

Original Research Article

Highly Active Antiretroviral Therapy Increased EFV-HAART increases Mortality, Locomotor Deficits and Reduced Reproductive Capacity in *Drosophila melanogaster*

ABSTRACT

Aims: This study was designed to evaluate the effects of Efavirenz-based highly active antiretroviral therapy (EFV_b-HAART, Efavirenz/Lamivudine/Tenofovir) with emphasis on survival, longevity, climbing ability, and reproductive capacity in *D. melanogaster*.

Methods: *D. melanogaster* (both sexes) 1-4 days old were exposed to different concentrations of EFV_b-HAART (range 10-1200 mg) in the fly food for initial 7 days to determine the LD₅₀, then 5 day fly exposure to 93.11 mg, 46.56 mg, 23.28 mg or 11.64 mg for negative geotaxis assay, and acetylcholinesterase (AChE) activity. Furthermore, 28-day fly survival and longevity were determined. Statistical significance was presumed at P<0.05.

Results: The LD₅₀ of EFV_b-HAART in *D. melanogaster* was 93.11 mg. The HAART exposed flies showed significantly ($P<.001$) increased mortality, significant ($P<.001$) decreased fly eclosion, acetylcholinesterase (AChE) activity and climbing ability compared to unexposed group at all experimental concentrations.

Conclusion: The decreased 28-day survival, longevity, climbing ability and reproductive capacity at all experimental concentrations may be attributable to the deleterious effects of EFV_b-HAART in *D. melanogaster*. Our findings suggest that long term use of EFV_b-HAART by HIV patients may be associated with accelerated aging, decreased life expectancy, quality of life (due to possible neurotoxicity) and reproductive competence, as evidenced by increased mortality, reduced longevity, AChE activity, and 100 % emergence failure respectively in *D. melanogaster*, and may require further study in humans.

We recommend that further research is required to expound the biochemical and molecular toxicodynamics of HAART in *D. melanogaster* with the view of ameliorating same.

KEYWORDS: *Drosophila melanogaster*, acetylcholinesterase, toxicodynamics, neurotoxicity.

1.0 INTRODUCTION

Antiretroviral drugs prevent the replication of human immunodeficiency virus (HIV), thereby slowing down the progression of the infection to acquired immune deficiency syndrome (AIDS)[1]. The world health organization (WHO) recommends the use of combinations of different classes of antiretroviral agents. These combined antiretroviral therapies (cART) called highly active antiretroviral therapy (HAART) consists of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and a Protease Inhibitor (PI), or a Non-Nucleoside/Nucleotides Reverse Transcriptase Inhibitors (NNRTI) [2,3].

HAART has transformed HIV infection into a manageable chronic disease; however, their inherent potential to cause short-term and long-term adverse effects tends to pose a peculiar health challenge [1,2]. Notably, antiretroviral toxicity has led to compromised compliance and discontinuation of HAART among HIV-infected individuals leading to treatment failure [5, 6]. These antiretroviral drug-related adverse effects may manifest in overt symptoms or initially only as laboratory abnormalities [4]. The spectrum of potential antiretroviral drug toxicity is broad, including renal toxicity, mitochondrial and metabolic effects, gastrointestinal symptoms, cardiovascular effects, hypersensitivity, skin reactions, insomnia, or other neuropsychiatric manifestations, and many other complications [2,3]. The antiretroviral

drugs inhibits several viral biochemical pathways such as nucleoside reverse transcriptase (NRT) and toxic to many biomolecules such as mitochondria that play an essential role in energy production in the form of adenosine triphosphate (ATP) [4,5]. Mitochondrial toxicity caused by Nucleotide Reverse Transcriptase Inhibitors (NRTIs) triggers a wide range of adverse effects, such as oxidative stress, lactic acidosis, myopathy, cardiomyopathy, peripheral neuropathy, pancreatitis, and possibly lipodystrophy syndrome [6,7].

Besides the overproduction of reactive oxygen species (ROS) seen in *in vitro* cell cultures exposed to antiretroviral drugs [12,13,14,15], *in vivo* human studies have confirmed that antiretroviral drug exposure results to varying degrees of oxidative damage [14] as evidenced by increase in lipid peroxidation [16], decreased catalase activity [17], decreased superoxide dismutase (SOD) activity [18], decreased thiol contents [18,15], increased sperm nuclear fragmentation rate [19, 20] and reduction in number of ovarian follicles [19]. Furthermore, *in vivo* studies in mice showed that exposure to cART significantly decreased sperm motility and viability, testicular glutathione, catalase, and superoxide dismutase but increases pro-oxidant like thiobarbituric acid reactive substance (TBARS) levels [21]. In view of the foregoing, the assessment of long term effects of antiretroviral therapy has garnered increasing attention [2].

(Your article is on Efavirenz. Stay focused on Efavirenz, Lamivudine and Tenofovir and not all of the ART drugs)

Drosophila melanogaster, a dynamic animal model, has carved a niche for itself in the field of biomedical research. Brief, the *Drosophila* model is treasured in human disease modeling, drug target, toxicological biomarker, as well as in the screening of therapeutic agents as drug candidates [10, 11]. The Flies are easy to maintain and propagate in the laboratory on simple fly food medium containing cornmeal, yeast, and antifungal agents. They grow and breed between 22-25°C, with a generation time of 12-14 days at this temperature. Their life-cycle consists of four developmental stages: embryo, larva, pupa and adult, which are all used in toxicology models [12,10].

This work aims at assessing the toxic effects (LD₅₀, Survival, Longevity, Climbing ability, and Reproductive capacity) of the fixed-dose Efavirenz based highly active antiretroviral therapy [EFV_b-HAART, Efavirenz EFV + Lamivudine 3TC + Tenofovir TDF] in *D. melanogaster* for the first time.

2.0 MATERIALS AND METHODS

2.1 Chemicals and Antiretroviral Drugs

The chemicals used were all of the analytical grades. Distilled water (CAS: 7732-18- 5), Randox Protein kit, 5'5' dithiobis(2-nitro benzoic acid) (DTNB), and acetylthiocholine iodide were sourced through Africa

Centre of Excellence in Phytomedicine Research and Development (ACEPRD), University of Jos, Plateau State, Nigeria.

The HAART [EFV 600 mg (CAS 154598-52-4) + TDF 300 mg (CAS 202138-50-9) + 3TC 300 mg (CAS 134678-17-4) mg per tablet, Batch number 3075041, NAFDAC number A4-5090, expiry date October 2020 and manufacture: Gilead Sciences Inc] used in this study was donated by the General Hospital Gboko, Benue State Nigeria. A total of thirty (30) tablets of the fixed-dose formulation were weighed to determine the average weight per tablet. The tablets were pulverized using porcelain mortar and pestle. The appropriate quantity of powder that will contain the desired amount of active ingredient were calculated and weighed using analytical balance (Mettler Model No. MT-200B), and freshly diluted with 1000 μ L distilled water before incorporation into cold fly diet. In all experiments, 1000 μ L distilled water was used as the negative control.

2.2 Animal Model

Wild *D. melanogaster* (Harwich strain) was obtained from the Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD), University of Jos, Jos, Nigeria. The fly stock was maintained at constant temperature and humidity (23 ± 1 °C; 60 % relative humidity, respectively) under 12 h dark/light cycles. The flies were fed on standard *Drosophila* medium composed of cornmeal (1% w/v), brewer's yeast (2% w/v), agar, and methylparaben (0.08% w/v).

2.3 Determination of 168 hrs LC₅₀

The determination of LC₅₀ was carried out following the methods described by (write out names in full, with year) [23] and [25], with slight modification. Sixty (60) flies of age range 1- 4 days were anesthetized under ice, counted and exposed to series of graded concentrations of EFV_b-HAART 10 mg, 20 mg, 40 mg, 50 mg, 100 mg, 150 mg, 200 mg, 400 mg, 800 mg, 1200 mg or 1000 μ L distilled water (as control) each per 10 g fly food respectively for 168 hrs (7 days). Mortality reading was scored every 24 hrs interval during this period. The mortality rate was subjected to dose-response simulation using Graphpad prism 7.04 for LC₅₀ determination. In all the experiments, HAART concentrations equivalent to 100 %, 50 %, 25 %, or 12.5 % of LD₅₀ were used for *D. melanogaster* exposure.

2.4 Survival and longevity studies of *D. melanogaster* Exposed to HAART

In this experiment, sixty (60) flies of both genders (1-4 days old) were exposed to 11.64 mg (12.5 % LD₅₀), 23.28 mg (25 % LD₅₀), 46.56 mg (50 % LD₅₀) or 93.11 mg (100 % LD₅₀ of EFV_b-HAART each per 10 g food in five replicates for 28 days as described by Abolaji *et al.*, [26]. The number of live and dead

flies was scored daily till the end of the experiment, and the survival rate was expressed as a percentage of live flies. For longevity assay, sixty (60) flies each were dosed with the concentrations as indicated above in five replicates for a lifetime, as described by Abolaji *et al.*, [26].

2.5 Five-Day Treatment for Negative geotaxis, Reproductive ability and AChE activity

From 28 day survival curves, taking a day with more than 70 % survival of EFV_b- HAART exposed flies; Five-day survival assay was conducted to assess short term effect of the cART on climbing performance, reproductive ability, and acetylcholinesterase activity of *D. melanogaster*. Sixty flies, age range 1-4 days old, were exposed to 11.64 mg, 23.28 mg, 46.56 mg or 93.11 mg each per 10 g fly food as described by Abolaji *et al.*, [26].

2.6 Negative Geotaxis (Behavioral Assay)

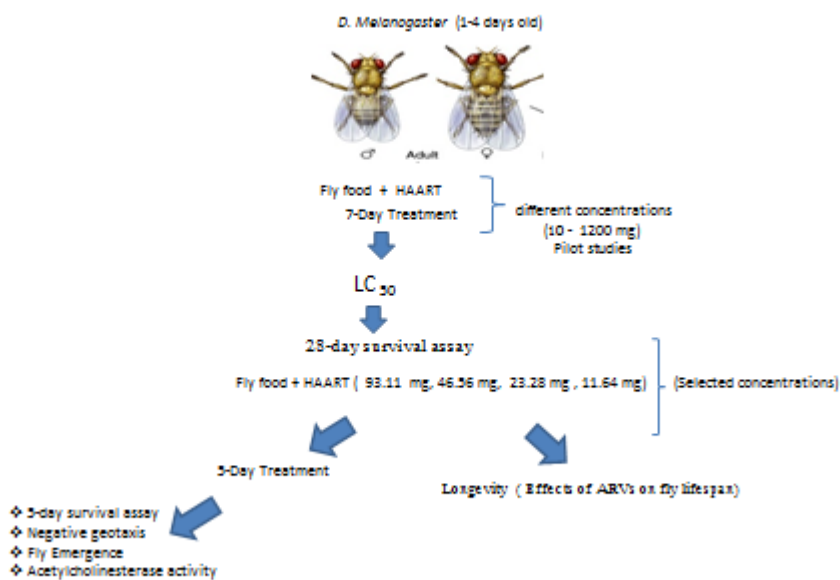
The locomotor (Climbing) performance of EFV_b-HAART exposed, and unexposed flies were investigated using the negative geotaxis assay [26]. Briefly, ten (10) cART exposed and unexposed flies were immobilized under mild ice anesthesia and placed separately in labeled vertical glass columns (length, 15 cm; diameter, 1.5 cm). After the recovery period (about 20 min), the flies were gently tapped to the bottom of the column. Following 6 s, the numbers of flies that climbed up to the 6 cm mark of the column, as well as those that remain below this mark were recorded. Data were expressed as the percentage of flies that escaped beyond the 6 cm mark in 6 s. The score of each group is an average of three trials for each group of treated and controlled flies

2.7 Reproductive Ability

The fertility of the flies, after exposure to EFV_b-HAART, was assessed using the reproductive ability assay [25] with slight modifications. Briefly, virgin flies (both sexes) were isolated (within 8 hours after eclosion) from their normal fly food, and treated with series of cART concentrations (11.64 mg, 23.28 mg, 46.56 mg or 93.11 mg of HAART each per 10 g fly food) for five days. Thereafter, the treated flies were pair mated in vials containing normal food using three different strategies for each treatment group. Five (5) pairs of flies were taken in each treatment group as follows: (1) HAART exposed males were paired with unexposed females, (2) Unexposed males were paired with HAART exposed females, and (3) Exposed male with exposed female. All the flies in each treatment group were transferred into fresh vials with normal food for 24 hours, and the number of eggs laid in each vial during this period was kept for 14 days for the emergence of adult flies. The mean number of flies emerged gives a measure of reproductive ability.

2.8 Acetylcholinesterase (AChE) activity

The five days EFV_b-HAART treated flies were anesthetized on ice, homogenized in 1:10 volumes 100 mM phosphate buffer saline (pH 7.4), and centrifuged using cold centrifuge (Eppendorf AG, 5227 R, Germany) at 4 °C for 10 min at 4000 rpm. The supernatant was collected and used for the determination of AChE activity following the method described by Ellman *et al.* [27] with slight modification. To the reaction mixture containing 285 µl of distilled water, 180 µl of 100 mM potassium phosphate buffer (pH 7.4), 60 µl of 10 mM DTNB, and 15 µl of sample, 60 µl of 8 mM acetylthiocholine was added. The change in absorbance was monitored at 412 nm for 2min at 10 s intervals, using a UV-VIS Spectrophotometer (Jenway 7315). The protein concentration of the whole fly homogenates was determined using total protein kit (Randox) according to the manufacturer's instructions. The data was calculated against blank and sample blank, and the results were corrected by the protein content. The enzyme activity was expressed as micromole/min/mg of protein.



Scheme 1. Summary of Experimental Design

2.9 Statistical Analysis

The data were expressed as mean±SEM (standard error of mean), and the statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's posthoc test to identify statistically different test groups. Survival curves were analyzed using the Log-rank (Mantel-cox), and Gehan-Breslow-Wilcoxon tests with the application of the Bonferroni corrected threshold for multiple curve

comparison (Graphpad Prism statistical software version 7.04). The results were considered statistically significant at $p < 0.05$.

3.0 RESULTS

3.1 168 hours LC_{50}

To select the concentrations of cART to be used in the main experiment, we exposed flies to a series of 10 different concentrations of EFV_b-HAART (10 mg -1200 mg) for 168 hours (7 days). The mortality rate of the exposed *D. melanogaster* for 168 hours showed 100 % mortality at 400 mg, 800 mg and 1200 mg /10 g food while there was no mortality recorded in the unexposed group. The 168 hrs LC_{50} was determined to be 93.11 mg as shown in figure 1.

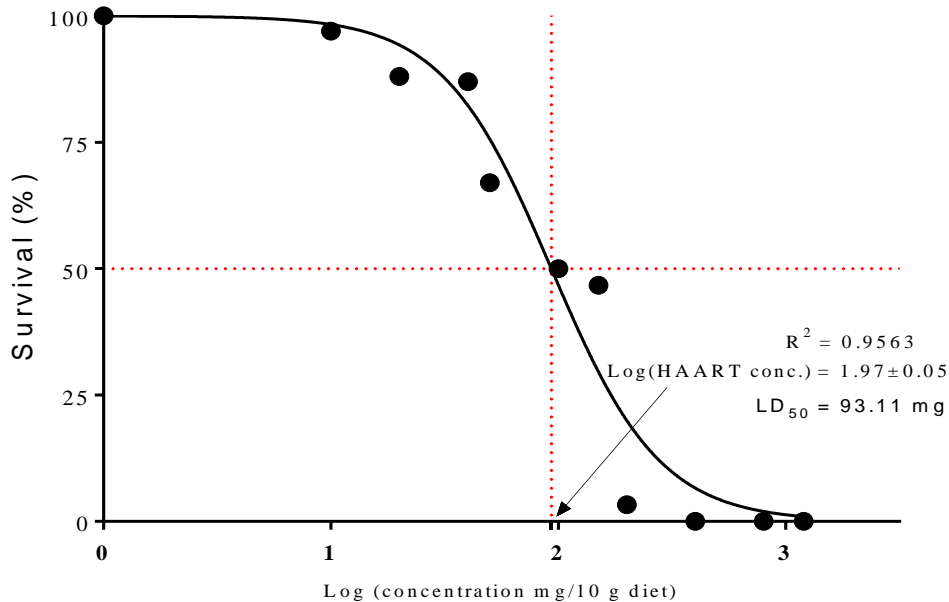


Figure 1. LD_{50} of Efavirenz-based highly active antiretroviral therapy (EFV_b-HAART) in *D. melanogaster*

3.2 Survival and longevity studies of *D. melanogaster* Exposed to HAART

The 28-day survival of EFV_b-HAART exposed *D. melanogaster* revealed a sharp, significant decrease ($p < 0.05$) in the survival proportion in a dose-dependent manner compared to the

control (1000 μ l Distilled H₂O/ 10 g fly food). The median survival time (MS) with corresponding hazard ratios (HR) for the concentrations used was 93.11 mg (MS 11 days, HR 4.95 \pm 0.88), 46.56 mg (MS 14 days, HR 3.37 \pm 0.61), 23.28 mg (MS=19 days, HR =2.74 \pm 0.51), 11.64 mg (MS = 20 days, HR = 2.32 \pm 0.51) (Figures 2A and B). The longevity study also revealed a significant reduction ($p < 0.05$) in the life span of EFV_b-HAART treated *D. melanogaster* compared to the control group. The 93.11 mg/10 g food fed flies survived up to 37 days representing 32.72% reduction in survival of the control group with 55 days survival. The calculated median lifespan (ML) with corresponding HR for the used concentrations and control was 93.11 mg (ML= 11 days, HR = 5.29 \pm 1.23), 46.56 mg (ML = 14 days, HR = 3.07 \pm 0.65), 23.28 mg (ML = 24 days, HR = 2.01 \pm 0.40) 11.64 mg (ML = 29 days, HR = 1.57 \pm 0.31) , and control (ML = 30 days) as shown in figure 3.

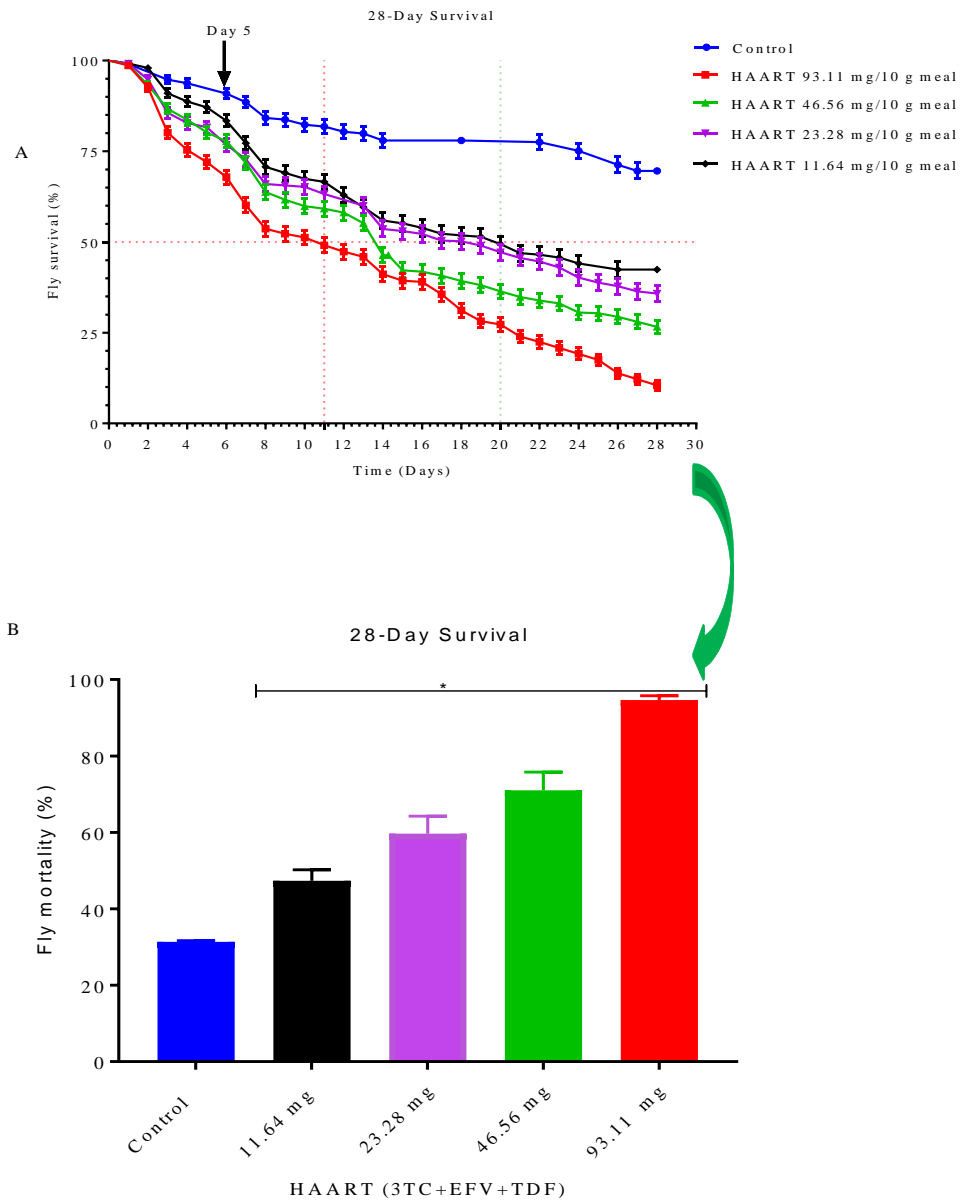


Figure 2. HAART exposure reduced survival rate of *D. melanogaster* after 28 days. (A) Survival curve analysis and (B) Chart of survival (%) of flies (both sexes) after 28 days exposure of *D. melanogaster* to 93.11 mg, 46.56 mg, 23.28 mg and 11.64 mg of HAART. Data are presented as mean±SEM of five independent biological replicates carried out in two separate experiments. *p<0.05 vs control.

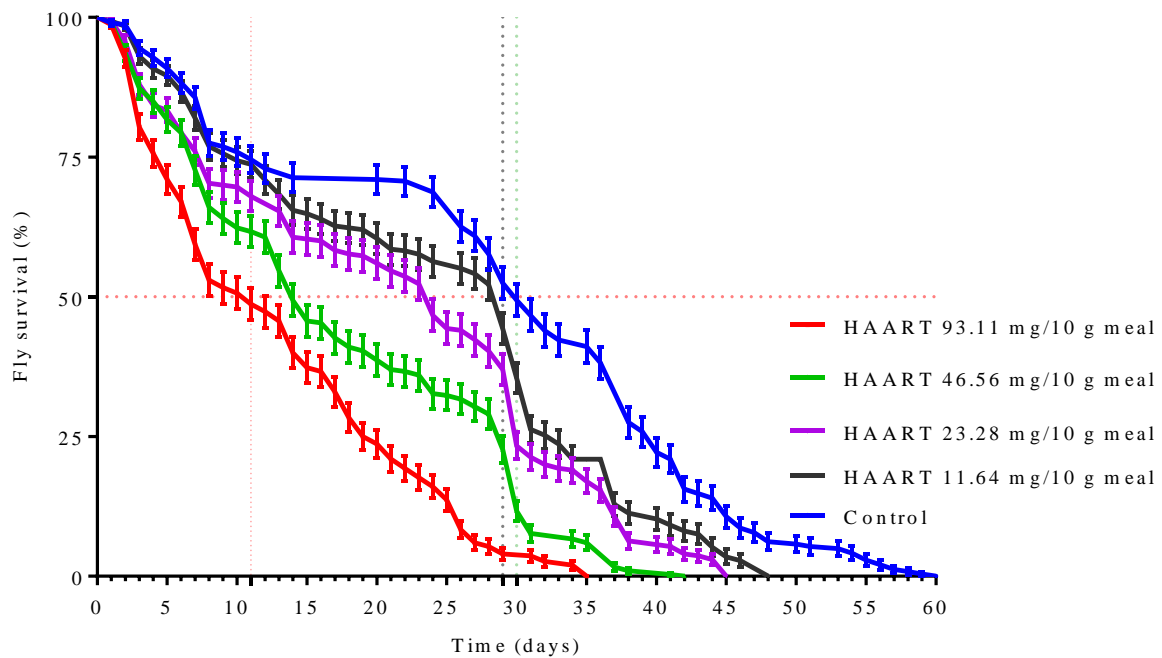
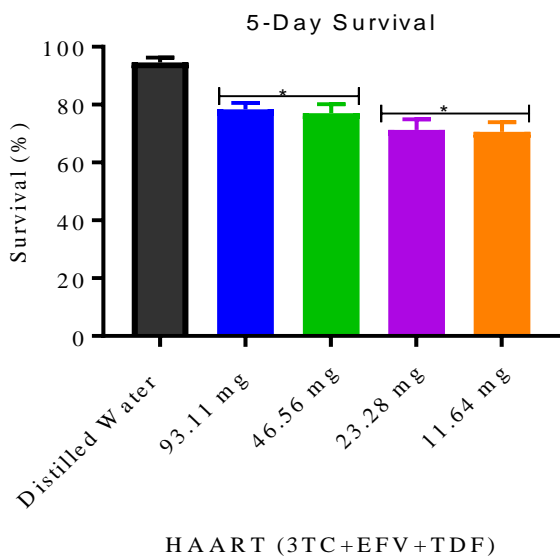


Figure 3. HAART exposure reduced life span of *D. melanogaster* in a dose dependent manner by 41.67% at 93.11 mg/10g meal, 28.33% at 46.56 mg /10 g meal, 25.00% at 23.28 mg /10 g meal, and 18.33% respectively.

3.3 Five-Day Treatment and Negative Geotaxis (Climbing Assay)

The survival of 5-day EFV_b-HAART treated flies revealed a significantly decrease ($p < 0.05$) survival proportion compared to the unexposed group. Similarly, the climbing performance of the treated flies was significantly impaired ($p < 0.05$) compared to the unexposed group (figures 4A and B).

A



B.

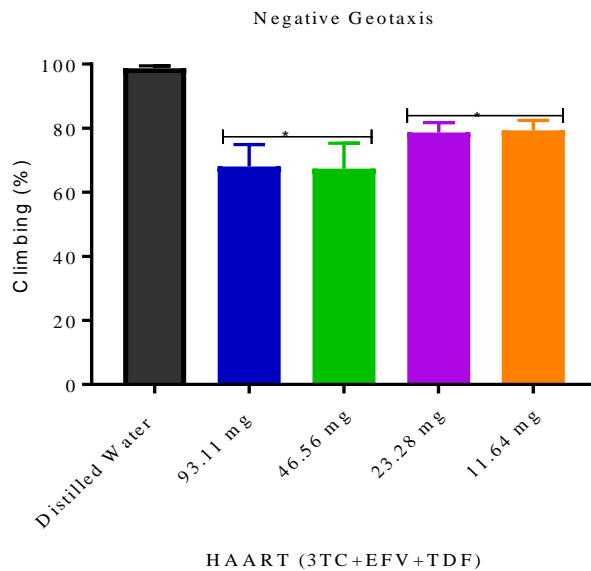


Figure 4. HAART significantly reduced survival and impaired climbing behaviour of *D. melanogaster* after 5 days of exposure. (A) Five days survival rate and (B) Negative geotaxis (% climbing rate) of flies treated with various concentrations of HAART. Data are presented as mean \pm SEM of five independent biological replicates. Each assay was carried out in two independent experiments. * $p < 0.05$ vs control.

3.4 Reproductive Ability and Acetylcholinesterase (AChE) activity

The experiment to assess reproductive ability revealed 100 % significant ($p < 0.05$) emergence failure at all concentrations of EFV_b-HAART exposed flies compared to unexposed group. We observed previously during our pilot studies that EFV_b-HAART naïve *D. melanogaster* flies produced a few larvae that pupated without eclosion in all test groups (data not shown). To further assess if this effect was gender-dependent, we exposed male flies to EFV_b-HAART and mated with unexposed female, exposed female mated with unexposed male and both gender exposed. The result revealed significant ($p < 0.05$) reduction in the groups with exposed male mated with unexposed female as well as exposed female mated with unexposed male. The diminished emergence was significantly ($p = 0.0001$) more pronounced in the group with exposed male while 100 % failure was recorded in the groups with both exposed genders. The result is presented in figures 5A and B. AChE activity of EFV_b-HAART treated flies was significantly reduced ($p < 0.05$) compared to control as shown in figures 6A and B.

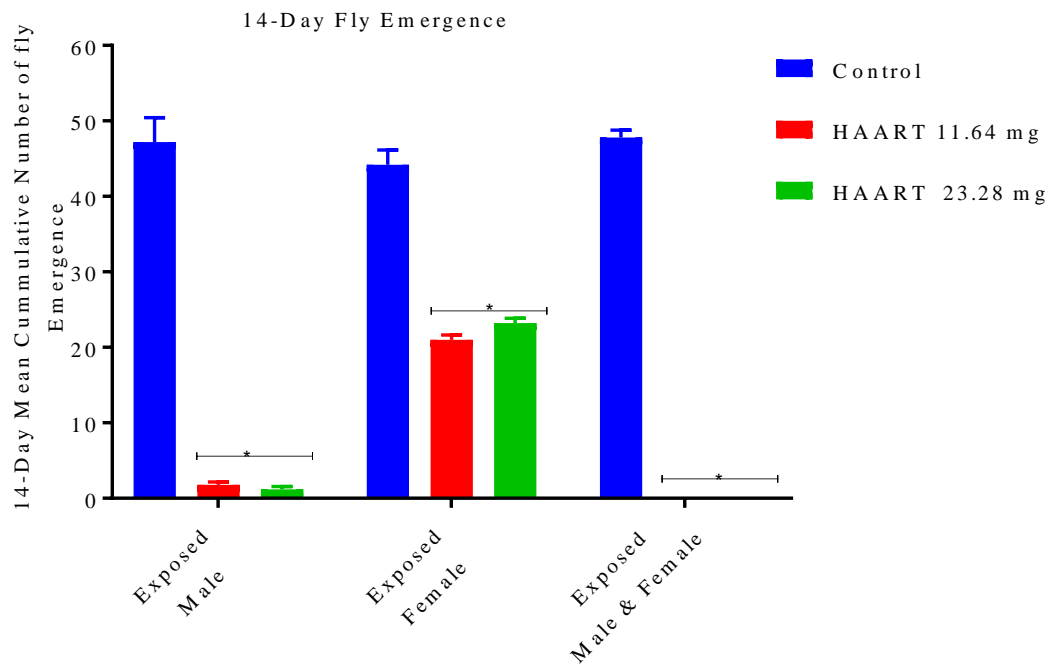
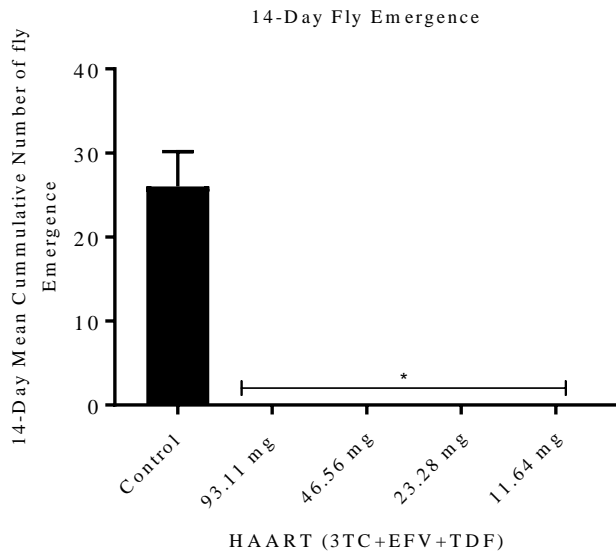


Figure 5. EFV_b-HAART exposure abolished 100% fly emergence while sex dependent exposure significantly ($p < 0.05$) reduced reproductive capacity of virgin *D. melanogaster* after 5 days of exposure. (A) Fly emergence after 5-day treatment of 1-4 day old flies (both sexes) with EFV_b- HAART. (B) Fly emergence after 5-day exposure of either virgin male or female or mixed sexes to EFV_b-HAART respectively. Data are presented as mean \pm SEM of five independent biological replicates of each concentration. Each assay was carried out in two independent experiments. * $p < 0.05$ vs control.

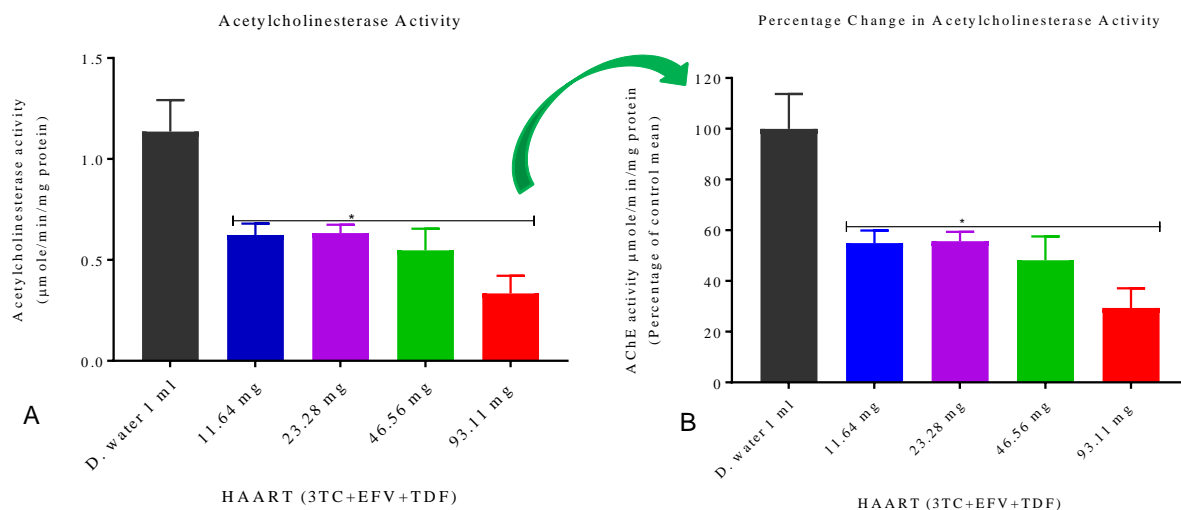


Fig 6. HAART significantly decreased the level of AChE in *D. melanogaster* after 5 days of exposure. (A) AChE levels after treatment of flies with Efavirenz based HAART for 5 days. (B) AChE of HAART treated flies presented as percentage of control mean. Data are presented as mean \pm SME of five independent biological replicates for each drug concentration. * $p < 0.05$ vs control.

4.0 DISCUSSION

HIV infection has created significant global health challenges, notably the emergence of resistant viral strains and the adverse side effects associated with prolonged use of combination antiviral therapy such as Efavirenz based HAART [2,14]. The purpose of this study was to evaluate the impact of Efavirenz-based HAART on survival, longevity, climbing ability and reproductive capacity in *D. melanogaster*.

Medications for the treatment of HIV may cause multiple toxicities such as hepatotoxicity [4], as well as central and peripheral nervous systems inflammation [28]. Nevirapine and Efavirenz have been shown to cross the blood-brain barrier and cause significant inhibition in the activity of creatine kinase, which plays a vital role in imparting cell energy homeostasis in the brain [29]. Generally, one of the theories propounded to explain antiretroviral drug toxicities partly involves mitochondria oxidative stress induction [21, 22], causing accelerated aging and lifespan reduction [31].

The LD₅₀ of EFV_b-HAART in *D. melanogaster* was determined to be 93.11 mg/10 g fly food, implying it is toxic to the flies. The sharp, significant reduction ($p < 0.05$) in the survival and longevity of the exposed flies in this study confirms the toxicity of EFV_b-HAART. It is pertinent to observed that, the reduced mean survival time on the survival curve ranged 11-20 days compared to the unexposed group whose mean survival was not defined within the experimental period of 28 days. The corresponding hazard ratios of the exposed groups ranged between 2.32 \pm 0.51 - 4.95 \pm 0.88. furthermore, the longevity curve showed a similar trend of toxicity with a reduced median lifespan ranged 11-29 days compared to the median lifespan of 30 days and maximum life span of 59 days of unexposed group. Since the increased mortality

and risk was consistently observed in all the cART exposed groups we assumed the drug is the culprit. It has also been reported that immune challenged *D. melanogaster* generally have shorter lifespan compared to the unchallenged *D. melanogaster* [32]. Our current findings in cART exposed *D. melanogaster* agrees with the earlier report [31] implicating HAART in accelerated aging and reduced life expectancy in humans.

We also discovered that EFV_b-HAART did not only reduce the life span of *D. melanogaster* but also impaired the climbing performance (negative geotaxis) significantly ($P < 0.05$). The results also revealed a significant decrease ($p < 0.05$) in the activity of AChE of EFV_b-HAART treated flies compared to the control, and this elaborates the positive correlation ($r = 0.74$, $P = 0.015$) between climbing activity and AChE Activity of *D. melanogaster*. The association between AChE activity and negative geotaxis in this study implied that 74% of the cART-induced toxicity, which caused a decreased AChE activity in the exposed *D. melanogaster*, might also explain the observed decrease in locomotor performance. We and other independent reports by Abolaji *et al.*, [26] and Sharma *et al.*, [33] showed that the decrease in AChE activities is directly proportional to decrease in the climbing performance of *D. melanogaster* exposed to toxicants. Pharmacological inhibition of AchE delays acetylcholine metabolism and hence prolongs the exposure of postsynaptic cells to released acetylcholine, and transiently potentiates responses at cholinergic synapses. This ultimately increased excessive post synaptic stimulation leading to blocked nerve conduction and impaired skeletal muscle coordination [34]. Also, since the AChE mutant flies has a short lifespan [34], the decreased AChE activity in our current study may have contributed to the reduced longevity in the EFV_b-HAART exposed *D. melanogaster*. Furthermore, the reduced AChE activity ($p < 0.05$) in the present study may imply a possible neurotoxicity [26] in agreement with the report by Apostolova *et al.*, [14] that Efavirenz (a component of the HAART in this study) may amplify HIV-associated neurocognitive disorder (HAND) or other neuropsychiatric disorders.

Some *in vitro* studies have shown the relationship between acetylcholine and fecundity [21,22]. Generally, the higher the activity of AChE, the lower the concentration of acetylcholine (ACh). It has been reported that elevated activity of acetylcholinesterase is crucial in female fertility by catalyzing the hydrolysis of the high level of acetylcholine in the Uterus protecting pregnancy and promoting productivity [35]. From our result, we recorded 100% emergence failure ($p = 0.0001$) when both genders were simultaneously exposed compared to unexposed group, significant reduction ($p = 0.0002$) in emergence of the group with only female exposed compared to control, and highly pronounced reduction ($p < 0.0001$) in emergence of a group with only male exposed. Similarly, it has been reported that significantly decreased AChE activity correlates positively with increased oxidative stress and acetylcholine concentrations which in turn impacts negatively on both male and female fertility [21-23]. Increased acetylcholine concentration via insecticides induced AChE inhibition, for example, impairs insects' spermatogenesis, sperm motility, and increased oxidative stress induced oocyte damage [35] due to increased segregation errors orchestrated by premature loss of cohesion [38]. Therefore, the observed emergence failure in HAART exposed *D.*

melanogaster in this study might be due to oxidative stress, decrease of AChE ($p < 0.05$), resulting to high concentration of ACh in flies uterus and general toxicity generated by the intake of EFV_b-HAART in their diet.

From the result, we discovered that the male gender reproductive ability was more significantly decreased ($P < 0.05$) than the female counterpart. This may imply that the spermatozoon of *D. melanogaster* is highly sensitive to EFV_b-HAART toxicity hence the more profound reduction in male reproductive competence than the female counterpart.

It is pertinent to note that in some reviews of antiretroviral drugs, most are reported to possess none or statistically insignificant fertility adverse effects [19]. However, combination therapies may result in peculiar toxicities arising from the synergistic interactions of the individual parent drugs or their metabolites, which may not be apparent when same drugs are taken singly [31][19]. Therefore, it is probable that adverse effects not seen on single Efavirenz, Lamivudine or Tenofovir disoproxil fumarate exposure may occur during exposure to the fixed dosed combination in HAART.

(Also discuss beside (alongside) established adverse effects of these drugs, both singly and in combination in HAART, in HIV patients, and the studies that established these adverse effects in such HIV patients)

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

We showed in this study some HAART-induced toxic events that may be potentially **fatal harmful** to humans using the *D. melanogaster* model for the first time. The 32.72% decrease in lifespan and 100% emergence failure in the HAART exposed flies is particularly worrisome. The impaired locomotor deficits and decreased acetylcholinesterase activity in *D. melanogaster* may also impact negatively on the quality of life on patients taking cART for the treatment of HIV due to possible neurotoxic consequences.

5.2 Recommendation

These findings stress the need for further research **to confirm these adverse-effects in HIV-patients by appropriate human-based studies, and** to elucidate the biochemical and molecular toxicodynamics of HAART in *D. melanogaster* with the view of ameliorating these toxicities.

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(Pay attention to style of listing References as recommended under ‘Authors’ guidelines’ at this Journal’s website)

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