

The effect of *Imperata cylindrica* (L.) P. Beauv. on Brain tissue Morphology of *Drosophila Melanogaster transgenic* flies overexpressing the paralytic Gene

¹Ssempijja Fred, ²Marta Vicente-Crespo, ¹Dare Samuel Sunday and ¹Edmund Eriya Bukenya

¹Department of Human Anatomy, Kampala International University-Western Campus, Bushenyi, Uganda.

²School of Medicine, St. Augustine International University, Kampala, Uganda.

ABSTRACT

Imperata cylindrica root extract is neuroprotective and is used by Luo people in Kenya and Uganda to manage epileptic fits (personal communication); the herb controls seizures in parabss flies by reducing Na⁺ and K⁺ channel activity. Brain anatomical changes, muscular activity as well as changes in learning and memory function that could arise in these flies from use of the herbas an anti-epileptic molecule during acute and chronic control of seizures were the objectives of our study. Learning and memory response was studied by an Aversive Phototaxic Suppression and brain histomorphological changes were analysed on brain tissues using Haematoxylin and Eosin plus Klüvers staining techniques. Our results have further confirmed the promise of *Imperata cylindrica* as an anti-epileptic drug and have shown that not only treats the convulsions but ameliorates associated electrophysiological and cognitive malfunctions. The extract also ameliorated the abnormal brain histomorphology after prolonged treatment. There was only relief of symptoms in acute exposure treatments unlike in chronic exposure treatments where the extract and sodium valproate cured the brain cellular biological defects.

Keywords: *Imperata cylindrica*, Brain tissue, Morphology, *drosophila Melanogaster* transgenic flies and paralytic gene

INTRODUCTION

Epilepsy is a neurological disorder related to seizures that have a habit of recurrence without an obvious trigger or cause. The seizures come as a result of simultaneous, excessive firing of nerve signals within the brain. Thus the syndrome is very difficult to treat yet endangering patients and/or indirectly impacting relatives as well [1]. The traditional plant *Imperata cylindrica* (*I. cylindrica*) commonly called the 'spear grass' is used by the Luo people to treat epileptic convulsions (personal

communication). In a study to determine the anti-epileptic effect of this plant on bang-sensitiveparalytic (parabss) *Drosophila melanogaster* (*D. melanogaster*) flies conducted by [2], the 'herb' was found to control epileptic seizures in these flies through reduction in the activity of Na⁺ and K⁺ channels. The therapeutic and toxic doses of *I. cylindrica* were also elucidated as were some of the behavioural effects of the extract in parabss flies [2].

MATERIALS AND METHODS

Study Design

The study design had several groups of *D. melanogaster*bss transgenic flies overexpressing the paralytic gene; these were tested in line with each specific objective. Each objective had three repeated independent experiments and each experiment contained three replicas. A maximum of 6 flies were used

in each replica; this was done to ensure quality and reproducibility in results.

Study Location

The study was conducted in the Institute of Biomedical Research (IBR) of KIU- WC; Uganda in Ishaka Municipality, Bushenyi District 350 Km from Kampala, Uganda. Histological techniques were performed at the Central diagnostic Laboratory at the College of Veterinary Medicine

Animal Resources and Bio-security, in the Joint National Animal Disease Center (J-NADIC), Makerere University, Kampala, Uganda.

Study Population

The study used laboratory-crossed *Drosophila melanogaster* flies. These were adults (5-23-days old) *D. melanogaster* bang-sensitive (bss) transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para). Experiments were performed on male flies only to ensure homogeneity of results. Two wild-type fly strains (GMR-GAL4 and UAS-para) were used in the study as control fly strains; these same strains were crossed in the laboratory to obtain the bss transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para) in accordance with a method [3].

Sampling Techniques

Each objective included single (acute) exposure and prolonged (chronic) exposure experiments. For any test that was performed there were three replicas (6 flies in each replica) which were randomly selected from a given treatment group; each test was repeated 3 times with different groups (three independent tests). All these aspects ensured quality and reproducibility in the results.

Method of Arriving at the Sample Size

To be able to perform a robust statistical analysis, large sample sizes are used [4]. Working with *D. melanogaster* allows to obtain fairly large sample sizes without incurring big expenses [5; 6]. The usual number of *Drosophila* flies used in each replica in experiments of exposure to drugs or medicinal herbs is 5-10 and the number of replicas per experiment being 3-5 [7].

Inclusion Criteria

The study involved use of male adult bss transgenic flies overexpressing the paralytic gene (GMR-GAL4>UAS-para) flies whose ages ranged from 5-23-days old. Male flies were recruited because they are always homozygous for the para gene responsible for seizures in *Drosophila*; female flies on the other hand can either be homozygous (infected) or heterozygous (carriers) for the paralytic (para) gene. Moreover, seizure characteristics in male and female bss flies overexpressing the para

gene are expressed differently; female bss flies overexpressing the para gene are more severely affected than their male counterparts because female flies have two alleles yet males have only one allele for the para gene [1].

Exclusion Criteria

The study excluded female flies. This was done to ensure that only flies which were homozygous (males) for the para gene were included in the study and those which might have been carriers for the gene (females) were eliminated; this ensured uniformity in the results since a single sex of flies was used. The study excluded fly larvae or flies that were too young or too old since these would interfere with cognitive abilities; larvae as well as flies younger than 5 days or older than 23 days were eliminated in the study.

Collection of *Imperata cylindrica* Samples

The plant materials were harvested from the banks of Rwizi River located in Mbarara Municipality, Mbarara district, Western Uganda. This area is located 295 Km by road from Kampala. A sample of the plant, roots and leaves were placed in a dry moisturized container and transported by road to the department of botany, Mbarara University of Science and Technology for botanical identification by Dr. Olet Eunice (Botanist). The plant was identified as *Imperata cylindrica* (L.) P. Beauv., Collection number: Fred Kalanzi #001. The plant roots from which the extract was obtained were stored in a fridge at Kampala International University, Western Campus in the Institute of Biomedical Research Laboratory for preservation.

Fly Stock

The control fly stocks used in this study included two wild-type *D. melanogaster* flies (GMR-GAL4 and UAS-para). In UAS-para (Upstream Activating Sequences-para) flies the para gene responsible for the bss phenotype is kept silenced and it is only triggered when crossed with an activator gene kept in GMR-GAL4 (glass multiple reporter-GAL4) system; therefore UAS-para flies are regarded as 'silencer' and GMR-GAL4 are regarded as 'activator' wild-type flies [3]. To obtain transgenic bss fly strains overexpressing the para gene for use in the study, female

virgin UAS-paraflies were crossed with male GMR-GAL4 flies in a method similar to that used by [3]. The larvae obtained from the cross were isolated from the adult flies to ensure that progeny is not mixed with the parents. Then these larvae were raised to obtain first generation adult GMR-GAL4>UAS-paraflies. The resultant F1 progeny were sexed to isolate the males from the females; the male flies were kept as the bss transgenic flies overexpressing the para gene. These transgenic flies were kept as the test stock flies for the study.

Determination of Activity of *Imperata cylindrica* Extract

The activity of the plant was determined by using the working concentrations that were established by Oginga, 2016, and the change in CT, PT and MRT before and after exposure to different concentrations of the extract was noted. To do this, 50 independent GMR-GAL4>UAS-paraflies were exposed to relatively safe doses of the plant extract as established in Oginga's study. In acute exposure treatments the safe dose range was established by Oginga as being below 1.0g/ml; therefore doses of 0.0, 0.6, 0.8 and 1.0g/ml of the extract were used to determine the effect of single exposure (2hr-exposure) on CT, PT and MRT. In prolonged exposure treatments (12-day exposure) the safe dose range was established by Oginga, 2016 as being

below 0.1g/ml; therefore we used doses of 0.0, 0.0125, 0.025 and 0.05 g/ml to determine the effect of prolonged exposure on CT, PT and MRT. All experiments were performed at room temperature (22- 25oC) unless stated otherwise [7].

Data Analysis and Statistical Measures

To test our scientific hypothesis, experimental results were analysed to test whether there was enough evidence to reject the null hypotheses: that the extract causes no brain histomorphological improvement on the transgenic *D. melanogaster* flies overexpressing the para gene, the extract does not ameliorate muscular activity of giant fibers in DLMs in the flies and that the extract does not improve learning and memory function in the transgenic flies. In general, Microsoft statistical computer package called 'Microsoft Excel Windows 2010' was used to analyze data obtained from the study and took the form of frequency tables, graphs and charts. A statistician was consulted in data analysis. Data on histomorphological changes was recorded in form of photomicrographs taken with a digital camera (Nikon digital sight DS Fi 1) mounted to the light microscope (Nikon Eclipse Ci, 104c type) and connected to a Toshiba computer with NIS-Elements F3.00, SP7; Build 547 software.

ETHICAL CONSIDERATIONS

Proper Handling of Flies

The fly strains and reagents used in the study were acquired by using the stipulated proper channels to get them (ordered from Bloomington Drosophila Stock Center) and ethics were followed to ensure that the flies were handled well and not intentionally injured or harmed. All procedures and techniques used in this study were in accordance with the care and use of laboratory animals from the Uganda National Council of Research

and approval was sought from the Postgraduate Ethics and Research Committee (PGERC) and Institutional Review and Ethics Committee (IREC) of KIU-WC. Less invasive techniques were employed to avoid causing pain on the flies; inflicting pain to live flies was minimized by sacrificing the flies under ether anaesthesia. Flies were provided with necessary requirements for their survival such as food, water and favorable environment.

RESULTS

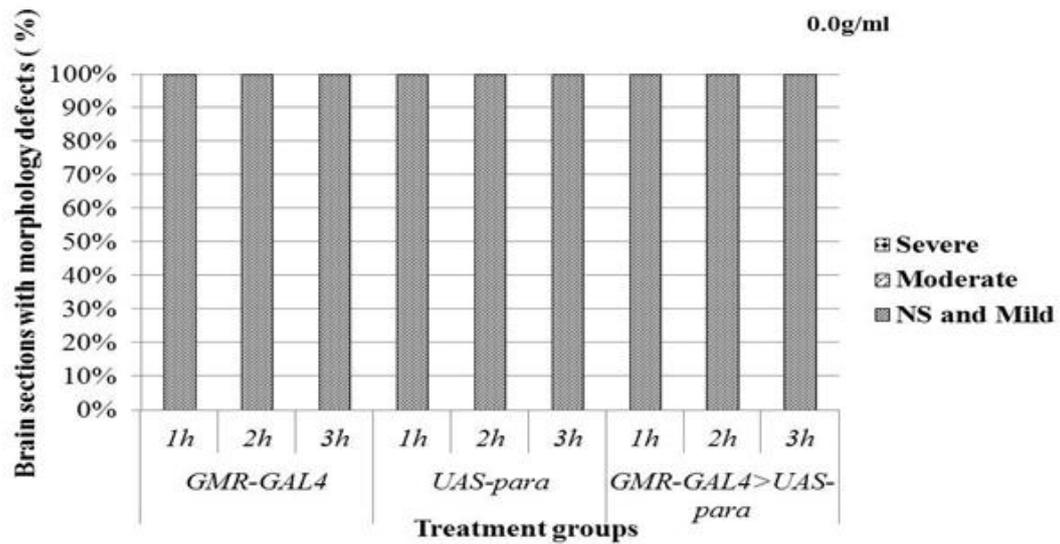


Figure 1: Brain sections showing general morphological defects of the flies at 0.0g/ml of *Imperata cylindrica* extract.

This data was from five *Drosophila melanogaster* flies in each treatment group; a total of 15 sections were

analysed from each group; NS- none significant.

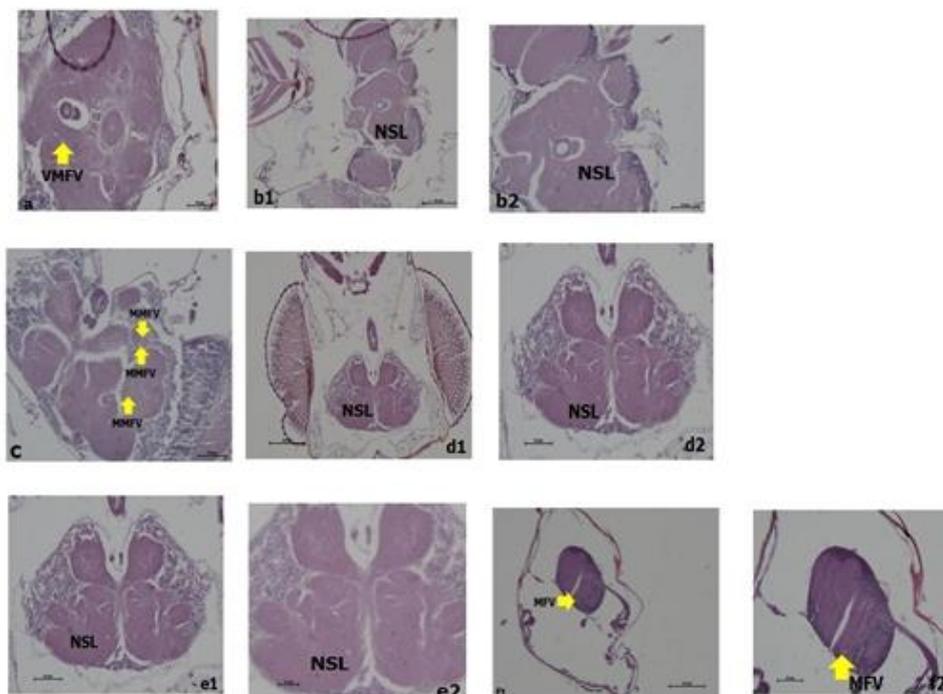


Figure 2. H and E micrographs (TS) from brain of GMR-GAL4 (a, b1 and b2), UAS-para (c, d1 and d2) and GMR-GAL4>UAS-para (e1, e2, f1 and f2) flies that got 0.0g/ml of *Imperata cylindrica* extract.

This data was from five *Drosophila melanogaster* flies in each treatment group. Three brain sections were obtained from each of those flies. Yellow arrow displays the focus of neurodegeneration. (a) shows very mild (small, non-prominent) focal vacuolation (VMFV) in the neuropil in 33% of flies and 67% with NSL x40 (spherical) after 1 hour of feeding; findings are similar after 2 hours of feeding but with 40% of flies VMFV and 60% with NSL. (b1) shows no pathology (NSL in 47% of flies), x20 and (b2) is a higher magnification showing no pathology (NSL) in the brain, x40 after 3 hours of feeding; in GMR-GAL4 flies; 53% of the rest had mild focal vacuolations (MFV) (c) Shows mild (small, non-prominent) multi-focal vacuolations (MMFV) in the neuropil at x40 after 1 hour of feeding. (d1) shows no pathology (NSL), x20 after 2 hours of feeding and (d2) is a higher magnification showing no pathology (NSL) in the brain, x40 after 2 hours of feeding; histological appearance is similar to that after 3 hours of feeding; in UAS-para flies (e1) shows no pathology in the neuropil (NSL) at x40 after 1 hour of feeding; histological appearance is similar to that after 3 hours of feeding (e2) is a higher magnification showing no

pathology (NSL) in the brain, x60 after 1 hour of feeding; histological appearance is similar to that after 3 hours of feeding. (f1) shows mild (small, non-prominent) focal vacuolation (MFV) in the peripheral neuropil, x20 after 2 hours of feeding (f2) is a higher magnification showing mild focal vacuolations (MFV) in the peripheral neuropil, x40 after 2 hours of feeding; in GMR-GAL4>UAS-para flies. In acute (single) exposure treatments, subjecting the fly models to 0.8g/ml of the extract caused different forms of general brain histomorphology (brain neurodegeneration) in the three strains with respect to time of exposure; in GMR-GAL4 flies (do not overexpress paralytic gene) there was very mild focal vacuolation after 1 or 2 hours of exposure then mild multifocal clear vacuolations in the neuropil after 3 hours of exposure to the extract; all these morphological defects are represented as none significant and mild brain neurodegeneration. In acute (single) exposure treatments, treatment GMR-GAL4 flies with 0.3mg/ml of sodium valproate led to observations similar to those seen in treatment of the flies with 0.8g/ml of the extract.

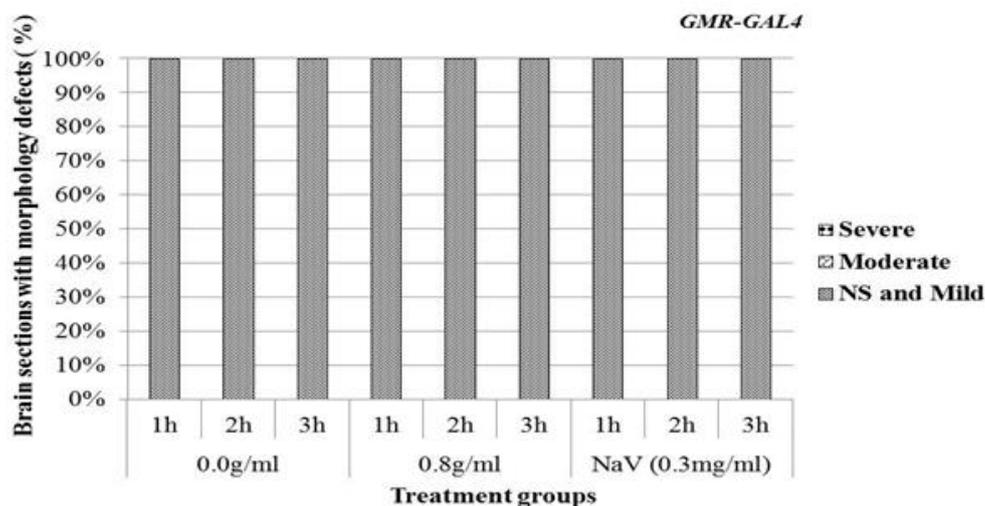


Figure 3. Brain sections showing general morphological defects of GMR-GAL4 flies in various treatment groups.

This data was from five *Drosophila melanogaster* flies in each treatment group; a total of 15 sections were analysed from each group; NS- none significant. In UAS-para flies (do not overexpress paralytic gene) we observed

mild multifocal vacuolations after 1 and 2 hours of exposure to extract; these then became mild focal vacuolation in the brain after 3 hours of treatment with the extract; these are all grouped together as none significant and mild brain

neurodegeneration. In acute (single) exposure treatments, treatment UAS-para flies with 0.3mg/ml of sodium valproate

led to observations similar to those seen in treatment of the flies with 0.8g/ml of the extract.

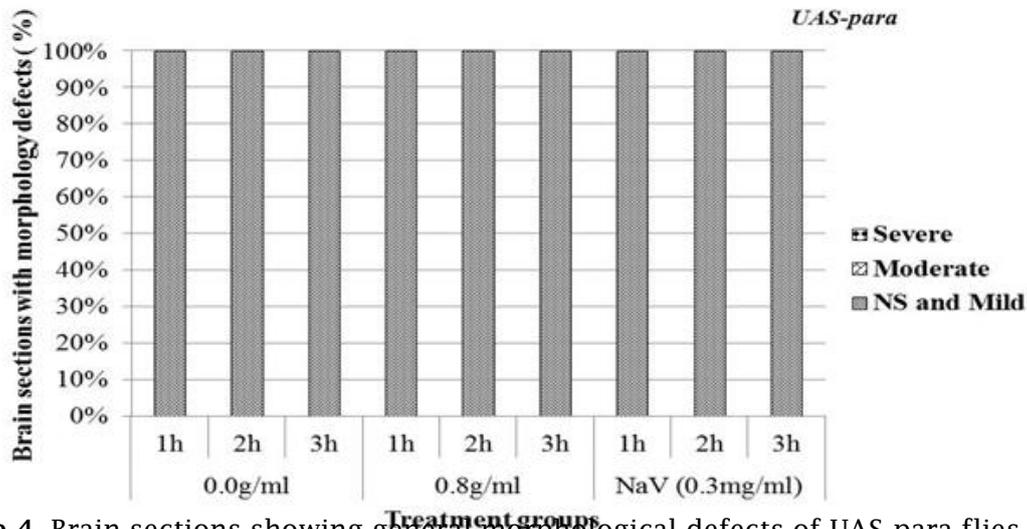


Figure 4. Brain sections showing general morphological defects of UAS-para flies in various treatment groups.

This data was from five *Drosophila melanogaster* flies in each treatment group; a total of 15 sections were analysed from each group; NS- none significant. In the flies overexpressing paralytic gene, exposure to the extract for 1, 2 and then 3 hours were observed to cause moderate multifocal vacuolations in the neuropil (moderate brain neurodegeneration). Exposure of flies that overexpress paralytic gene to the standard anti-epileptic drug

(0.3mg/ml of sodium valproate) was noted to cause moderate multifocal vacuolations (moderate brain neurodegeneration) in 47% of the flies and severe multifocal vacuolations (severe brain neurodegeneration) in 53% of the flies at 2 hours; then moderate multifocal vacuolations (moderate brain neurodegeneration) after 3 hours of treatment with the standard antiepileptic drug.

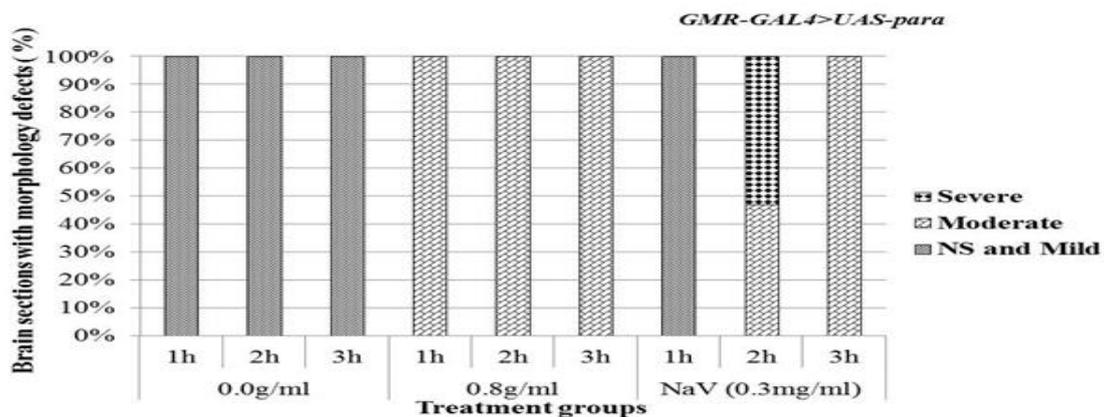


Figure 5. Brain sections showing general morphological defects of GMR-GAL4>UAS-para flies in various treatment groups.

This data was from five *Drosophila melanogaster* flies in each treatment group; a total of 15 sections were

analysed from each group; NS- none significant.

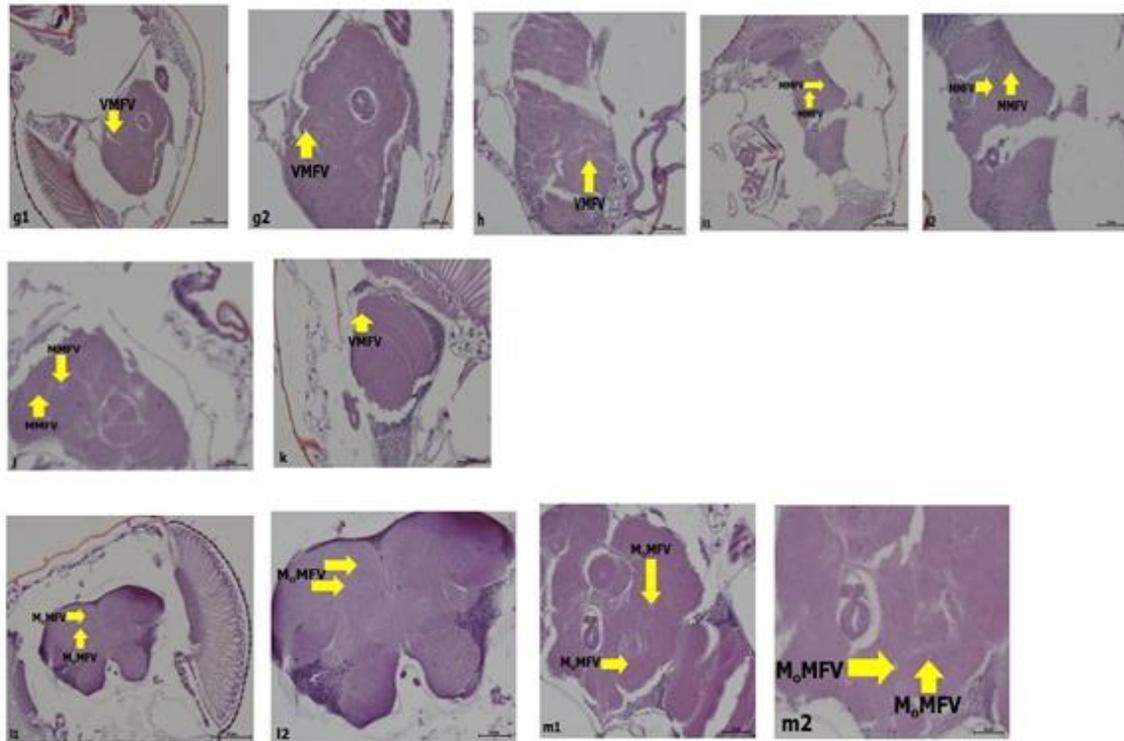


Figure 6: H and E micrographs (TS) from brain of GMR-GAL4 (g1, g2, h, i1, 12), UASpara (j and k) and GMR-GAL4>UAS-para (i1, i2, m1 and m2) flies that got 0.8g/ml of *Imperata cylindrica* extract.

This data was from five *Drosophila melanogaster* flies in each treatment group. Three brain sections were obtained from each of those flies. Yellow arrows display various focal points of neurodegeneration. (g1) shows very mild (very small) focal vacuolation (VMFV) x20 after 1 hour of feeding (g2) is a higher magnification showing very mild focal vacuolation (VMFV) in the brain, x40 after 1 hour of feeding. (h) Shows very mild (very small, non-prominent) focal vacuolation (VMFV), x40 after 2 hours of feeding. (i1) shows mild (small) multifocal vacuolations (MMFV) in the neuropil, x20 after 3 hours of feeding (i2) is a higher magnification showing mild multifocal vacuolations (MMFV) in the neuropil, x40 after 3 hours of feeding; in GMR-GAL4 flies. (j) shows mild multifocal (small, non-prominent) vacuolations (MMFV), x40 after 1 hour of feeding;

histological appearance is similar to that after 2 hours of feeding (k) Shows very mild (very small, non-prominent) focal vacuolation (VMFV)- not significant, x40 after 3 hours of feeding; in UAS-para flies. (l1) moderate multifocal (large, prominent) vacuolations (MoMFV) in the neuropil x20 after 1 hour of feeding; histological appearance is similar to that after 2 hours of feeding. (l2) is a higher magnification showing moderate multifocal vacuolations (MoMFV) in the neuropil, x40 after 1 hour of feeding; findings are similar to that after 2 hours of feeding. (m1) shows moderate (large, prominent) multifocal vacuolations (MoMFV), x40 after 3 hours of feeding (m2) shows a higher magnification with moderate multifocal vacuolations (MoMFV), x60 after 3 hours of feeding; in GMR-GAL4>UAS-para flies.

DISCUSSION

General Brain Tissue Morphology

In acute exposure treatments, the flies that do not overexpress the para gene and those that overexpress the para gene (GMR-GAL4>UAS-para; glass multiple

reporter GAL4>Upstream. Activating Sequences-para) had similar levels of general brain tissue morphology (none significant and mild neurodegeneration). Therefore, overexpression of the para

gene did not lead to significant brain histopathology in acute phases of the study. In chronic exposure treatments, the flies that do not overexpress the para gene showed none significant and mild brain neurodegeneration whereas those that overexpress the para gene (GMR-GAL4>UAS-para; glass multiple reporter-GAL4>Upstream Activating Sequences-para) showed moderate forms of brain neurodegeneration after 6 and 12 days which then worsened to the severe forms of neurodegeneration after 18 days. Thus overexpression of the para gene leads to abnormalities in general brain morphology (neurodegeneration) which increases with time and age of the flies as suggested previously in parabss models for epilepsy [8]. The observed cellular brain defects were found as being associated with physiological neuropathological features such as cognitive impairment and abnormal muscular activity in parabss flies [9; 1] an observation that was also found to be true in our current study. It is also interesting to note that the progressive brain neurodegenerative defects seen in our transgenic flies is in concurrence with the results of our study where the defects in muscular activity and in learning and memory of the transgenic flies were observed to be aggravated with increasing time and age of the flies. However, some findings from our study are in contradiction with those from [8], where they also found moderate and severe forms of neurodegeneration in brain sections of 5-day old parabss mutant flies which was not the case in our study where we used transgenic flies overexpressing paralytic gene. This could be an indicator that the effect of the paralytic gene brings about severe defects in the established model (parabss) as compared to the transgenic flies overexpressing the para gene. This opinion is in line with the preliminary work of our study where we found that the transgenic flies used in the study had 70-75% seizure values compared to parabss. The methanolic extract of *Imperata cylindrica* (0.8g/ml) and sodium valproate (0.3mg/ml) are not toxic to brain morphology in flies that do not overexpress paralytic gene but are toxic to brain morphology of flies overexpressing paralytic gene in acute

exposure treatments. This could be due to presence of intact homeostatic mechanisms owing to the cytochrome enzymes in the normal flies [10] or it could be due to presence of normal cell polarity in and asymmetric cell division in brain tissue which make these cells able to respond to the processes that regulate proliferation and degeneration in the brain tissue of the normal flies but not in the transgenic flies [11]. It could also suggest that overexpression of paralytic gene might be associated with a deficiency in homeostatic control mechanisms as occurs in some forms of mutant flies [12]. In the transgenic flies overexpressing the para gene, treatment with the extract and sodium valproate for a few hours does not improve brain cellular morphological defects (neurodegeneration) but was instead toxic yet this same treatment was associated with improvement in muscular activity in dorso-longitudinal muscles (DLMs) and ameliorated the cognitive defects. This suggests that the extract could be relieving symptoms but not curative in acute phases of the treatment. In general, treatment of the normal flies that do not overexpress the para gene with 0.025g/ml of the extract and 0.15mg/ml of sodium valproate are not toxic in brain tissue morphology in prolonged exposure treatments a phenomenon similar to that seen in acute exposure treatments. However, both the extract and sodium valproate were toxic in brain tissue morphology after 18 days of treating GMR-GAL4 flies. The form of toxicity seen here was lipofuscinosis a pigment which indicates wear and tear of brain tissue due to aging [13; 14]. Whether it is possible for prolonged use of *I. cylindrica* extract and the standard antiepileptic drug to cause the brain of this strain of wild-type flies to age faster than in the other strains remains a mystery. This finding needs to be investigated further before we recommend the use of the herb as an antiepileptic molecule. *Imperata cylindrica* and sodium valproate rescued the progressive brain neurodegeneration seen in older flies in prolonged exposure treatments of the transgenic flies. This suggests presence of neuroprotective (curative) compounds in the extract and sodium valproate that

reversed the brain neurodegeneration in flies overexpressing para gene after prolonged treatments. This finding concurs with a study to determine the effect of *I. cylindrica*, where the methanolic extract from rhizomes of the herb showed extensive neuro-protective (curative) activity against glutamate-induced neurotoxicity in cultures of rat cerebral cortex cells [15]. In addition, *I. cylindrica* and sodium valproate have been depicted to have neuroprotective potential in *Drosophila* models of seizures [16]. Prolonged treatment of the transgenic flies with sodium valproate was at first curative to the abnormal brain cellular neurodegeneration after 6 and 12 days of treatment; but treatment with sodium valproate for a further 18 days caused brain cellular neurodegeneration to reappear. The neurodegenerative effect of sodium valproate witnessed in the current study concurs with most studies in mammalian and fly models which suggest that prolonged exposure to standard antiepileptic drugs including sodium valproate predispose the individuals to further brain histomorphological defects in mutant epileptic mice and fly models, creates behavioral and cognitive challenges plus alteration of neurochemistry, and reduction of brain weight [17; 18]. However, prolonged use of sodium valproate caused

neurodegeneration but neither cognitive nor muscular activity defects in our study and the reason for these toxicity findings need further investigation. This ability to ameliorate brain tissue neurodegeneration in prolonged exposure treatments was also associated with improvement of muscular activity, amelioration in learning and memory after treating the transgenic flies with the extract and sodium valproate for several days as was determined in our study. As already suggested, this is an indicator of curative potential of the extract and NaV on the cellular defects in the flies' brains as was proposed in the hypothesis of this study. This curative potential of the herb could be one factor that led to the improvement in life-span and frequency in resting membrane potential in muscles and might have caused negative geotaxis of the mutant fly models for seizure (parabss) observed in previous studies by [2]. This anatomical curative potential as a factor in improving neurophysiological defects is derived from presence of brain morphological defects in parabss flies which are associated with impairment in muscular activity as well as defects in cognitive functioning [9;19]; and therefore improvement in the anatomical defects would result in better: muscular activity response as well as learning and memory levels [20].

CONCLUSION

The results of this study show that the transgenic flies overexpressing the para gene have similar levels of muscular activity, show reduction in learning and memory performance and are associated with both progressive neurodegenerative and demyelination defects; therefore these flies reproduce the phenotype of the established *Drosophila melanogaster* model for epilepsy (parabss) and are therefore a model for seizures. *Imperata cylindrica* and sodium valproate ameliorate the progressive

histomorphological defects in flies overexpressing paralytic gene and this cellular curative effect is seen in prolonged exposure treatments where it increases with time. *I. cylindrica* extract seems to be relieving symptoms in acute exposure treatments and curative by improving brain cell biology which in turn ameliorates muscular basal membrane potential, learning and memory defects following prolonged exposure treatments.

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