-phenylethyl) chromone **(1)** and 5-hydroxy-2-[2-(2hydroxyphenyl)ethyl]chromone **(2)** were evaluated using high-performance liquid chromatography (HPLC) technique following the reflux extraction method described previously.[77] Briefly, standard solutions (1 mg/mL of standards) were prepared by diluting stock solutions with acetonitrile (ACN) to make 2, 4, 6, 8, and 10 µg/mL of **1** and 2.5, 5, 10, 15 µg/mL of **2**, and quantification of the chromones in the herb was performed using high-performance liquid chromatography (HPLC) following the reflux extraction method as follows: 10 g of plant powder was weighed and refluxed with 70% methanol for 3 h at 45°C, the extract obtained was filtered through Whatman number 1 filter papers (Whatman® qualitative filter paper, Grade 1, WHA1001325, Darmstadt, Germany) and the solvent removed *in vacuo*. After preparing the methanol extract 2.5 mg were dissolved in 1 mL of ACN for HPLC analysis (injection volume, 20 µL, flow rate, 1 mL/min, temperature, 22- 30°C, detection at 330 nm, and acetic acid was used to reduce the tailing). Separation was done on a C_{18} column with a mobile phase of 0.5% (v/v) AR grade acetic acid (CDH, India) in distilled water (A), and ACN (B) under the best gradient elution determined to give a stable baseline peak and resolution of **1** and **2**. **1** and **2** were identified by analysing peaks at corresponding retention times by spiking

with standards of the same corresponding compounds isolated and characterized earlier from *I. cylindrica* into methanol extract.[77] The amounts of **1** and **2** in the sample were quantified using standard spectrophotometric analytical methods (PerkinElmer's Lambda 35 UV/Vis, USA). The data were expressed as milligrams of chromones per gram of each fraction.

In Vivo Anticonvulsant and Antiepileptic Effects of I. cylindrica Root Extract on Mutant Flies

Determination of Bang-sensitivity Behavior and Seizure Parameters of Flies. To determine the bang sensitivity and seizure characteristics of the flies, we subjected the mutant flies ($para^{bss1}$) and wild-type controls (*OrR* and *CS*) in different groups to mechanical stress, then observed for the occurrence of bang sensitivity and measured the time taken during convulsions, paralysis, to recovery from the paralysis.[62,63] Bang sensitivity and convulsion parameters (mean convulsion time, CT; mean paralysis time, PT; and mean recovery time, RT, were assessed following previous methods.[49,62,63,78] Briefly, on the day of the behavioral assessment, each fly was transferred into an empty plastic cylindrical 10 mL vial capped with a cotton plug, the fly was mechanically stimulated by placing an inverted vial on a benchtop vortex, then vortexed at high speed (maximum speed of 3200 rpm for 10 s, at room temperature of 22- 30°C) with a vortex mixer (Benchmark Scientific, BMK-BV1000, USA). The durations spent during convulsions, paralysis, and recovery for each animal to right itself (recover) were recorded as CT, PT, and RT respectively (QIANZICAI Stopwatch 382 ®). CT is the time taken while the fly is experiencing a convulsion (seizures, shaking, and lying on its back); paralysis is the second phase after a convulsion; PT is the time between the end of a convulsion and recovery; recovery is when the fly is standing and exhibiting normal mobility or flight; RT is the time taken by a fly that exhibits bang-sensitive behavior to recover completely from the convulsion.[49,62,63,78] The mean of the convulsion, paralysis, and recovery times (CT, PT, and RT) of the 50 flies in each group were calculated as follows:

$$\text{Mean CT} = \frac{\Sigma\text{CT for the 50 flies}}{50}$$

$$\text{Mean PT} = \frac{\Sigma\text{PT for the 50 flies}}{50}$$

$$\text{Mean RT} = \frac{\Sigma\text{RT for the 50 flies}}{50}$$

Determination of learning and Memory Pass Rates of Flies. The learning and memory assays were performed at 22–30°C using the Aversive Phototaxic Suppression (APS) assay previously described[57,79] with a modified T-maze set-up.

Learning Assay. On the day of the behavioral assessments (learning and memory tests), flies were anaesthetized using CO_2 anesthesia with Fly CO_2 anesthesia setup (Genesee Scientific, 59-114/54-104M, USA), secluded from various treatment groups in groups of 10 (ten) flies per plastic fly vial (Genesee Scientific, 32-116, USA), and in 10 replicates (n = 100 flies in each experimental group). The secluded flies were positioned in an empty polystyrene vial with a water-dampened filter paper for six hours to induce starvation for ensuring a good perception of the flies to the aversive taste and to allow complete recovery before the conduction of the assay. Our modified T-maze was designed by attaching a lighted chamber (15mL plastic centrifuge tube connected to a light source) and a dark chamber (15mL plastic centrifuge tube wrapped in aluminum foil) on each side of the center column using adapter connectors. Quinine solution (1 μM, 180 μL) was added to filter paper using a 200 μL pipetter and placed in the light chamber. Then each fly was trained 10 times to the APS assay to enable learning of the aversive quinine taste stimulus as described previously.[57,79] Immediately after the training of the fly for APS, ten test trials were conducted for each fly, and in each test trial, the light was switched on, the fly was pushed into the dark chamber using a small paint brush, then 10 s allowed to pass before the trained fly was left to move to the lighted chamber containing the aversive (quinine) stimulus. Failure of the fly to move to the lighted vial or chamber within 10 s was recorded as "pass" (equivalent to "task learned through reinforcement"). For each fly, the average 'pass' over the ten consecutive test trials was recorded as PC0, this was the learning indicator (learning pass) value of that fly.[57,79] The percent learning pass rate of the 100 flies in each group was calculated as follows:

$$\%\text{Learning pass rate} = \frac{\Sigma\text{PC0 of the 100 flies} \times 100\%}{100}$$

Memory Pass rate. After the initial learning tests and PC0 recordings, the flies from each experimental group were put back into their original food vials (plastic fly vials; Genesee Scientific, 32-116, USA) and kept for six hours. The memory-retaining capability of each fly was assessed on the same flies that were used in the learning tests. Memory-retaining capacity was recorded 6 h after the initial training session of the flies in the learning experiments. To do this, each fly was again subjected to ten (10) test trials as described in the learning assay.[57,79] The number of times out of the10 test trials that each fly evaded the lighted chamber within 10 s indicated the memory 'pass' for that fly and was recorded as PC6 (6 h after the initial training), and this was the indicator (memory pass) value of short-term memory of the fly.[57,79] The percent memory pass rate of the 100 flies in each group was calculated as follows:

$$\%\text{Memory pass rate} = \frac{\Sigma\text{PC6 for the 100 flies} \times 100\%}{100}$$

Histopathological Examination of Brain Tissues of Flies. Histopathological tests were performed using standard histological methods as previously described.[78,80-82] Briefly, on the day of histological assessments, *Drosophila melanogaster* flies were anesthetized with CO_2 (Fly CO_2 anesthesia setup; Genesee Scientific, 59-114/54-104M, USA), and placed on a CO_2 perfused pad for

collecting. The flies were decapitated under a dissecting stereo microscope (Leica Microsystems, Leica M60 CMO), and the heads were fixed in 10% neutral buffered formalin (NBF) fixative at room temperature (22- 30°C) for 24 h, after which tissues were processed using routine histology techniques by dehydrating with graded alcohols, clearing with xylene, and embedding into paraffin wax using an automated tissue processor (Histokinette-SLEE MAINZ, MTP type). Every tissue was randomly sectioned into six, 6 μm-thick transverse histological sections using a rotary microtome (SLEE MAINZ, CUT4062), and the sections were then placed on slides and stained with hematoxylin and eosin (H &E) and combined Luxol fast blue (LFB) and Nissl (Klüver's) staining techniques following standard protocols.[78,80-82] The stained sections were mounted in mounting media and qualitative histological examination of the sections was done and photographed with a light microscope (Nikon Eclipse Ci-L Upright Microscope**,** New York, USA), at a magnification of 200x or 400x, digital camera (Nikon DS-Fi1c Digital Camera**,** New York, USA), and imaging software (Nikon NIS- NIS-Elements F Ver4.60.00 Imaging software), for image analysis and documentation.

A qualitative examination of the tissues was done using previously described methods[64,83-85] where brain neurodegeneration was categorized as normal, moderate, or severe based on the size and frequency of brain vacuolations in H and E stained-sections following previous methods.[84,85] The non-myelinating Schwann cells in mammals are comparable to the 'ensheathing' glial cells within the CNS of *Drosophila* which encase axons and neuropil of the flies,[86,87] therefore, LFB in Klüver LFB stain was used to demonstrate axonal degeneration of neurons rather than axonal demyelination.[88] The nature of the Nissl substance and nerve tracts (axons) were demonstrated using the Klüver LFB-stained tissues following previous methods,[64,83] where the nerve tracts were shown by blue color and the Nissl substance was shown by magenta (violet) colour. A weak LFB stain (light blue patchy areas) indicated axonal degeneration of nerve tracts, while a weak cresyl echt violet stain (light violet) indicated abnormalities in Nissl substance,[64,83] supplementary file 2**.**

Statistical Analysis

The data was recorded and organized into an excel spreadsheet (version 219), then imported into Graph Pad Prism Version 6 software for statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) and two-way ANOVA for convulsion and learning/memory parameters respectively and Tukey's multiple comparison tests were used to determine sources of variation, and significant differences ($P < 0.05$) at a 95% confidence interval were indicated with different superscript letters (a, b, c, d, e, f, and g). The information was expressed as mean ± SD and presented in graphs and tables. For easy graphical visualization and representation of brain histopathological findings of the flies, histological data were also graphically represented using the GraphPad Prism software, and the grouped interleaved bars were used to represent the general brain morphology, and grouped stacked bar graphs were employed to represent the brain axonal morphology of flies.

Results

Phytochemical Composition of I. cylindrica Root Extract

Phytochemical analysis of the methanol *I. cylindrica* root extract showed the availability of saponins, flavonoids, tannins, cardiac glycosides, polyphenols, alkaloids, and chromone derivatives (**1** and **2)**. The standard spectrophotometric analytical methods showed that the levels of phytochemicals in the methanol extract were in the order flavonoids > polyphenols > chromones > saponins > tannins > cardiac glycosides > alkaloids. Therefore, flavonoids, polyphenols, and chromones were in the highest concentration in the methanol extract of our herb, (**Table 3**).

Juvenile-Adult and Old-Adult bss Paralytic Mutant Flies Portray Acute and Chronic Bang-Sensitivity and Seizure Phenotypes

Bang-sensitive assays revealed that the wild-type control flies (*OrR* and *CS*) were unaffected by mechanical stress (did not experience bang-sensitivity), while the mutant control flies of acute and chronic studies in the same groups (I and VI) displayed bang-sensitivity. This indicates that juvenile adults (10-day-old post-eclosion) and old-adults (16-28-day-old post-eclosion) mutant flies show acute and chronic bang-sensitivity, unlike the wild-type controls which show normal phenotypes (Figure 1A).

The bss paralytic mutant *Drosophila* ($para^{bss1}$) were used in our subsequent convulsion experiments to determine their convulsion characteristics (convulsion, paralysis, and recovery times), and the wild-type controls that showed neither bang-sensitivity nor convulsion phenotypes were only used for purposes of controlling the experiments. Bang-sensitivity tests revealed that the mean CT, PT, and RT of juvenile-adult mutant controls in group I of the acute experiments were 20.46 s, 42.26 s, and 63.6 s respectively, and the average CT, PT, and RT of the old-adult mutant controls in group VI of the chronic tests were 20.74 s, 45.24 s, and 58.98 s respectively, with no significant ($P > 0.05$) difference between the acute and chronic CT and PT, but with significant ($P < 0.05$) difference between the acute and chronic RT **(Figure 1A** and Supplementary file 3: Table 1**).**

Table 3. Total Phytochemical Content in the Methanol Root Extract of *I. cylindrica*.

Phytochemical compound	Content (mg/g Extract)
Tannins	1.5 ± 0.06
Polyphenols	20.17 ± 1.00
Flavonoids	80.3 ± 0.10
Cardiac glycosides	1.06 ± 0.03
Saponins	8.17 ± 0.02
Alkaloids	0.9 ± 0.03
Chromone **(1)**	5.8 ± 0.01
Chromone **(2)**	3.8 ± 0.12

Values represent means ± standard deviations (SD) for triplicate experiments.

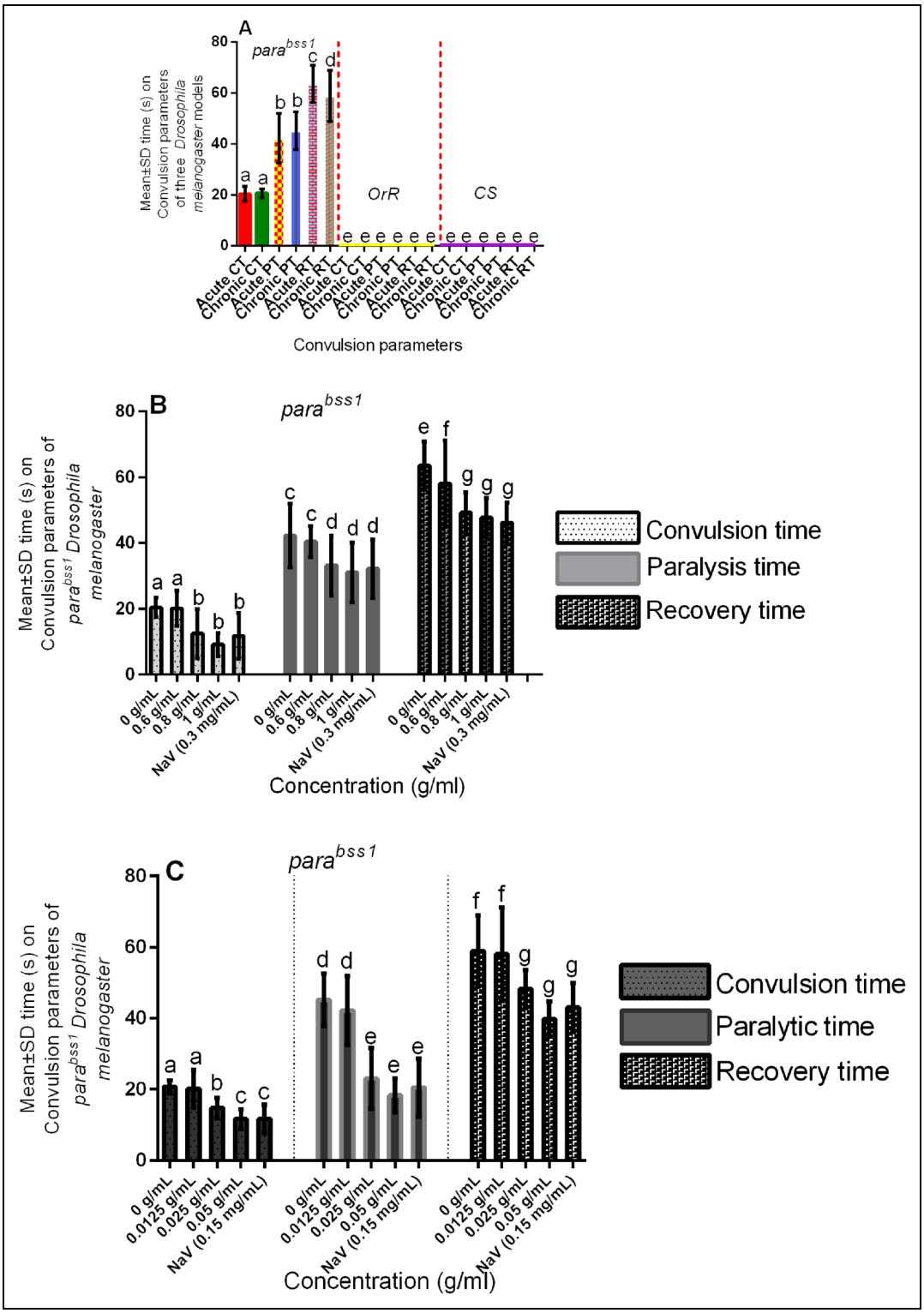


Figure 1. Acute and chronic effects of methanol *I. cylindrica* root extract on bang-sensitivity and seizure phenotypes of male juvenile-adults (10-day-old) and old-adults (22-day-old) bang-sensitive mutant *Drosophila* ($para^{bss1}$). A. Acute and chronic seizure parameters (CT, PT, and RT) of juvenile-adults and old-adults; mean-time (y-axis) plotted for the three parameters. B. Acute (2-h) extract treatment alleviates acute seizures of juvenile adults. C. Chronic (12-day) extract treatment alleviates chronic seizures of old adults; mean time (y-axis) plotted against different treatment groups. *OrR* and *CS* = wild-type controls; $para^{bss1}$ = mutant (test) model. Each value is expressed as mean ± SD; n = 50. Different letters (a, b, c, d, e, f, and g) meant significant differences among groups, ($p < 0.05$). Abbreviations: BSS = bang-senseless, CS = Canton-Special, CT = mean convulsion time, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic, PT = mean paralysis time, RT = mean recovery time. Juvenile-adult and old-adult *bss* mutant flies showed bang-sensitivity and seizure phenotypes unlike the wild-type controls (A). The acute bang-sensitivity and seizure phenotypes of mutant flies were alleviated by acute (2 h) extract feeding in a dose-dependent pattern similar to SV (B). The chronic bang-sensitivity and seizures of mutant flies were alleviated by chronic (12 days) extract feeding similar to SV (D).

I. cylindrica Root Extract Controlled Acute and Chronic Bang-Sensitivity and Convulsion Phenotypes of bss Paralytic Mutant Flies Following Short-Term and Prolonged Treatments

The mean CT, PT, and RT of the untreated mutant control or negative control group (group I) were significantly ($P < 0.05$) elevated compared to the moderate and high-dose extract test groups (groups III and IV) in acute experiments, with no significant ($P > 0.05$) difference between SV group (group V) compared to the moderate and high-dose extract test groups, but with significant ($P < 0.05$) difference between the low-dose group (II) compared to SV, moderate and high-dose extract test groups (III, IV, and V), (Figure 1B, Table 4, and Supplementary file 3: Table 2). The results indicate that short-term ingestion of the extract similar to SV protects against acute seizures of the mutants in a dose-dependent fashion, with 0.8 mg/ml and 1 g/mL extract, and 0.3 mg/mL SV being effective seizure-controlling doses compared to 0.6 g/mL extract.

The mean CT, PT, and RT of the untreated mutant controls (group VI) were significantly ($P < 0.05$) elevated compared to the moderate and high-dose extract test groups (groups VIII and IX) in chronic experiments, with no significant ($P > 0.05$) difference between SV group (X) compared to the moderate and high-dose extract test groups (VIII and IX), but with significant ($P < 0.05$) difference between the low-dose group (VII) compared to SV group, moderate and high-dose extract test groups (VIII, IX, and X), (Figure 1C, Table 4, and Supplementary file 3: Table 2). The results indicate that prolonged ingestion of the extract similar to SV protects against chronic seizures of the mutants in a dose-dependent fashion, with 0.025 mg/mL and 0.050 g/mL extract, and 0.15 mg/mL SV being effective seizure-controlling doses compared to 0.0125 g/mL extract.

Juvenile-Adult and Old-Adult bss Paralytic Mutant Flies have Acute and Chronic Learning and Memory Impairments

APS assay revealed the average learning and memory pass rates of wild-type controls as being78% and 71% respectively and were significantly ($P < 0.05$) higher than the learning and memory pass rates of the mutant controls (40% and 35% respectively) in group XI of the acute tests, with no significant ($P > 0.05$) difference in the average learning and memory pass rates of the mutant flies at 1, 2, or 3 h within the mutant controls of group XI (Figure 2A, Table 5 and Supplementary file 3: Table 3). This indicates that juvenile-adult (10-day-old) mutant flies show acute non-progressive deficits in learning and memory performance compared to the wild-type control group in the acute phases of the study.

Also, the assay revealed that the average learning and memory pass rates of wild-type controls (78% and 71% respectively) were significantly ($P < 0.05$) higher than that of the mutant controls (35% and 33% respectively) in the group

Table 4. Multiple Comparisons of Acute and Chronic Effects of Methanol *I. cylindrica* Root Extract on Acute and Chronic Seizure Parameters of Male Juvenile-Adult (10-day-old) and old-Adult (22-day-old) Bang-Sensitive Mutant *Drosophila* ($para^{bss1}$), n = 50.

Tukey's Multiple Comparisons Tests	Convulsion Time	Paralysis Time Adjusted P-Value	Recovery Time
Acute (2 h)			
0 g/ml versus 0.6 g/ml	0.9036	0.8300	0.0035
0 g/ml versus 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
0 g/ml versus 1 g/ml	< 0.0001	< 0.0001	< 0.0001
0 g/ml versus NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
0.6 g/ml versus 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
0.6 g/ml versus 1 g/ml	< 0.0001	< 0.0001	< 0.0001
0.6 g/ml versus NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
0.8 g/ml versus 1 g/ml	0.2786	0.7451	0.8718
0.8 g/ml versus NaV (0.3 mg/ml)	0.9036	0.9036	0.3478
1 g/ml versus NaV (0.3 mg/ml)	0.5746	0.9036	0.8718
Chronic (day 12)			
0 g/ml versus 0.0125 g/ml	> 0.9999	0.7297	> 0.9999
0 g/ml versus 0.025 g/ml	0.0026	< 0.0001	< 0.0001
0 g/ml versus 0.05 g/ml	< 0.0001	< 0.0001	< 0.0001
0 g/ml versus NaV (0.15 mg/ml)	< 0.0001	< 0.0001	< 0.0001
0.0125 g/ml versus 0.025 g/ml	0.0128	< 0.0001	< 0.0001
0.0125 g/ml versus 0.05 g/ml	< 0.0001	< 0.0001	< 0.0001
0.0125 g/ml versus NaV (0.15 mg/ml)	< 0.0001	< 0.0001	< 0.0001
0.025 g/ml versus 0.05 g/ml	0.6704	0.0491	< 0.0001
0.025 g/ml versus NaV (0.15 mg/ml)	0.6704	0.8816	0.0192
0.05 g/ml versus NaV (0.15 mg/ml)	> 0.9999	0.9646	0.5974

$p < 0.05$ meant significant differences among the groups. Abbreviations: BSS = bang-senseless, CT = mean convulsion time, NaV = sodium valproate, Para = Paralytic, PT = mean paralysis time, RT = mean recovery time.

XIV of the chronic tests, with no significant ($P > 0.05$) difference in the mean learning and memory pass rates of the mutant flies at 6, 12, or 18 days within the mutant controls of group XIV (Figure 2C, Table 5 and Supplementary file 3: Table 4). This indicates that old-adult (16-28-day old) mutant flies show chronic non-progressive deficits in learning and memory performance compared to the wild-type control group in the chronic phases of the study.

I. cylindrica Root Extract Controlled Acute and Chronic Learning and Memory Impairments of bss Paralytic Mutant Flies Following Short-Term and Prolonged Treatments

The average learning and memory pass rates of untreated mutant controls or negative controls (group XI) were significantly ($P < 0.05$) decreased compared to the extract test group

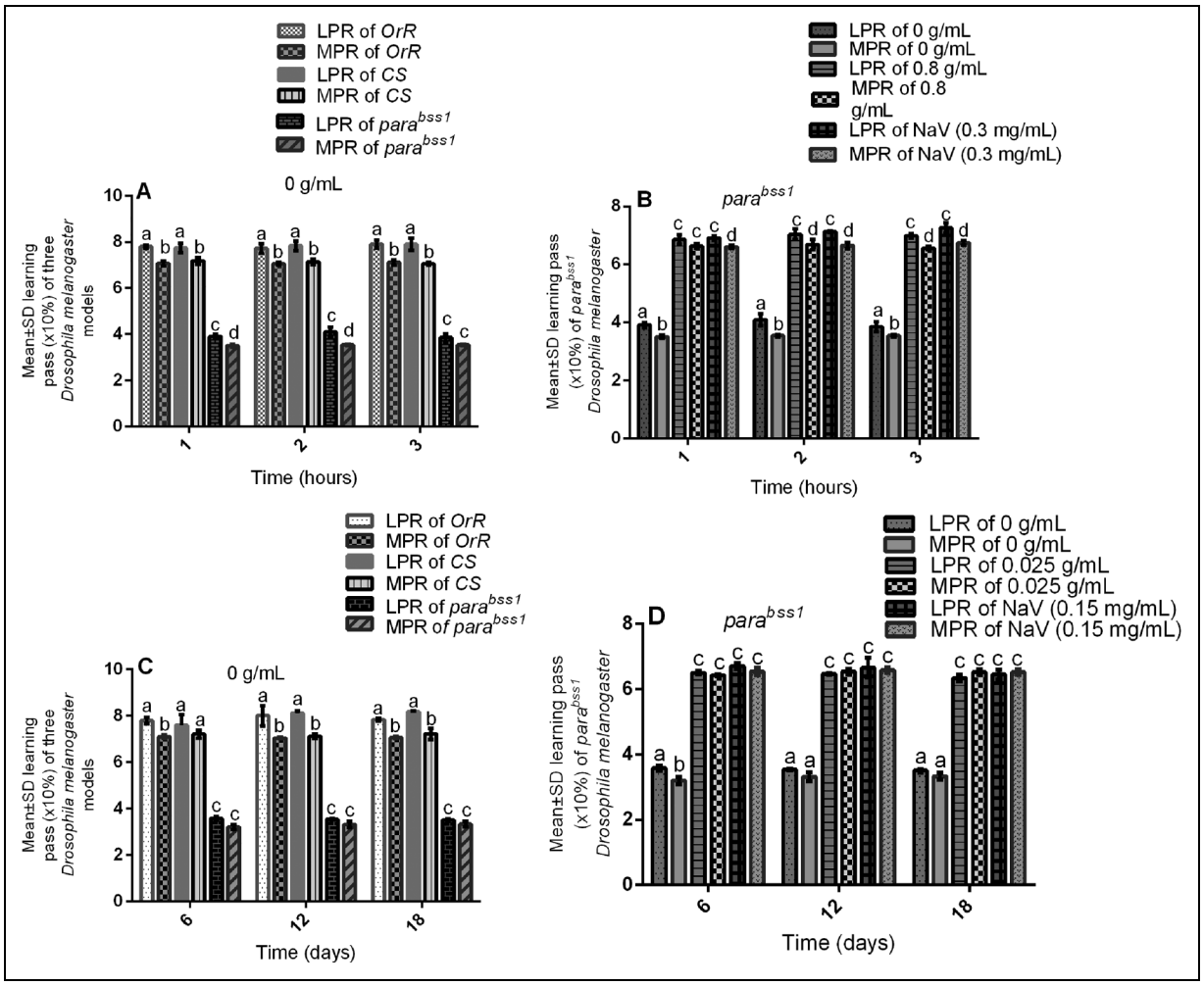


Figure 2. Acute and chronic effects of methanol *I. cylindrica* root extract on acute and chronic learning and memory deficits of male juvenile-adults (10-day-old) and old-adults (16–28-day-old) bang-sensitive mutant *Drosophila* (*para*^{bss1}). A. Acute learning and memory deficits of juvenile-adult bang-sensitive mutant *Drosophila*. B. Acute (1–3-h) extract treatment alleviates acute learning and memory deficits of juvenile-adult mutant flies; mean learning and memory pass rate for mutant flies (y-axis) plotted against time (hours) with respect to the concentration of extract. C. Chronic learning and memory deficits in old-adult bang-sensitive mutant *Drosophila*; mean learning and memory pass rate (y-axis) plotted against time (days). D. Chronic (6–18-day) extract treatment alleviates chronic learning and memory deficits in old-adult mutant flies; mean learning and memory pass rate for mutant flies (y-axis) plotted against time (days) with respect to the concentration of extract; mean learning and memory pass rates (y-axis) plotted against time with respect to different treatment groups. *OrR* and *CS* = wild-type controls; *para*^{bss1} = mutant (test) model. Each value is expressed as mean ± SD; n = 100. Different letters (a, b, c, and d) meant significant differences among groups, ($p < 0.05$). Abbreviations: BSS = bang-senseless, CS = Canton-Special, LPR = learning pass rate, MPR = memory pass rate, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic. Juvenile-adult and old-adult *bss* mutant flies showed impaired learning and memory function compared to the wild-type controls with normal learning and memory function (A & C). The acute learning and memory functions of the mutant flies were rescued by acute (1–3 h) extract feeding in a duration-independent pattern similar to SV (B). The chronic learning and memory functions of the mutant flies were rescued by chronic (6–18 days) extract feeding in a duration-independent pattern similar to SV (D).

(XII) of acute experiments, with no significant ($P > 0.05$) difference between the SV group (XIII) and extract test group (XII), and with no significant ($P > 0.05$) difference of learning and memory pass rates after 1, 2, or 3 h of treatment in the extract test group (XV), (Figure 2B, Table 6 and Supplementary file 3: Table 3). The results indicate that short-term ingestion of 0.8 g/mL extract (similar to 0.3 mg/mL SV) alleviates the acute learning and memory deficits of the mutant flies in a time-independent fashion during the acute phase of the study.

The mean learning and memory pass rates of untreated mutant controls (group XIV) were significantly ($P < 0.05$) decreased compared to the extract test group (XV) in chronic tests, with no significant ($P > 0.05$) difference between the SV group (XVI) and extract test group (XV), and with no significant ($P > 0.05$) difference of learning and memory pass rates following 6, 12, or 18 days of treatment in the extract test group (XV), (Figure 2D, Table 6 and Supplementary file 3: Table 4). The results indicate that prolonged ingestion of 0.025 g/mL extract (similar to 0.15 mg/mL SV) protects against the chronic learning and memory deficits of the mutant flies in a duration-independent pattern during the chronic phases of the study

The acute and chronic APS assays in mutant and wild-type control groups showed significantly ($P < 0.05$) higher average learning pass rates compared to the corresponding average memory pass rates within each experimental group (Figure 2A-D, Table 5, and Table 6). This indicates that in general terms, the learning performance of flies is higher than their memory performance in both acute and chronic phases of the study, this disparity is probably due to the method used to obtain the two parameters.

Table 5. Multiple Comparisons on Acute and Chronic Learning and Memory Deficits of Male Juvenile-Adults (10-day-old) and Old-Adults (16–28-day-old) Bang-Sensitive Mutant and Wild-Type Control *Drosophila* at 0 g/ml. n = 100.

Tukey's Multiple Comparisons Tests	Learning and Memory Pass Rate		
	Adjusted P-Value		
	Time		
Acute Treatment	1hr	2hrs	3hrs
LPR of *OrR* versus MPR of *OrR*	< 0.0001	< 0.0001	< 0.0001
LPR of *OrR* versus LPR of *CS*	0.9948	0.9322	> 0.9999
LPR of *OrR* versus MPR of *CS*	< 0.0001	0.0003	< 0.0001
LPR of *OrR* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *OrR* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *OrR* versus LPR of *CS*	< 0.0001	< 0.0001	< 0.0001
MPR of *OrR* versus MPR of *CS*	0.9322	0.9681	0.9968
MPR of *OrR* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *OrR* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *CS* versus MPR of *CS*	0.0005	< 0.0001	< 0.0001
LPR of *CS* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *CS* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *CS* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *CS* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR $para^{bss\,1}$ vs. MPR of $para^{bss\,1}$	0.0163	0.0005	0.1057
Chronic treatment	**Day 6**	**Day 12**	**Day 18**
LPR of *OrR* versus MPR of *OrR*	0.0006	< 0.0001	0.0001
LPR of *OrR* versus LPR of *CS*	0.7608	0.9767	0.2656
LPR of *OrR* versus MPR of *CS*	0.0050	< 0.0001	0.0036
LPR of *OrR* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *OrR* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *OrR* versus LPR of *CS*	0.0252	< 0.0001	< 0.0001
MPR of *OrR* versus MPR of *CS*	0.9767	0.9963	0.8794
MPR of *OrR* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *OrR* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *CS* versus MPR of *CS*	0.1341	< 0.0001	< 0.0001
LPR of *CS* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of *CS* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *CS* versus LPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
MPR of *CS* versus MPR of $para^{bss\,1}$	< 0.0001	< 0.0001	< 0.0001
LPR of $para^{bss\,1}$ vs. MPR of $para^{bss\,1}$	0.1707	0.6895	0.8794

OrR and *CS* = wild-type controls; $para^{bss\,1}$ = mutant (test) model. $p < 0.05$ meant significant differences among the groups. Abbreviations: BSS = bang-senseless, CS = Canton-Special, LPR = learning pass rate, MPR = memory pass rate, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic.

Table 6. Multiple Comparisons on the Acute and Chronic Effect of Methanol *I. cylindrica* Root Extract on Acute and Chronic Learning and Memory Deficits of Male Juvenile-Adult (10-day-old) and old-Adult (16–28-day-old) Bang-Sensitive Mutant *Drosophila* (*para*$^{bss\,1}$). n = 100.

Tukey's Multiple Comparisons Tests	Learning and Memory Pass Rate		
	Adjusted P-Value		
	Time		
Acute Treatment	1hr	2hrs	3hrs
LPR of 0 g/ml versus MPR of 0 g/ml	0.0024	< 0.0001	0.0300
LPR of 0 g/ml versus LPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus MPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus LPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus MPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus LPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus MPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus LPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus MPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
LPR of 0.8 g/ml versus MPR of 0.8 g/ml	0.2315	0.0107	0.0014
LPR of 0.8 g/ml versus LPR of NaV (0.3 mg/ml)	0.9925	0.9313	0.0735
LPR of 0.8 g/ml versus MPR of NaV (0.3 mg/ml)	0.1394	0.0040	0.1627
MPR of 0.8 g/ml versus LPR of NaV (0.3 mg/ml)	0.0735	0.0007	< 0.0001
MPR of 0.8 g/ml versus MPR of NaV (0.3 mg/ml)	0.9998	0.9992	0.4221
LPR of NaV (0.3 mg/ml) versus MPR of NaV (0.3 mg/ml)	0.0394	0.0003	< 0.0001
Chronic treatment	**Day 6**	**Day 12**	**Day 18**
LPR of 0 g/ml versus MPR of 0 g/ml	0.0042	0.1907	0.4882
LPR of 0 g/ml versus LPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus MPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus LPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
LPR of 0 g/ml versus MPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus LPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus MPR of 0.8 g/ml	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus LPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
MPR of 0 g/ml versus MPR of NaV (0.3 mg/ml)	< 0.0001	< 0.0001	< 0.0001
LPR of 0.8 g/ml versus MPR of 0.8 g/ml	0.9669	0.9669	0.3718
LPR of 0.8 g/ml versus LPR of NaV (0.3 mg/ml)	0.2715	0.3718	0.8389
LPR of 0.8 g/ml versus MPR of NaV (0.3 mg/ml)	0.9986	0.8389	0.3718
MPR of 0.8 g/ml versus LPR of NaV (0.3 mg/ml)	0.0543	0.8389	0.9669
MPR of 0.8 g/ml versus MPR of NaV (0.3 mg/ml)	0.8389	0.9986	> 0.9999
LPR of NaV (0.3 mg/ml) versus MPR of NaV (0.3 mg/ml)	0.4882	0.9669	0.9669

$p < 0.05$ meant significant differences among groups. Abbreviations: BSS = bang-senseless, CS = Canton-Special, LPR = learning pass rate, MPR = memory pass rate, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic.

Juvenile-Adult and Old-Adult bss Paralytic Mutant Flies Show Acute and Chronic Brain Neurodegeneration

Histopathological examination of H & E-stained brain tissues found no significant brain lesions (focal brain vacuolations < 3 μm in diameter) in wild-type controls of group XI, while mutant (negative) controls of the group (XI) showed moderate brain neurodegeneration (multifocal vacuolations, 3-5 μm in diameter) in acute tests, with a similar nature of brain histopathology in mutant controls of group XI at 1, 2, or 3 h (Figure 3A and 4, and Supplementary file 3: Table 5). This indicates that juvenile-adult (10-day-old) mutant flies show acute non-progressive brain neurodegeneration compared to the juvenile-adult wild-type controls which depict normal general brain histology in the acute phases of the study.

Histopathological examination of H & E-stained brain tissues showed no significant brain lesions (focal brain vacuolations < 3 μm in diameter) in wild-type controls of group XIV, while mutant controls of the group (XIV) showed age and duration-dependent progressive brain neurodegeneration during the chronic phase of the study, as follows: 45% of animals showed moderate brain neurodegeneration and 55% of them showed severe brain neurodegeneration on day 6; 40%, moderate brain neurodegeneration and 60%, severe brain neurodegeneration on day 12; and 100%, severe brain neurodegeneration on day 18 (Figure 3C and 5, and Supplementary file 3: Table 6). This indicates that old-adult (16-28-day old) mutant flies show chronic age-dependent progressive brain neurodegeneration, unlike old-adult wild-type controls that depict normal general brain histology during chronic phases of the study.

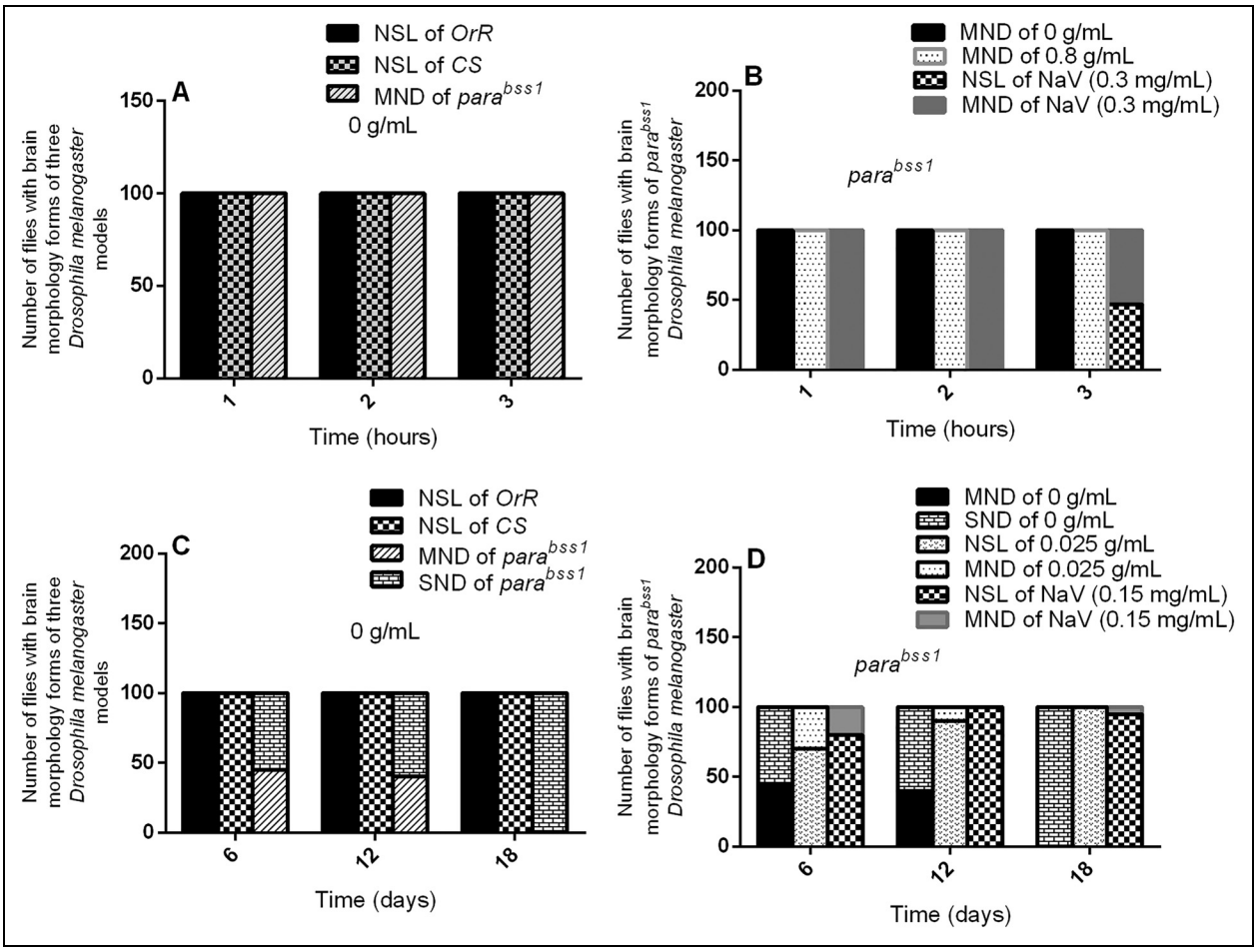


Figure 3. Acute and chronic effects of methanol *I. cylindrica* root extract on acute and chronic brain neurodegeneration in male juvenile-adult (10-day-old) and old-adult (16–28-day-old) bang-sensitive mutant *Drosophila* (*para*^{bss1}). A. Acute brain neurodegeneration of juvenile-adult bang-sensitive mutant *Drosophila*. B. Effect of acute (1–3-h) extract treatment on acute brain neurodegeneration of juvenile-adult mutant flies. C. Chronic neurodegeneration of old-adult bang-sensitive mutant *Drosophila*. D. Effect of chronic (6–18-day) extract treatment on chronic brain neurodegeneration of old-adult mutant flies. The number of flies having each type of brain morphology (y-axis) plotted against time concerning different treatment groups; n = 100. *OrR* and *CS* = wild-type controls; *para*^{bss1} = mutant (test) model. Abbreviations: BSS = bang-senseless, CS = Canton-Special, NaV = sodium valproate, NSL = no significant lesion, MND = moderate neurodegeneration, OrR = Oregon R, Para = Paralytic, SND = severe neurodegeneration. 100% of juvenile-adult (10-day-old) *bss* mutant flies showed moderate brain neurodegeneration versus the wild-type controls of the same age with normal brain morphology/NSL (A). The acute brain neurodegeneration of mutants was not alleviated by acute extract feeding versus alleviation by acute feeding with SV (B). 100% of old-adult (16–28-day-old) *bss* mutant flies showed moderate-severe brain neurodegeneration versus the wild-type controls of the same age with NSL (C). The chronic brain neurodegeneration of mutants was rescued in 100% of mutant flies by chronic (18 days) extract feeding similar to feeding with SV (D).

I. cylindrica Root Extract Lessened Brain Neurodegeneration of bss Paralytic Mutant Flies Following Prolonged but not Short-Term Treatment

The acute brain histopathological features (with H & E-stained tissues) of untreated mutant controls (group XI) were similar to the extract test group (XII) in acute tests, with a marked difference between histopathological findings of SV group (XIII) and extract test group (XII), ie, SV group showed improved brain histology from moderate brain neurodegeneration to normal brain forms in 47% of the animals after 3 h of treatment with SV (Figure 3B and 4, and Supplementary file 3: Table 5). Therefore, short-term ingestion of 0.8 g/mL extract (unlike SV) does not reduce the acute brain neurodegeneration of juvenile-adult mutant flies during the acute phase of the study.

The brain histopathological features (with H & E stain) of untreated mutant controls (group XIV) were markedly defective compared to the extract test group (XV) in the chronic phase of the study. The extract test group (XV) showed a noticeable alleviation of brain neurodegeneration in a duration-dependent fashion as follows: 70% of animals, showed no significant brain lesions, and 30%, had moderate brain neurodegeneration on day 6; 90%, had no significant brain lesions, and 10%, moderate brain neurodegeneration on day 12; 100%, no significant brain lesions on day 18. SV group (XVI) showed similar brain

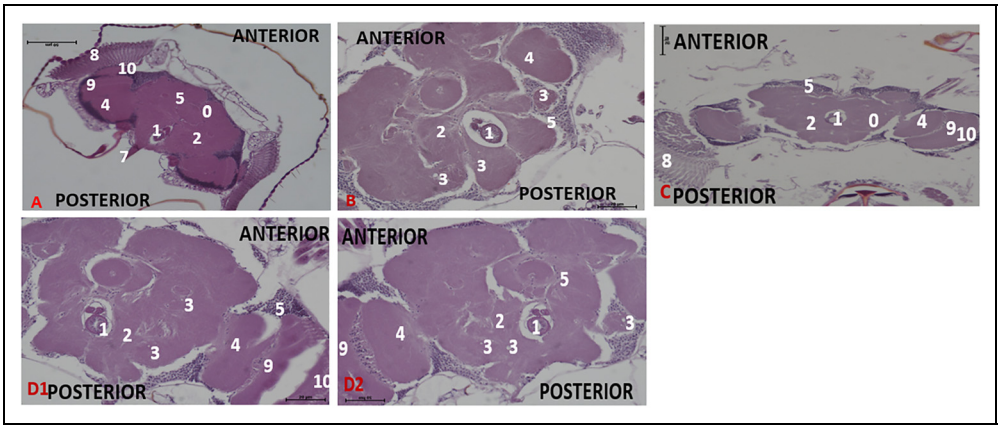


Figure 4. Effect of acute treatment with methanol *I. cylindrica* root extract on acute brain neurodegeneration of male juvenile-adults (10-day-old) bang-sensitive mutant *Drosophila* ($para^{bss\,1}$). n = 100, (H&E; transverse sections at level of mid-brain, x200 or x400). Bar, 20 µm or 50 µm. A. Wild-type control, x200. B. Mutant control, x400. C. Standard control, x200. D. Methanol extract test, x200 (D1), x400 (D2). Categories of brain neurodegeneration: NSL (vacuolations <3 µm in diameter) = Normal; MND, (vacuolations 3–5 µm in diameter); SND, (vacuolations >5 µm in diameter). *OrR* and *CS* = wild-type controls; $para^{bss\,1}$ = mutant (test) model. 0 = No significant lesion in the neuropil, 1= Oesophagus; 2= Central brain; 3= Moderate vacuolations in neuropil; 4= Optic lobe; 5= Cell bodies; 7= Ventral nerve cord; 8= Compound eyes; 9= Medulla; 10= Lamina. Abbreviations: BSS = bang-senseless, CS = Canton-Special, NaV = sodium valproate, NSL = no significant lesion, MND = moderate neurodegeneration, OrR = Oregon R, Para = Paralytic, SND = severe neurodegeneration. 100% of juvenile-adult (10-day-old) *bss* mutant flies showed moderate brain neurodegeneration (B) versus wild-type controls of the same age with NSL (A). The acute brain neurodegeneration of mutants was not alleviated by acute extract feeding (D) versus alleviation by acute feeding with SV (C). See the bar graphs in Figure 3 above for a summary.

histological features as the extract test group (XV) during the chronic phases of the study as follows: 80% of animals, no significant brain lesions and 20% moderate brain neurodegeneration on day 6; 100%, no significant brain lesions on day 12; 95% no significant brain lesions and 5% of the animals regressed to moderate brain neurodegeneration on day 18, (Figure 3D and 5, and Supplementary file 3: Table 6). The results indicate that prolonged ingestion of 0.025 g/mL extract (like SV) alleviates chronic brain neurodegeneration of old-adult mutant flies in a duration-dependent fashion during the chronic phase of the study. Also, prolonged administration with SV (beyond 12 days) may be detrimental to the improved brain histology of the mutant flies.

Juvenile-Adult and Old-Adult bss Paralytic Mutant Flies Portray Acute and Chronic Brain Axonal Degeneration

Histopathological examination of axonal morphology and Nissl substance on Klüver Luxol Fast Blue-stained tissues found no significant lesions in axons and Nissl substance of wild-type controls of group XI, while mutant controls of the group (XI) showed axonal degeneration in the brain but with normal Nissl substance during the acute phase of the study, and with no differences in axonal morphology and Nissl substance of mutant flies at 1, 2, or 3 h within mutant control group (XI), (Figure 6A and 7, and Supplementary file 3: Table 5). This indicates that juvenile-adult (10-day-old) mutant flies show acute non-progressive axonal degeneration in the brain, compared to the juvenile-adult wild-type controls that depict normal axonal morphology in the acute phase of the study.

Histopathological examination of Klüver Luxol Fast Blue-stained tissues for axonal morphology and Nissl substance showed no significant lesions in axons and Nissl substance of wild-type controls in group XIV, however, the mutant controls in the group (XIV) showed brain axonal degeneration but with normal Nissl substance during the chronic phase of the study, and with no marked differences in nature of axon morphonology and Nissl substance of mutant flies at 6, 12, or 18 days within the mutant controls (group XIV), Figure 6C and 8, and Supplementary file 3: Table 6). This indicates that old-adult (16-28-day old) mutant flies show chronic age-independent non-progressive brain axonal degeneration compared to old-adult wild-type controls which depict the normal axonal structure and Nissl substance in chronic phases of the study.

I. cylindrica Root Extract Alleviated Brain Axonal Degeneration of bss Paralytic Mutant Flies Following Prolonged but Not Short-Term Treatment

The acute brain axonal degeneration seen with Kluvers LFB-stained tissues of untreated mutant controls (group XI) was similar to the extract test group (XII) of the acute study, with no differences between SV group (XIII) and extract test group (XII), (Figure 6B and 7, and Supplementary file 3: Table 5), indicating that short-term ingestion of 0.8 g/mL extract (like 0.3 mg/mL SV) did not alleviate the acute brain axonal degeneration of juvenile-adult mutant flies during the acute phase of the study.

The chronic axonal degeneration was found in untreated mutant controls of the chronic study (group XIV), whereas

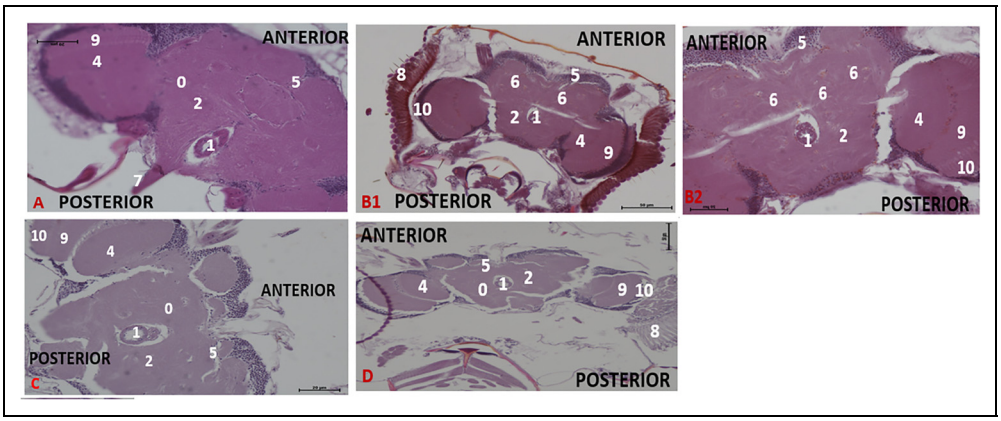


Figure 5. Effect of chronic treatment with methanol *I. cylindrica* root extract on chronic brain neurodegeneration of male old-adult (16–28-day-old) bang-sensitive mutant *Drosophila* ($para^{bss\,I}$). n = 100, (H&E; transverse sections at level of mid-brain, x200 or x400). Bar, 20 µm or 50 µm. A. Wild-type control, x400. B. Mutant control, x200 (B1), x400 (B2). C. Standard control, x400. D. Methanol extract test, x200. Categories of brain neurodegeneration: NSL (vacuolations <3 µm in diameter) = Normal; MND, (vacuolations 3–5 µm in diameter); SND, (vacuolations >5 µm in diameter) = brain histopathology. *OrR* and *CS* = wild-type controls; $para^{bss\,I}$ = mutant (test) model. 0 = No significant lesion in the neuropil, 1= Oesophagus; 2= Central brain; 4= Optic lobe; 5= Cell bodies; 6= Moderate to severe vacuolations in neuropil; 7= Ventral nerve cord; 8= Compound eyes; 9= Medulla; 10= Lamina. Abbreviations: BSS = bang-senseless, CS = Canton-Special, NaV = sodium valproate, NSL = no significant lesion, MND = moderate neurodegeneration, OrR = Oregon R, Para = Paralytic, SND = severe neurodegeneration. 100% of old-adult (16–28-day-old) *bss* mutant flies showed brain neurodegeneration of moderate or severe type (B) versus the wild-type controls of the same age with NSL (A). The chronic brain neurodegeneration of mutants was rescued in 100% of mutant flies by chronic (18 days) extract feeding (D) in a similar way to SV feeding (C). See the bar graphs in Figure 3 for a summary.

the extract test group (XII) of the chronic study showed normal axonal morphology in 100% of treated mutant animals after days 6, 12, or 18 of treatment, and similar changes in axonal morphology were observed with the SV group (XVI), (Figure 6D and 8, and Supplementary file 3: Table 6), indicates that prolonged ingestion of 0.025 g/mL extract (like 0.15 mg/mL SV) alleviates the chronic brain axonal degeneration of old-adult mutant flies in a duration-independent pattern during the chronic phase of the study.

Discussion

The number of epilepsy cases has increased tremendously within the past few years and between 2019 and 2020 around 50 million people had the condition globally and almost 80% of these live in low- and middle-income countries.[2,3] *Imperata cylindrica (L.) P. Beauv* (Poaceae) is a famous neuro-medicinal, anticonvulsant, and antiepileptic herb in traditional therapy due to its receptor inhibition and antioxidant properties,[32,34-38] however, to the best of our knowledge little is known regarding the neuroprotective efficacy of *I. cylindrica* as an antiepileptic herb and few studies have demonstrated the neuroprotective potential of the plant on glutamate-induced neurotoxicity in rat cerebral cortical cells.[37,38] The study demonstrated that phytochemicals of *I. cylindrica* root extract had neuroprotective roles on convulsions, cognitive deficits, and brain histopathology of *Drosophila melanogaster* bang senseless mutant models of epilepsy. This is vital because little is known about the neuroprotective role of the phytochemicals,[35,36,42] and in this study, the plant phytochemicals are thought to have upregulated endogenous antioxidants and inhibited receptor or voltage-gated sodium ion channels (VGSCs) which are linked to increased antioxidant status, decreased inflammation and apoptosis, increased tissue repair, and improved cell biology in the brain of the mutant flies faced with epilepsy-like neuropathological features.

The high contents of phytochemicals isolated from the methanol *Imperata cylindrica (L.) P. Beauv* (Poaceae) root extract (flavonoids, polyphenols, chromones **1** and **2**, saponins, tannins, cardiac glycosides, and alkaloids) are similar to those isolated previously from ethanolic, methanolic, and aqueous root and rhizome extracts of *Imperata cylindrica* (Poaceae or Gramineae**)**, but previous data also demonstrated lignans, coumarins, and reducing sugars in the plant.[34,35,66] Plant antioxidants have alleviative properties and can be utilised in the control and treatment of neurodegenerative diseases[89,90] and epilepsy phenotypes[91,92] due to genetic mutations in humans and lower organisms such as *D. melanogaster*, rodents, zebrafish (*Danio rerio*), and nematodes (*C. elegans*).[89,90] Polyphenols, flavonoids, and chromones were in the highest concentration in the extract which concurs with previous studies involving ethanolic and methanolic extracts of rhizomes of *I. cylindrica* Beauv. (Gramineae) var *major*, (Nees) C. E. Hubb.[37,93] and these have strong antioxidant properties.[94,95] Therefore, the herb can be utilized in the development of therapeutic molecules[47,96-100] due to the neuroprotective attributes of flavonoids, polyphenols, and chromones that have been demonstrated in brain cells of rodents[37-39] via antioxidant and receptor inhibition mechanisms[37-39] and these mechanisms are linked to antiseizure, antiepileptogenic, and cognitive deficit alleviation attributes in rodents with neurological disorders.[40,41]

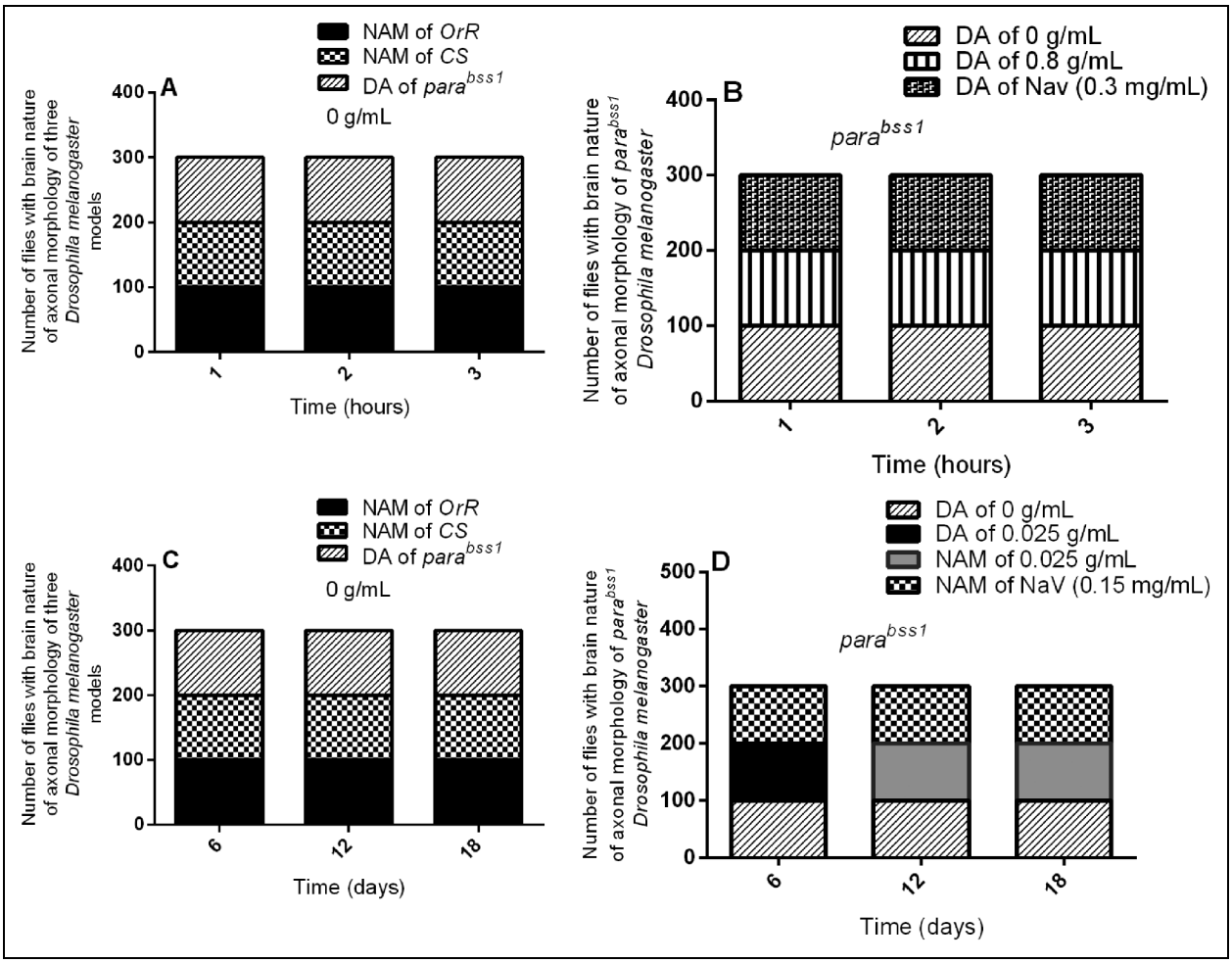


Figure 6. Acute and chronic effects of methanol *I. cylindrica* root extract on acute and chronic axonal degeneration in the brain of male juvenile adult (10-day-old) and old-adult (16–28-day-old) bang-sensitive mutant *Drosophila* ($para^{bss1}$). A. Acute axonal degeneration in the brain of juvenile-adult bang-sensitive mutant *Drosophila*. B. Effect of acute (1–3-h) extract treatment on acute axonal degeneration in the brain of juvenile-adult mutant flies. C. Chronic axonal degeneration in the brain of old-adult bang-sensitive mutant *Drosophila*. D. Effect of chronic (6–18-day) extract treatment on chronic axonal degeneration in the brain of old-adult mutant flies. The number of flies with each type of brain axonal morphology (y-axis) plotted against time for different treatment groups; n = 100. *OrR* and *CS* = wild-type controls; $para^{bss1}$ = mutant (test) model. Abbreviations: BSS = bang-senseless, CS = Canton-Special, DA = degenerated axons, NAM = normal axonal morphology, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic. 100% of juvenile-adult (10-day-old) *bss* mutant flies showed axonal degeneration in the brain versus wild-type controls of the same age with normal brain axonal morphology (A). Acute brain axonal degeneration of mutants was neither alleviated by short-term extract feeding nor short-term feeding with SV (B). 100% of old-adult (16–28-day-old) *bss* mutant flies showed brain axonal degeneration versus wild-type controls of the same age with normal brain axonal morphology (C). Chronic axonal degeneration of mutants was rescued in 100% of mutant flies by prolonged (6–18 days) extract feeding, similar to feeding with SV (D).

The current study has demonstrated that bang-senseless (bss) *D. melanogaster* mutant models ($para^{bss}$) depict marked acute and chronic abnormal brain phenotypes (age-dependent progressive brain neurodegeneration and axonal degeneration) and significant neuropathological symptoms (acute and chronic convulsions and cognitive deficits or learning and memory deficits) in juvenile and old-adults arising from a gain-of-function point mutation in a *para* sodium channel gene at the *para* locus (up-regulates and increases the activity of the gene) in neurons of the current mutant *D. melanogaster* models of epilepsy ($para^{bss1}$), unlike the wild-type controls (*Oregon R and Canton-Special*) which showed normal brain morphology, cognitive functions, and without bang sensitive and convulsion behaviors. Therefore, the abnormal brain phenotype and neuropathological features that were demonstrated in the mutant flies ($para^{bss1}$), validate a mutation in the *para* gene (overexpression of *para*) in the neurons of brain tissues of juvenile and old-adult *D. melanogaster* bss mutants[15,16] and is similar to the expression of a *para* gene demonstrated in neuronal brain tissues of adult *D. melanogaster* when *UAS-para* is driven by *ELAV-GAL4* driver,[101-104] and the expression of the mutant genes has been linked to human-like brain neurodegenerative disorders in *Drosophila*.[101,103,105,106]

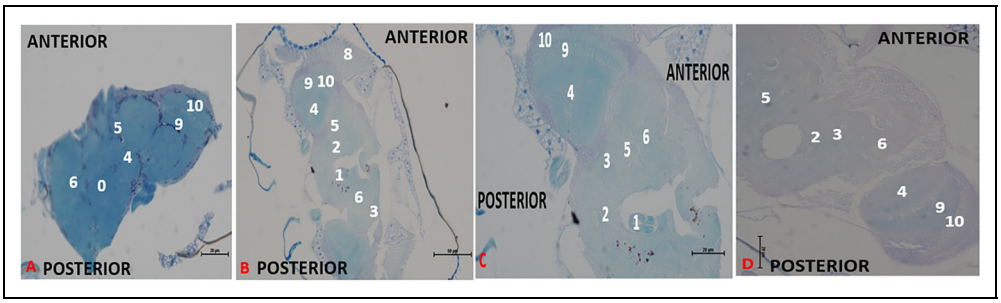


Figure 7. Effect of acute treatment with methanol *l. cylindrica* root extract on acute axonal degeneration in the brain of male juvenile-adult (10-day-old) bang-sensitive mutant *Drosophila* (*para*$^{bss\,1}$). n = 100, (Klüver Luxol Fast Blue; transverse sections, x200 or x400). Bar, 20 µm or 50 µm. A low-intensity patchy Klüver LFB stain indicates axonal degeneration/lesion in the Nissl substance. Axons are depicted in blue colour and Nissl substance is shown in magenta/ violet colour. A. Wild-type control, x400 in peripheral neuropil. B. Mutant control, x200 at the level of mid-brain. C. Standard control, x400 at the level of mid-brain. D. Methanol extract test, x400 at the level of mid-brain. NAM, a consistent LFB stain (no light blue patchy areas) = Normal morphology of axons; DA (weak LFB stain/light blue patchy areas) = axonal degeneration. *OrR* and *CS* = wild-type controls; *para*$^{bss\,1}$ = mutant (test) model. 0= No significant lesion in axonal structure, 1= Oesophagus; 2= Central brain; 3= Axonal degeneration; 4= Optic lobe; 5= Cell bodies; 6= No significant lesion in Nissl substance; 8= Compound eyes; 9= Medulla; 10= Lamina. Abbreviations: BSS = bang-senseless, CS = Canton-Special, DA = degenerated axons, NAM = normal axonal morphology, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic. 100% of juvenile-adult (10-day-old) *bss* mutant flies showed axonal degeneration in the brain (B) versus wild-type controls of the same age with normal axonal morphology (A). Acute axonal degeneration of mutants was neither alleviated by acute extract feeding (D) nor acute feeding with SV (C). See the bar graphs in Figure 6 above for a summary.

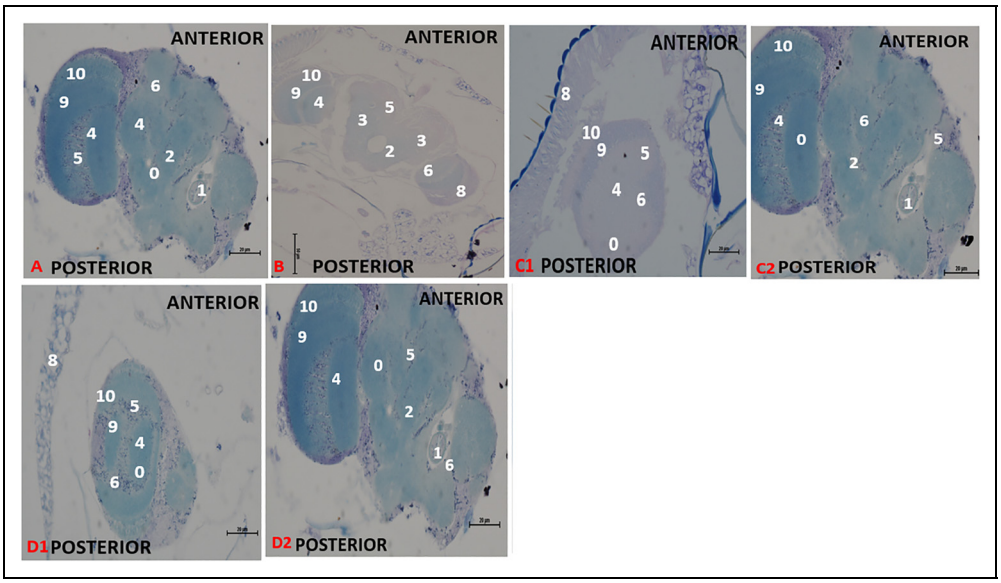


Figure 8. Effect of chronic treatment with methanol *l. cylindrica* root extract on chronic axonal degeneration in the brain of male old-adult (16–28-day-old) bang-sensitive mutant *Drosophila* (*para*$^{bss\,1}$). n = 100, (Klüver Luxol Fast Blue; transverse sections, x200 or x400). Bar, 20 µm or 50 µm. A low-intensity patchy Klüver LFB stain indicates axonal degeneration/lesion in the Nissl substance. Axons are depicted in blue colour and Nissl substance is shown in magenta/ violet colour. A. Wild-type control, x400 at the level of mid-brain. B. Mutant control, x200 at the level of mid-brain. C. Standard control, x400 in peripheral neuropil (C1), and at the level of mid-brain (C2). D. Methanol extract test, x400 in peripheral neuropil (D1), and at the level of mid-brain (D2). NAM, a consistent LFB stain (no light blue patchy areas) = Normal morphology of axons = Normal; DA (weak LFB stain/light blue patchy areas) = axonal degeneration. *OrR* and *CS* = wild-type controls; *para*$^{bss\,1}$ = mutant (test) model. 0= No significant lesion in axonal structure, 1= Oesophagus; 2= Central brain; 3= Axonal degeneration; 4= Optic lobe; 5= Cell bodies; 6= No significant lesion in Nissl substance; 8= Compound eyes; 9= Medulla; 10= Lamina. Abbreviations: BSS = bang-senseless, CS = Canton-Special, DA = degenerated axons, NAM = normal axonal morphology, NaV = sodium valproate, OrR = Oregon R, Para = Paralytic. 100% of old-adult (16–28-day-old) *bss* mutant flies showed axonal degeneration in the brain (B) versus wild-type controls of the same age with normal axonal morphology (A). Chronic axonal degeneration of mutants was rescued in 100% of mutant flies by prolonged (6–18 days) extract feeding (C) in a similar way to SV feeding (D). See the bar graphs in Figure 6 for a summary.

Altered gene expression and tissue-specific expression of specific neurodegenerative mutant genes such as epilepsy mutant genes[21,107] in *D. melanogaster* cause neuropathological phenotypes of the neurodegenerative disorder (in our case, epilepsy)[21,108-110.] The tissue-specific expression of mutant genes and transgenes in neurons has been linked to neuropathological phenotypes such as neurodegeneration, defects in mean lifespan, and locomotion in *D. melanogaster* models of neurodegenerative diseases and epilepsy.[101-106,108,109,111] The expression of tissue-specific mutants and transgenes provides robust and precise models with which to study human neurodegenerative diseases, epilepsy, and possible preventive and curative treatments for neurological disorders.[21,108-110]

The neuropathological findings of the current study concur with previous studies that demonstrated bang-sensitive behaviour, convulsions in mutant paralytic *D. melanogaster* models of seizures overexpressing *para* gene ($para^{bss1}$) due to a mutation of the gene[15,16] and resulting reduction of seizure-stimulation threshold arising from increased expression of the VGSCs in the neurons of the mutants.[15,16] The impaired learning and memory functions demonstrated in the current $para^{bss1}$ mutant *Drosophila* compared to the wild-type controls are similar to the cognitive phenotypes previously shown in the model ($para^{bss1}$),[15-17] and concur with other findings that have shown cognitive deficits in *Drosophila* models of neurodegeneration (*ELAV-GAL4 > UAS-GFP and ELAV-GAL4 > UAS-TAU*).[57] Cognitive deficits in bang-sensitive paralytic *D. melanogaster* are attributable to a mutation of the *para* gene in neurons of the flies which disrupts the metabolic pathways and neuronal activities leading to bang-sensitive and seizure behaviors that originate in the brain of the flies (epileptogenic zone),[15,53,83,112] and the seizures and their effects spread to secondary brain centers including the motor centers and learning and memory centers in mushroom bodies (cognitive centers) hence distorting the learning and memory functions (cognitive deficits) in the brain of *D. melanogaster*.[113] The study demonstrated that in general, the current $para^{bss1}$ mutants have less magnitudes of memory function than the corresponding levels of learning function in all the study groups, the observed general differences in the two parameters come from the method (APS assay) that was employed to assess the parameters, ie, memory tests were performed 6 h after performance of the learning tests without re-training the flies to the task learnt.[57]

The current $para^{bss1}$ mutants show progressive age-related brain neurodegeneration (neurovacuolation) and axonal degeneration concurring with previous studies on bss and excitability *Drosophila* ($para^{bss}$, jus, *eas*, *tko*, *ses B*) that found age-related brain neurodegeneration due to gene mutations in neurons of the flies.[22,114,115] The axonal degeneration found in the current mutant models is typical of axonal-type neurodegenerative disorder (axonopathy) found in *Drosophila* models of human genetic neurodegenerative disease,[116-118] and axonal degeneration is a cause of disease pathogenesis, disability, and loss of function in the CNS during epilepsy.[119-121] It is characterized by a reduction in the density and number of nerve tracts as demonstrated previously in *D. melanogaster* bang-sensitive mutants ($para^{bss}$, *ses B, eas*, *jus*, *tko*),[115] and is due to a mutation of the *para* gene at the *para* locus in neurons of bss para *D. melanogaster* models of epilepsy which induces defective nerve fibers.[20,83,84,115,122] Similarly, mammalian forms of genetic epilepsy due to gene mutations in VGSC β subunit genes (SCN1B-SCN4B) and α subunit genes (*SCN1A*, *SCN2A*, *SCN3A,* and *SCN8A*) in the brain have been linked to neurodegeneration and pathology found in epilepsy.[9-11,123]

The neurodegeneration (degeneration of nerve tracts) in the brain of the bang senseless paralytic flies distorts electrical activity and conduction of impulses,[124-126] contributes to the development of seizure and epileptic phenotypes (bang-sensitivity, convulsions, learning, and memory deficits) observed in the brain and peripheral parts of the current mutants, features of which are consistent with previous studies.[124-126] In conclusion, the mutant flies of the study ($para^{bss1}$) display fascinating epileptic phenotypes and behaviors similar to mutants of the BS paralytic family such as sdaiso,[7,8] easPC,[80] and tko^{25t} mutants, as well as other *Drosophila* models of human seizures[10,15,17,22,114,115] ie, progressive age-related brain neurodegeneration and axonal degeneration of the mutant flies, are linked to bang-sensitive behavior, seizures, and defective cognitive functions, consistent with CNS neurodegenerative and axonopathy features of bss paralytic *D. melanogaster* seizure models.[10,17,83,114,124-127]

To the best of our knowledge, the study is the first of its kind to demonstrate the staining affinity of CNS axons for combined Luxol fast blue (LFB) and Nissl (Klüver's) stain in *Drosophila* models indicating that *Drosophila* neurons and axonal sheaths could be containing biological compounds mimicking the phospholipids or lipoproteins of the myelin sheath and Schwann cells of myelinated neurons. However, *Drosophila* axons are non-myelinated and not surrounded by Schwann cells,[117] instead, they are encased in 'ensheathing glial cells' within the CNS of *Drosophila* ,[87] and although they are non-myelinated and not surrounded by Schwann cells,[117,128,129] the current data concurs with previous studies which demonstrated Schwann-like (*Drosophila*) or Schwann cells (mammals) to be existing in unmyelinated axons in form of grooves but not surrounding the axons, closely interdependent with the axons to provide structural integrity and function of the axons.[117,130-132] Also, Schwann cells have been reported to secrete neurotrophic factors and myelin-related proteins.[130,133] The non-myelinating Schwann cells of mammals are comparable in function to the 'ensheathing' and 'wrapping' *Drosophila* glial cells of the CNS and peripheral nervous system of *Drosophila* respectively and are responsible for encasing axons and neuropil of the flies.[86,87,134] Future studies should focus on the utility of combined Luxol Fast Blue and Nissl (Klüver's) staining technique in experimental examination of the basic neuronal structure in the CNS of *Drosophila* to establish the staining properties of the nervous tissues of *Drosophila*.

Acute and chronic extract treatment significantly reduced the neuropathological features of the mutant flies (convulsions,

learning and memory deficits, and brain histopathology) in the extract test animals and the standard control group treated with SV in duration and dose-dependent fashions compared to the mutant (negative) control group, and the values of the parameters (CT, PT, RT, learning/memory pass rate, brain, and axonal neurodegeneration) of the extract test and standard control groups were similar to (not significantly different from) the wild-type control group indicating that our *I. cylindrica* extract induces significant neuroprotective activities (alleviates the neuropathological features) in the nervous tissue of the mutant flies, with a similar neuroprotective capacity to the standard control drug of our study (SV).

However, the acute and chronic age-related brain neurodegeneration and axonal degeneration of the mutant flies were alleviated by prolonged (6-18 days) extract treatment but not by short-term (1-3 h) treatment and the standard antiepileptic (SV) depicted better alleviative potential on the brain histopathology than the plant extract since the former modulated the brain histological defects of the mutant flies following both short-term (3 h) and prolonged (6-12 days) treatments compared to the extract which showed modulatory potential only after prolonged treatment (6-18 days). The standard antiepileptic showed a side-effect on brain morphology since further feeding with SV (18 days) caused brain histopathological features in the previously improved brain histology of the mutant flies indicating that although antiepileptic molecules have marked beneficial roles, they may cause detrimental side effects on the CNS, especially in cases of chronic antiepileptic therapy.[135,136]

The neuroprotective attributes of the current herb (*I. cylindrica*) are consistent with previous findings that demonstrated the phytochemicals of the herb to be possessing neuroprotective attributes on the neuropathology of rat brain cells[37,38] and is in tandem with previous studies that suggest plant-based nonenzymatic antioxidants notably phenols, flavonoids, chromones, and glycosides to be contributors neuroprotection on brain cell damage by the provision of significant antioxidant activities (antioxidation mechanism controls oxidative stress markers in the brain of animals) that are linked to improved brain cell biology,[37,39] thereby inducing anticonvulsant and antiepileptogenic pathways,[40,137] and alleviate cognitive deficits of animals.[41] The antiepileptic similarity of the herb compared to standard anticonvulsant (SV) indicates that the extract similar to standard antiepileptics can alleviate the bang-sensitive behaviour of *Drosophila* models of epilepsy by improving brain cell biology, seizure-control, and cognitive-function modulatory pathways in concurrence with previous findings.[15,61,127]

Again, the potential of our extract to modulate the brain histopathology of the mutant flies is in tandem with a review by Trojnar et al, (2002) who demonstrated novel standard antiepileptics (lamotrigine, tiagabine, and topiramate) as having neuroprotective effects on CNS morphology of epileptic animals,[138] and concurs with previous studies that reported similar neuroprotectant potential in antioxidant compounds and antiepileptic drugs on epileptic animals.[137,139] However, our findings contradict with findings of Qiao et al, (2000) where Vigabatrin demyelinated the brain of epileptic rats[140] indicating that antiepileptic molecules have side effects.[135] The differences and similarities between the current data and those by Trojnar et al, (2002), Qiao et al, (2000), and Cavanna et al, (2010) are striking but not surprising since antiepileptics can depict neuroprotective, neutral, or detrimental CNS activities in epileptic models.[135,138,140]

Oxidative stress has been strongly related to the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease (PD), and epilepsy.[115,141-146] Bang-sensitive *Drosophila* models of epilepsy are vital models for oxidative stress-related neurological defects, and the abnormal phenotypes in the models such as $para^{bss}$, *jus*, *eas*, and *tko* are attenuated by feeding with an antioxidant.[115] In addition, attenuation and rescue of neurological defects by natural antioxidants have been reported in *Drosophila* models of epilepsy[143] and *Drosophila* is an emerging model organism for plant-derived antioxidants.[147-149] Most importantly, the potential role of natural antioxidants from plant extracts as neuroprotectants for treating and preventing neurodegenerative diseases and epilepsy has been shown in vitro, in vivo, and in clinical settings.[89,137,150] In addition, hyperactivity of the VGCs due to mutation (s) in the *para* gene (gain-of-function mutation in the *para* sodium channel gene at the *para* locus) in neuronal brain tissues of bang-sensitive (BS) *Drosophila* models of epilepsy is strongly linked to the neuropathological features of epilepsy (brain histopathology, seizures, and cognitive deficits).[15,16]

The brain has very high metabolic activities and is the most aerobically active organ in the body, the organ is very susceptible to oxidative stress, causing oxidative-induced brain injuries and neuronal hyperexcitability,[139] such as in bang-sensitive *Drosophila* and the defects can be rescued via the antioxidant pathways (feeding with neuroprotectant antioxidants),[115,139] and by treating with molecules that inhibit the VGSCs.[151,152] Therebefore, the proposed mechanisms by which *I. cylindrica* provides its neuroprotective attributes are the 'antioxidant' and 'receptor inhibition' mechanisms[115,139,151,152] arising from the main phytochemicals of the plant (polyphenols, flavonoids, and chromones), these provide important biological activities within the brain cells including the improvement of antioxidant status, tissue repair, decreased inflammation and apoptosis, improvement of brain cell biology,[37,39] and binding and inhibition of VGSCs and receptors (eg, 5-HT2B) by chromones **1** and **2.**[37,38,153] These in turn result in the alleviation of brain histopathology, seizure control, and alleviation of cognitive deficits in our mutant *D. melanogaster* model of epilepsy. This concurs with previous studies that have shown novel therapeutic strategies including 'natural antioxidant therapy' using plant extracts to be able to prevent epileptogenesis, modify epileptic phenotypes, and attenuate the associated neurological deficits and defects in epileptic animals.[89,90,154] Also, similar findings from a previous study showed attenuation of neuromotor deficits by natural antioxidants of a plant (*Decalepis hamiltonii*) root extract in a transgenic *Drosophila* model of Parkinson's disease.[155] The current findings concur with previous data where antioxidants promoted brain regeneration and axonal growth and repair in animal models of seizure disorders through the inhibition of oxidative stress and promotion of antioxidant pathways[156,157]

and by epigenetic modulation.[158] These regenerative potentials are linked to the rescue of the defective brain electrophysiology, improvement of brain metabolic pathways, and establishment of improved conduction of impulses in the motor brain centers and mushroom bodies of *Drosophila* brains,[83,159] which causes cessation of seizures and improves cognitive functions in the flies.[160]

Given the weight of the flies tested (1 mg per fly) and that they can consume between 20–100 μg a day and twice this amount when starved for 14–17 h,[161] the doses used can be extrapolated to the human context eg 0.8 g/mL utilized in acute therapy, and 0.025 g/mL used in chronic therapy correspond to 16 g/kg and 0.5 g/kg respectively when projected to a human adult.

The current study had some limitations: it did not focus on the evaluation of the neuroprotective molecular mechanisms via the antioxidant signaling and receptor binding inhibition mechanisms, did not emphasize the gender-related differences associated with the abnormal phenotypes and effect of the herb on the phenotypes of the mutant flies, and only focused on the effect of the herb on a genetic form of epilepsy while leaving out acquired/induced convulsions like the antimuscarinic and pentylenetetrazole kindling models that are often studied[137,162]; a prospective study will be conducted to assess the practical attributes of antioxidant and receptor inhibition pathways related to the neuroprotective effects of *I. cylindrica* root extract using bss *Drosophila*, other genetic models and kindling models of convulsions.

Conclusion

The juvenile-adult and old-adult mutant flies ($para^{bss1}$) show age-related progressive brain neurodegeneration and axonal degeneration and associated acute and chronic seizure and cognitive deficit phenotypes. The methanol root extract of *I. cylindrica* in a similar magnitude to sodium valproate exerts neuroprotective properties on the neuropathological features of the mutant flies by proposed antioxidant and receptor inhibition mechanisms arising from the main phytochemicals of the herb (Polyphenols, flavonoids, and chromones **1** and **2**) which increase the antioxidant status and tissue repair, decrease inflammation and apoptosis, improve brain cell biology and cause inhibition of VGSCs and receptors in the brain cells of the mutant flies. This results in the improvement of brain histology, seizure control, and improvement of cognitive functions in our mutant *D. melanogaster* model of epilepsy.

Therefore, the methanolic extract contains high amounts of non-enzymatic antioxidants (phenols, flavonoids, and chromones **1** and **2**) and non-nitrogenous ligands for receptor and VGSC inhibition (chromones **1** and **2**) reflecting huge antioxidant, receptor binding, and inhibition, and neuroprotective powers of *I. cylindrica* root extract in genetic cases of epilepsy associated with a gain-of-function point mutation in the *para* sodium channel gene at the *para* locus in $para^{bss1}$ mutant lines. Thus, we recommend more experimental and clinical studies regarding this extract in treating genetic forms of epilepsy.

Abbreviations

BS	Bang-sensitive
BSS	Bang senseless
CS	Canton-Special
CNS	Central nervous system
OrR	Oregon R
Para	Paralytic
SV	Sodium valproate
VGSC	Voltage-gated sodium ion channel.

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Authors' Note

The datasets used and/or analyzed during the current study and the Supplemental materials are available at: https://figshare.com/s/b484daeacf410f8fb9ea.

Authors' Contributions

Fred Ssempijja: conceptualization, Principal Investigator, methodology, data collection, validation, data analysis and interpretation, writing—original draft preparation, and writing—review and editing. Samuel Sunday Dare: data analysis and interpretation, writing—review and editing, and supervision of this study. Edmund E. M. Bukenya: validation, writing—review and editing, and supervision of this study. Keneth Iceland Kasozi: validation, data analysis and interpretation, writing—original draft preparation, and writing—review and editing. Ritah Kenganzi: methodology, data collection, validation, writing—original draft preparation, and writing—review and editing. Edgar Mario Fernandez: conceptualization, methodology, and writing—review and editing. Marta Vicente-Crespo: conceptualization, methodology, validation, writing—review and editing, and supervision of this study. All authors have read and agreed to publish the version of the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The experimental protocol was approved by the Institutional Review Ethics Committee of Kampala International University, Western Campus, Uganda (study protocol code: MSC-ANA-001/142-DU), and carried out following the guidelines given by the Uganda Council for Higher Education (UCHE).

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Supplemental Material

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