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Afr. J. Biomed. Res. Vol. 23 (September, 2020); 397- 406

Research Article

Moringa oleifera Ameliorates Age-Related Memory Decline and Increases Endogenous Antioxidant Response in *Drosophila melanogaster* Exposed to Stress

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ABSTRACT

Age-related dementia has been estimated to double every 20 years. Despite the nutritional value of *Moringa oleifera* Lam., its effect on age-related memory decline and its anti-ageing bioactive fractions are yet to be fully elucidated. In the present study, the crude methanol leaves extract of *Moringa oleifera* Lam. (MEMO) and its different fractions were screened for anti-ageing bioactivity in the fruit fly, *Drosophila melanogaster* w^{1118} whiteflies. A day-old virgin flies were reared in food supplemented with different concentrations of the methanol leaves extract of *Moringa oleifera* at 1, 2, and 5mg/ml respectively. A sequential bioassay-guided fractionation approach was subsequently adopted. Treated flies were subjected to lifespan, reproductive performance, climbing, memory and oxidative stress resistance assays according to established protocols. There was a dose-dependent effect of the leaves extract on lifespan, reproductive performance, climbing activity and oxidative stress resistance as age progressed ($P < 0.05$). Mean survival times of treated male and female flies increased by 12.3±1.2, 15.5±1.0 and 26.6±0.4%, and 12.0±1.1, 17.5±0.8 and 24.3±0.8% respectively, relative to the control. There was a significant increase in memory scores of the 50-day old aged flies in the 5mg/ml group $P < 0.0001$. The *Moringa* Ethyl acetate (EA) fraction F2 gave the highest anti-ageing bioactivity in the flies ($P < 0.05$). The leaves extract showed an ameliorative effect on age-related memory decline and an increase in oxidative stress resistance in *Drosophila melanogaster* w^{1118} white flies without the commonly found adverse effect in reproductive performance.

Keywords: *Moringa oleifera Lam; oxidative stress resistance; age-related memory; anti-ageing; Drosophila melanogaster; antioxidant.*

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Received: November, 2019; Accepted: June, 2020

Abstracted by:

Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

INTRODUCTION

Ageing is a continuous deterioration in cellular function owing to amassed free radical-induced damage to macromolecules (Lopez-Otin *et al.*, 2013). It is accompanied by an increased loss of functionality and fragility (Ferrucci and Zampino, 2020). The highest rate of accelerated ageing is occurring in the least developed countries of the world as a result of socioeconomic stress factors (Nagarajan, Teixeira and Silva, 2020). Age-related dementia has also been anticipated to double, every-20 years. This situation will increase to about 115million by the year 2050 (Yasamy *et al.*, 2013).

The free radical theory of ageing (FRTA) proposed that accumulation of free radical damage to cellular macromolecules is the major fundamental influence of ageing and the major determinant of lifespan (Harman, 1956, 1968). A general phenomenon associated with ageing is the

accumulative damage caused by reactive oxygen species (ROS) production in the mitochondria (Ziegler, Wiley and Velarde, 2015). Being a complex biological process, it is influenced by many factors. However, it is flexible and greatly influenced by nutrition (Lee *et al.*, 2015).

Natural antioxidants are endogenous antioxidant system enhancers, they increase the ability of organisms to scavenge free radicals (Nimse and Pal, 2015), they prevent cellular oxidative stress, in the process mediating oxidant and antioxidant balance (Salehi *et al.*, 2020). *Moringa oleifera* Lam. is one of the most potent antioxidant and nutritious plants on earth (Mahmood, Mugal and Ul Haq, 2010).*Moringa* leaves extract possesses high levels of polyphenols which enhances its antioxidant activity by scavenging free radicals. Plant antioxidants and phytochemicals possess protective effect on the brain during exposure to oxidative stress in a model of *Drosophila melanogaster* (Park *et al.*, 2012).

The fruit fly, *Drosophila melanogaster* was used in the present study because the cellular processes of ageing are remarkably similar to that of mammals, including the processes of age-related memory decline (Kim, 2007). Their brain is able to undergo higher order learning, they are evolutionarily adapted to recall learned events and information. The fruit fly, as a model organism can be used in certain brain function assays (Haddadi *et al.*, 2014). Despite the reported nutritional value of *Moringa*, its anti-ageing bioactive fractions and effect on age-related memory decline are yet to be fully elucidated. Therefore, this study aimed to evaluate the anti-ageing effect of the crude methanol leaves extract of *Moringa oleifera* (MEMO) and its different fractions using *Drosophila melanogaster* as a model organism.

MATERIALS AND METHODS

Reagents: All chemicals and reagents used in this study were of analytical grade from Sigma-Aldrich and Sigma Zanyo: Methanol, 30% hydrogen peroxide, Triton X-100, Catalase from bovine liver (2000-5000 units/mg protein, product no.C9322-5G), Nipagin (methyl-p-hydroxybenzoate), n-Hexane, Chloroform, Ethyl acetate, Acetone, Propionic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), L-Ascorbic acid and Paraquat (PQ) (1,1-dimethyl-4-4-bipyridynium dichloride), 3-Octanol (OCT), 4-methyl cyclohexanol (MCH), paraffin oil, pyrogallol, Thiobarbituric acid, Acetylthiocholine iodide, 5, 5'–dithiobis 2-nitrobenzoic acid (DTNB). Silica gel powder (0.040-0.063mm) for column chromatography, M = 60.08g/mol.

Plant Collection, Authentication and Extraction: Fresh mature leaves of *Moringa oleifera* were harvested from Jinja, Uganda, at a private plot. The plant was identified as *Moringa oleifera* Lam. by Madam Olive Wanyanna of Makerere University, Uganda. Accession number 42223 was allocated to the plant voucher which was deposited at the University herbarium.

The fresh leaves were washed with distilled water and air-dried under a shade and then powdered using an electric mill (Philips). The resulting weight of the powder was 300 g. *Moringa* powder was subjected to cold extraction using 70% v/v Methanol. The extract was sieved using a muslin cloth and filter paper, the filtrate was then concentrated at 40^{o}C using a rotatory evaporator. The extract, a dark-green concentrate, was stored in an airtight container, wrapped in a foil paper, at 4^{o}C for further use. The crude extract obtained was 21.46g (equivalent to a yield of 7.2% w/w). % Yield = (Final weight of crude extract / original weight) x 100).

After testing the crude methanol leaves extract of *Moringa oleifera* (MEMO) on longevity, oxidative stress resistance, climbing activity, reproductive performance, learning and memory, the crude extract was subjected to a successive extraction by different extraction solvents in their increasing order of polarity to obtain five Moringa-extracts. Each obtained extract was dried into concentrated form before the next extraction with subsequent solvent. The longevity effect of the five fractions was evaluated. Ethyl acetate fraction gave the highest bioactivity. Through a column chromatographic technique, Ethyl acetate fraction was subjected to chromatographic portions (Krishnamurthy *et al.*, 2015). The resulting *Moringa*-Ethylacetate fractions (F1-10) were subjected to free radical scavenging activity, the one with the significant activity was tested for oxidative stress resistance in the flies.

Fly stock husbandry: The *Drosophila melanogaster* w^{1118} (*white*) strain was used in the present study. Virgin males were separated from virgin females. Flies were maintained at 25^{o}C under a 12/12hour light and dark cycle in a digital fly incubator (SPX-150A), on a standard culture cornmeal medium, and raised at a standard density of 10 flies per fresh food medium changed twice a week (Kinghorn *et al.*, 2015). Each experiment included five replicas of ten flies each, repeated on three independent occasions. A day-old synchronized w^{1118} virgin flies were treated with food supplemented with 0, 1, 2, and 5mg/ml methanol leaves extract of *Moringa oleifera* (MEMO). The concentrations used in the present study were obtained based on preliminary toxicity investigations. Flies were maintained on standard cornmeal agar media supplemented with the extract at concentrations of 0, 1, 2, and 5mg/ml respectively. Adults that emerged from these culture media were monitored to obtain the age groups; young (5days old), mid-age (25days old) and aged flies (50days old) used in the memory assay.

Toxicity analysis of the leaves extract was evaluated in the flies. Flies were fed with food supplemented with the extracts at the following concentrations; 1, 2, 5, 10, 20, 40, 100, 500, and 1000 mg/ml. The number of live and dead flies were counted and recorded after every 24hrs. The result obtained gave a baseline for choosing the selected extract concentrations used in this research work.

Induction of Oxidative stress: *Hydrogen peroxide Oxidative stress induction***:** Flies were reared in food supplemented with the different concentrations of *Moringa oleifera* for fourteen (14) days. Thereafter, they were starved for 3hrs and placed in food vials containing 5% Hydrogen Peroxide. The flies were then monitored for lifespan (Slack *et al.*, 2010).

*Paraquat induced Oxidative stress***:** Flies were reared in food supplemented with the different concentrations of *Moringa*

oleifera for fourteen (14) days. Thereafter, they were starved for 3 hours, placed in vials containing filter paper which was impregnated with a mixture of 15mM Paraquat and 5% Sucrose, for 3 hours every day. Flies were then monitored for lifespan (Jahromi *et al.*, 2013).

Longevity assay: The rate of survival of treated flies was evaluated by a daily counting of live flies until all the flies died. Log-rank tests was used to evaluate the survival analysis. Percentage (%) increase in mean survival time = (T/C-1) X 100, where T is the mean survival time of treatment group and C is the mean survival time of the control group.

Climbing activity assay: The climbing performance of flies was carried out with the aid of a negative geotaxis assay, in which the percentage of flies that reached the 25cm mark of a 25ml pipette in 45seconds was calculated. The experiment was repeated three times at each time point (Kinghorn *et al.*, 2015). After 24hr of Paraquat exposure, climbing activity was evaluated (Jahromi *et al.*, 2013).

Reproductive performance: The treated flies were made to undergo reproductive performance assay. Females and males (10 each) were left in the experimental food vials to mate for ten days and then removed from the food vials to monitor the eggs laid. The number of offsprings that emerged from the eggs laid were counted and recorded every 24hrs for ten days.

Fly whole-body homogenization for biochemical assays: The flies were manually grounded and homogenized in ice-cold phosphate buffer saline 2 mL (0.1 M, pH 7.4) using a mini-pestle and tube. They were centrifuged at 2,500g for 10 min at 4^{o}C and the supernatant was filtered. The resulting homogenate was kept at 4^{o}C for the biochemical assay.

Head homogenization for brain chemistry analysis
The flies were placed on ice to deactivate them. Fly heads were decapitated and placed on ice. They were immediately homogenized in ice-cold 1 ml sodium-phosphate buffer (0.1 M, pH 8.0). The homogenates were centrifuged at 2,500g for 10 min at 4^{o}C, the resulting supernatant was filtered through a mesh, and the resulting sample homogenate was used for biochemical assays.

Catalase activity : Catalase (CAT; EC 1.11.1.6) activity was determined according to the method of Aebi, (Aebi, 1984), by monitoring the clearance of H_2O_2 at 25^{o}C at 240nm, in a reaction mixture containing 1800µL of 50mM Phosphate buffer (pH 7.0), 180µL of 300mM H_2O_2, 20µL sample homogenate. The reaction was monitored for 2min (10s intervals) by taking absorbance at 240nm using a UV-visible spectrophotometer. Catalase activity (expressed as mM of H_2O_2 consumed /min/mg of protein) = (ΔA) / (ε.d.t.c), where ε = 39.4 M-1 cm-1 (H_2O_2 extinction coefficient), d = 1 cm path length, t = 2 min (incubation time), c = protein content in the test sample (mg).

Superoxide dismutase (SOD) activity: Superoxide dismutase activity was determined by the earlier described method (Marklund and Marklund, 1974), by monitoring the inhibition of pyrogallol auto-oxidation. The reaction was started by adding 0.5 ml of 2 mM pyrogallol in a total volume of 3 ml reaction mixture containing 200µl sample and 0.1 M Tris HCl buffer (pH 8.2). Change in absorbance was monitored at 412 nm. The specific activity of the enzyme was expressed as units; wherein 1 unit was equal to 50 % inhibition of pyrogallol autoxidation

Lipid peroxidation activity: Lipid peroxidation was measured as previously described (Ohkawa, Ohishi and Yagi, 1979). Lipid peroxidation assay in the fly homogenates was carried out by Thiobarbituric acid reaction. Briefly, lipid peroxidation was measured by using TBA (Thiobarbituric acid). About 500µL of the homogenate was mixed with 1.5ml of 0.8 % w/v TBA and 8 % w/v sodium lauryl sulphate, and 1.5 ml of Acetic acid (20 %, pH 3.5).The absorbance of malondialdehyde (MDA) produced was measured at 532 nm and quantified as malondialdehyde equivalents. Protein concentrations in the fly homogenates were measured using the standard protocol of Lowry et al, 1951 and lipid peroxidation marker MDA was quantified as microgram/mg protein.

Acetylcholinesterase activity: The assay reaction mixture consisted of 20µl of 10mM DTNB; 5, 5-dithiobis 2-nitrobenzoic acid, 50µl sample and 1 ml phosphate buffer (0.1 M, pH 8.0), 20µl of 8mM acetylthiocholine iodide was added to the mixture. Absorbance changes were monitored at 412nm for 3min.The enzyme activity was expressed as µmoles of substrate hydrolyzed/min/mg protein (Ellman *et al.*, 1961).

Learning assay: An olfactory aversive learning test was adopted. The flies were conditioned to associate an odour with an aversive mechanical shock and then tested for the choice between this odour and another odour in a T-maze. This assay is in two phases, the flies were trained in the first phase, and tested in the second phase (Malik and Hodge, 2014). They were simultaneously exposed to an odour A (CS+) and a mechanical shock (US) in a training tube, thereafter, they received another odour B (CS-) without a mechanical shock. The odourants used in this study are 4-methylcyclohexanol (MCH) and 3-octanol (OCT). Dilutions are made such that 4-methylcyclohexanol (1:67) and 3-octanol (1:100) were mixed in mineral oil in the specified ratios respectively. 30µl of the diluted odourants were used (Malik and Hodge, 2014). The flies were transferred into an empty food vial and exposed to 3 conditioning cycle which comprised of a 30 seconds exposure to one odourant followed by six bouts of mechanical shock at 5 seconds intervals with a vortex shaker, then by another 60 seconds of humidified air rest period, then a 30 seconds exposure to another odourant without a shock, and completed by 60 seconds exposure to humidified air rest period (Burger, Buchel and Tadeusz, 2010). Learning measures were taken by introducing the flies to the T-maze apparatus, they were moved to the point of the T-maze where they were maintained for 90 sec for choice making. They were then exposed to both odours at the same time to make a choice. The assay was conducted for 120 seconds.

Memory assay: After the training, the flies were transferred from the T-maze into their respective food vials in the dark at 25 °C to determine the different memory phases of interest, short, mid and long term memories respectively. They were introduced into the T-maze at the central chamber for 90 seconds and allowed to make choices between the two odours, thereafter, the flies at the right or left arm of the T-maze were counted. Five cycles of training with 15 min intervals was used in the long term memory. Memory scores, calculated as performance indexes was given as Performance index (PI) = (# CS-flies - # CS+flies) / (# total flies) (Malik and Hodge, 2014).

Qualitative Phytochemical screening and *in vitro* antioxidant activity: Phytochemical screening of MEMO and its five fractions were carried out according to standard established procedures (Kasolo *et al.*, 2010). The extract was prepared in concentrations of 10, 20, 50 100, and 200µg/ml, and the free radical scavenging activity of the varying concentrations was measured using 2, 2-diphenyl-1-picrylhydrazyl DPPH and the Absorbance was measured at 517nm, Ascorbic acid was used as a standard control (Kanti, Chatterjee and Ghosh, 2015). The percentage inhibition exerted by the extract on the DPPH free radical was calculated thus; % DPPH free radical scavenging = (A0 – A1) / A0 x 100.

Statistical analysis

The result data were expressed as Mean ± Standard Deviation (SD), with the aid of Graph Pad Prism version 6 Statistical software. Survival analysis was carried out with log-rank tests. Reproductive performance and climbing analysis were done using ANOVA, Dunnet's test, memory scores were analyzed using ANOVA, Tukey's test. The DPPH free radical scavenging activity was analyzed using linear regression. The obtained results at $p < 0.05$ were considered as statistically-significant.

RESULTS

Supplementation with *Moringa oleifera* methanolic extract increases lifespan, climbing activity and reproductive performance.

The result obtained from the toxicity analysis of the *Moringa* leaves extract showed that the toxic lethal doses are 100, 500, and 1000 mg/ml respectively. This revealed that higher concentrations of *Moringa oleifera* leaves extract are toxic. The outcome of this finding was the baseline for choosing the concentrations used in this study which are 1, 2, and 5mg/ml respectively.

To test if dietary supplementation with methanol leaves extract of *Moringa oleifera* (MEMO) can facilitate healthy ageing, treated *Drosophila melanogaster* whiteflies were exposed to different concentrations of the extract at 1, 2, and 5mg/ml. There was a concentration-dependent positive effect of the extract on longevity, climbing activity and reproductive performance (Fig 1), with the significant concentration being the 5mg/ml ($p < 0.05$). The percentage increase in mean survival times calculated from the survival curve (Fig 1A&B), were 12.3 ± 1.2, 15.5 ± 1.0 and 26.6 ± 0.4%, and of the females by 12.0 ± 1.1, 17.5 ± 0.8 and 24.3 ± 0.8% with respect to the control group, in the 1, 2, and 5mg/ml treated flies respectively (Table 3). In the climbing activity, only the 5 mg/ml group showed a significant effect ($p < 0.05$) relative to the control. In the reproductive performance analysis the 1, 2, and 5 mg/ml groups showed significant effects relative to control group ($p < 0.05$) for each of the treatment groups.

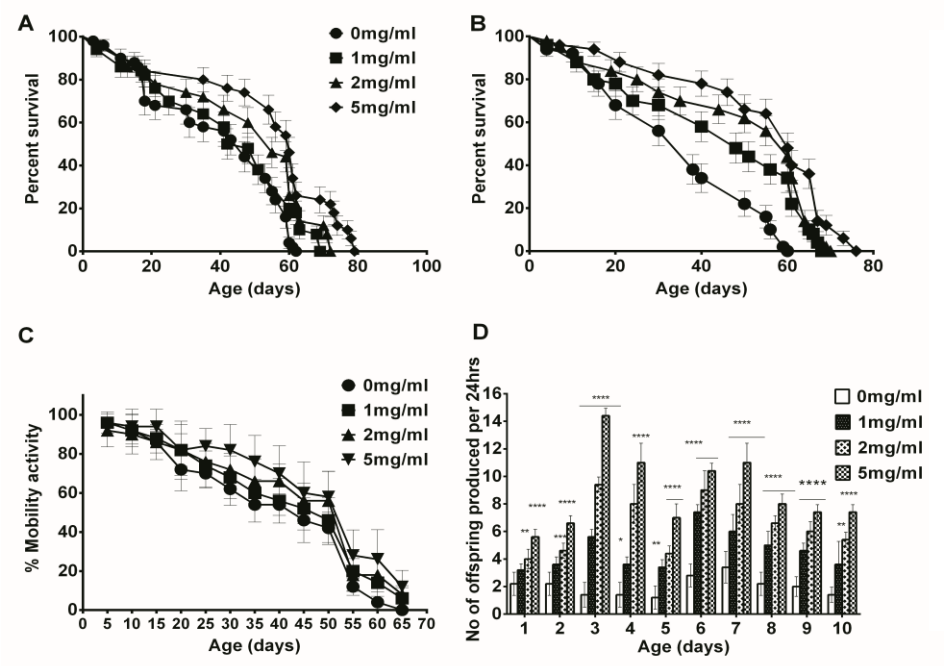

Figure 1.
***Moringa oleifera* supplementation increases lifespan, climbing activity and reproductive performance.**
*Survival of male (A) and female (B) flies fed on different concentrations of Moringa oleifera extract showed a significant increase in lifespan in a concentration dependent manner compared to the control (n = 50 flies per treatment group, p < 0.0001), Log rank (Mantel-Cox) test. (C) The health span promoting effect of Moringa oleifera was evident by a significant increase (in the 5mg/ml group) in the climbing activity with age compared to control, two-way ANOVA, Tukey's test (n = 50 flies per treatment group, p< 0.05). (D) The effect of the extract on reproductive performance showed a concentration dependent effect on the number of offspring produced per 24hr (n = 50 flies per treatment group, p < 0.05) ANOVA, Tukey's test. * means p < 0.05; ** means p < 0.01; **** means p < 0.0001; **** means p < 0.0001*

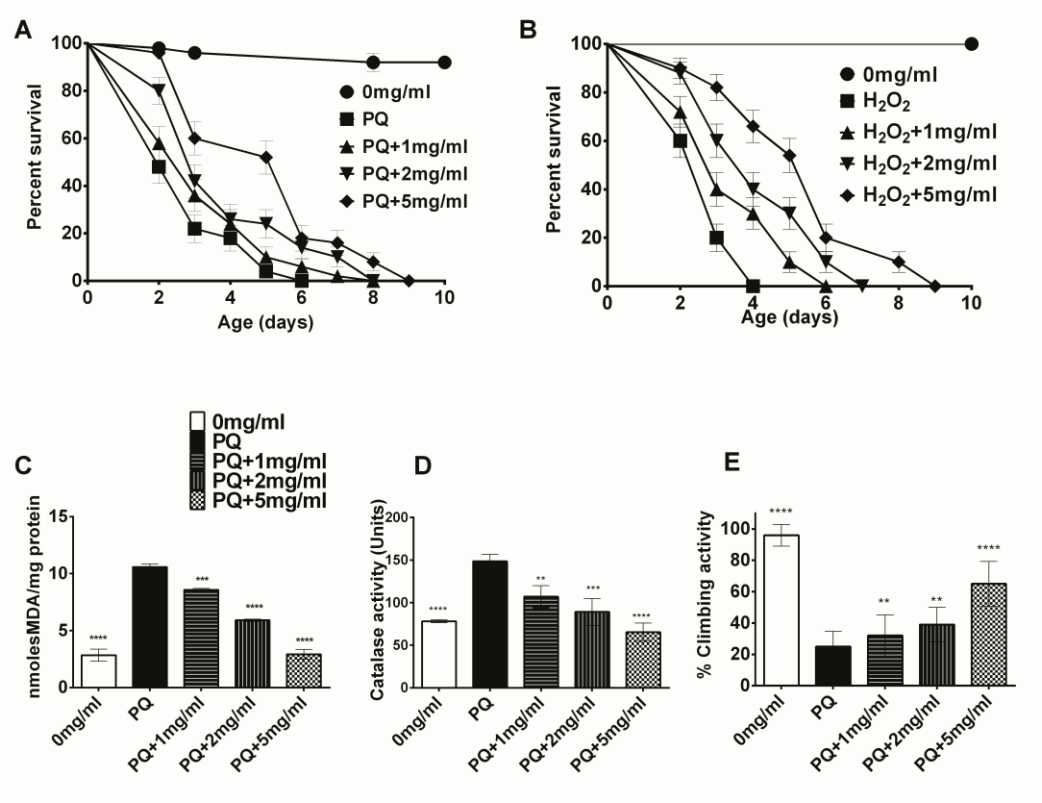

Figure 2.
Moringa oleifera* increases resistance to Oxidative stress. (A)** There was a concentration dependent effect of the extract on oxidative stress resistance after exposure to 15mM Paraquat 5mg/ml, $p < 0.05$ and **(B)** 5% H_2O_2. There was a significant increase in lifespan of treatment groups compared to the oxidative stress groups ANOVA , Dunnett's test. ($n = 50$ flies per treatment group, $p < 0.05$ in the 5mg/ml group). **(C)** There was a significant decrease in MDA levels of treated flies when compared to the Paraquat group. Mean ± SD ($p < 0.001$, 1mg/ml group and $p < 0.0001$ in the 5mg/ml group), Dunnet's test. **(D)** There was a significant increase in oxidative stress resistance in a dose dependent manner in the treatment group ($p < 0.01$ in the 1mg/ml, $p < 0.001$ in the 2mg/ml group, and $p < 0.0001$in the 5mg/ml group). **(E)** *Moringa oleifera* mitigates Paraquat induced locomotor deficit. In flies. One way ANOVA, Dunnet's test ($n = 100$ flies per group, $p < 0.01$ in the 1 and 2mg/ml group, $p < 0.0001$in the 5mg/ml group). ** means $p < 0.01$; *** means $p < 0.001$; * means $p < 0.0001$).

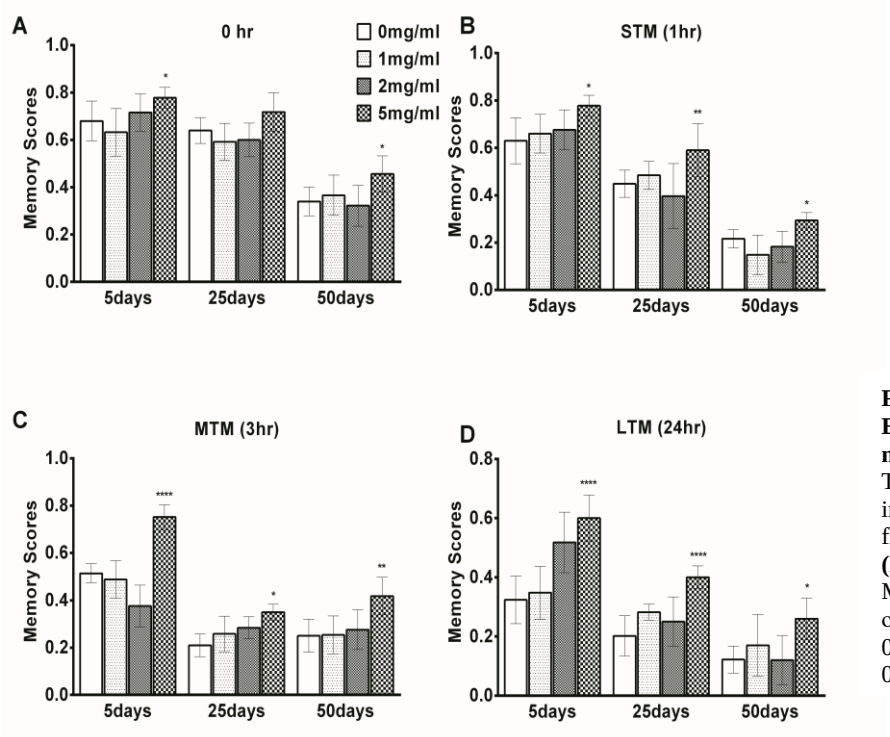

Figure 3.
Effect of *Moringa oleifera* on learning and memory as age progressed.
The 5mg/ml extract caused a significant improvement in learning **(A)** and memory of the flies as age progressed in the Short Term Memory **(B)**, Mid Term Memory **(C)** and Long Term Memory **(D)** compared to the control group and other treatment groups. Tukey's post hoc test $P < 0.05$. $n = 50$ flies per treatment group. * means $p < 0.05$; ** means $p < 0.01$; **** means $p < 0.0001$.

Supplementation with *Moringa oleifera* methanolic extract increases resistance to oxidative stress and is associated with changes in the endogenous antioxidant response system

Flies pre-treated with the leaves extract were exposed to oxidative stress. There was an increase in lifespan during exposure to oxidative stress, which was induced by hydrogen peroxide and Paraquat (Fig 2A and B). The general well-being of the flies under sustained oxidative stress was tested using the climbing activity assay. Flies pre-treated with the extract showed significant ameliorated performance in the Paraquat-induced locomotor deficit, only in the 5mg/ml treated groups, ($p < 0.0001$). Catalase activity in fly homogenates revealed the ability of extracts to mitigate the effect of Paraquat induced oxidative stress and its modulatory effect. There was a significant increase in oxidative stress resistance in flies. Lipid peroxidation reaction was significantly reduced in the treated flies, (Fig 2C&D).

Supplementation with Moringa oleifera methanol leaves extract ameliorates age-related memory decline

To test the effect of Moringa oleifera leaves extract on learning and memory as age progressed, flies were subjected to olfactory learning and memory assay with age. The methanol leaves extract of Moringa oleifera (MEMO) treated flies showed a general increase in learning and memory as age progressed, two-way ANOVA and Tukey's multiple comparison's tests n = 50 flies/treatment group. There was a significant increase in memory scores of treated flies with respect to the control group, $P < 0.0001$, in the aged flies of 50days old.

Supplementation with Moringa oleifera methanolic extract increases Acetylcholinesterase activity and is associated with changes in endogenous antioxidant response in the brain

Ordinarily, brain antioxidant enzymes tend to decrease with age. The *Moringa* 5mg/ml extract treated group showed a significant increase in brain acetylcholinesterase activity as age progressed (5days; $P = 0.0006$, 25days; $P = 0.0463$, 50days; $P = 0.0040$, Fig. 4A). The levels of lipid peroxidation in the brain were reduced compared to the control when flies were pre-treated with 5mg/ml of *Moringa oleifera* extract, $P = 0.0007$, 25days; $P = 0.0078$, 50days; $P < 0.0001$ (Fig 4B). There was a significant increase in the activity of catalase enzyme as age progressed, most especially in the aged 50day old flies, 5days; $P = 0.0233$, 50days; $P < 0.0001$ (Fig. 4C). There was equally an increase in the SOD activity in the 50day old, $P = 0.0008$ (Fig. 4D).

***Moringa oleifera* Ethylacetate fraction enhances longevity and its F2 fraction increases resistance to oxidative stress**

To identify the fraction of *Moringa oleifera* which may be responsible for its longevity effect, a bioassay-guided fractionation was adopted. The Ethylacetate fraction gradually increased the longevity of flies compared to other groups (Fig 5A), $P < 0.05$. There was an increase in free radical scavenging activity of the same Ethylacetate fraction (Fig 5B), and there was an increase in resistance to oxidative stress by the Ethylacetate fraction compared to other extract groups (Fig 5C). In a preliminary investigation, ten fractions were obtained from the *Moringa* Ethylacetate fraction. On testing the free radical scavenging activity of the ten fractions of the *Moringa*-Ethylacetate fraction, the F2 gave the highest free radical scavenging activity against DPPH, therefore the F2 fraction was tested for oxidative stress resistance. There was a dose-dependent increase in oxidative stress resistance in the Ethylacetate F2 treated flies (Fig. 5D). The suspected anti-ageing bioactive fraction of *Moringa* is thus the F2 fraction of *Moringa* Ethylacetate fraction.

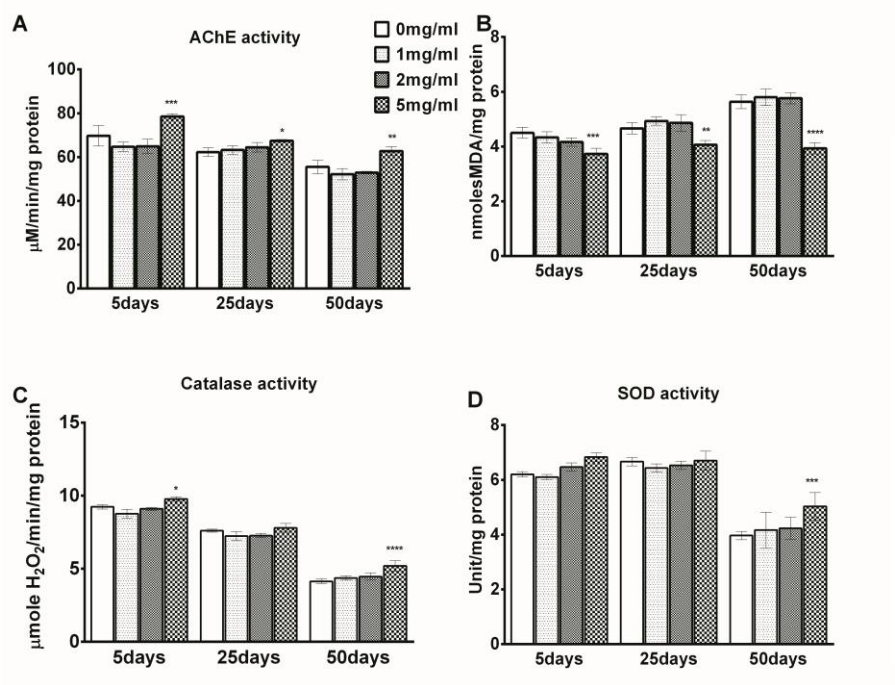

Figure 4
Effect of *Moringa oleifera* on brain biochemical parameters as age progressed.
There was a significant change in the acetylcholinesterase activity of the 5mg/ml group, $p < 0.01$ relative to control **(A)**, the extract caused a significant reduction in lipid peroxidation with age in the 50 day old flies ($p < 0.0001$) **(B)**, there was a significant increase in catalase activity in the aged flies of 50days, $p < 0.0001$ **(C)** and a significant increase in superoxide dismutase activity in the aged flies compared to the control **(D)** ($p < 0.001$, n = 3). * means $p < 0.05$; ** means $p < 0.01$; *** means $p < 0.001$; **** means $p < 0.0001$

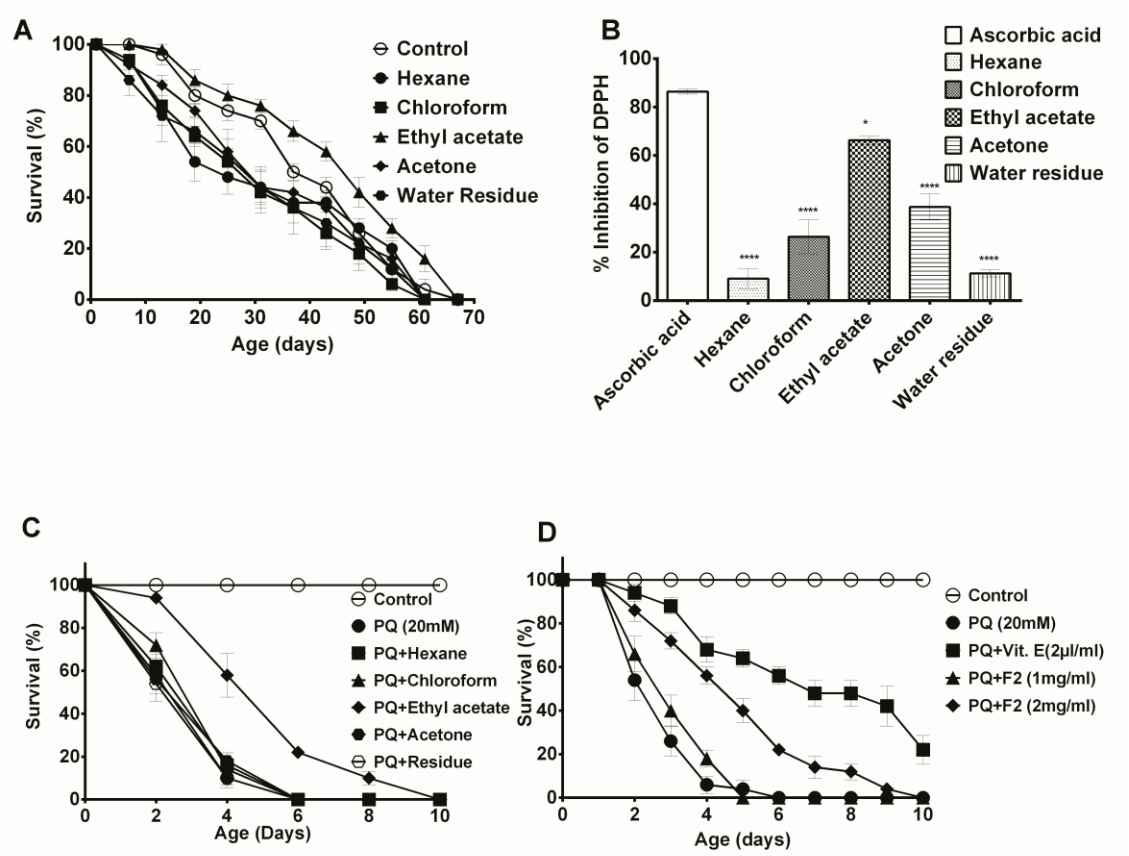

Figure 5
Effect of different fractions of *Moringa oleifera* on longevity and oxidative stress resistance.
Ethylacetate fraction of *Moringa oleifera* increases longevity ($p < 0.0001$) **(A)**, the *Moringa* Ethylacetate fraction gave the highest free radical scavenging activity, ($p < 0.05$) **(B)** and the highest oxidative stress resistance ($p < 0.05$) **(C)**. The F2 of the *Moringa* Ethylacetate fraction increased resistance to Paraquat induced oxidative stress and longevity with respect to other treatment groups and Vitamin C positive control ($p < 0.05$, n = 50 flies/group), **(D)** Tukey's multiple comparison test. * means $p < 0.05$; **** means $p < 0.0001$

Table 1:
Phytochemical screening of the Methanol Leaves Extract of *Moringa oleifera* Lam and its 5 fractions.

S/N	Phytochemicals	Crude Methanol extract	n-Hexane extract	Chloroform extract	Ethyl acetate extract	Acetone extract	Hydro-methanol residue
1	Flavonoids	++	_	+	++	+	_
2	Polyphenolic compounds	+++	_	+	++	_	+
3	Steroids	++	_	_	++	_	_
4	Tannin	++	_	+	+	+	_
5	Alkaloid	++	_	+	+	+	+
6	Reducing sugar	++	_	+	+	+	_
7	Saponins	++	_	+	+	+	+
8	Cardiac glycoside	+++	+	_	++	_	_
9	Terpenoids	++	+	+	+	_	+
10	Coumarin	++	_	_	_	+	_
11	Free Amino acids	+++	_	_	+	+	_
12	Proteins	+++	_	_	+	+	+
13	Anthraquinone	_	_	_	_	_	_

Keys: - (Absent), + (Mild), ++ (Moderate), +++ (High), n = 3.

Qualitative Phytochemical Screening and DPPH free radical scavenging activity

The qualitative phytochemical screening of the methanol leaves extract of *Moringa oleifera* Lam. showed the presence of alkaloid, reducing sugars, saponin, phenolic compounds, cardiac glycosides, terpenoid, coumarin, flavonoid, free amino acids, steroids and proteins, but no anthraquinones (Table 1). The free radical scavenging activity reported in this study is evident by an increase in percentage inhibition of the extract from 3.23 ± 0.6 to 61.45 ± 1.2 in concentrations of 10 to 200µg/ml (Table 2). The concentration at which 50% of the DPPH was scavenged by the *Moringa* leaves extract (IC_{50}) was 133.12 µg/ml with respect to a positive control Ascorbic acid which gave a value of 86.71µg/ml.

Table 2:
DPPH free radical scavenging activity of the Methanol leaves extract of *Moringa oleifera* Lam. and Ascorbic acid.

Sample	Concentration (µg/ml), mean ± SD					IC50
	10	20	50	100	200	
Ascorbic acid	11.69±1.8	17.50±1.2	46.61±0.9	68.06±1.0	86.6±1.3	86.71µg/ml
Moringa oleifera	3.23± 0.6	10.48±1.0	37.02±1.0	49.92±1.1	61.45±1.0	133.12µg/ml

Data is expressed as mean ± SD, n=5.

Table 3:
Percentage increase in mean survival time of *Moringa* treated flies.

	Mean Survival Time			
	Control	**1mg**	**2mg**	**5mg**
Male MST	63 ± 0.6	70.8 ± 1.2	72.8 ± 1.0	79.8 ± 0.4
Female MST	61.6 ± 0.8	69 ± 1.1	72.4 ± 0.8	76.6 ± 0.8
% increase in MST (M)		12.3 ± 1.2	15.5 ± 1.0	26.6 ± 0.4
% increase in MST (F)		12.0 ± 1.1	17.5 ± 0.8	24.3 ± 0.8

*Percentage increase in mean survival time of treated flies = (T/C-1)*100. T= Mean survival time of treatment group. C= Mean survival time of control group.*

The DPPH free radical scavenging activity of the Methanol leaves extract of Moringa oleifera Lam. and Ascorbic acid is shown in Table 2. The result revealed a concentration-dependent effect. The IC50 values of the extract and Ascorbic acid are 133.12µg/ml and 86.71µg/ml respectively, these are the concentrations at which 50% of the DPPH radical was scavenged by the extract.

Table 3 shows the percentage increase in mean survival time of *Moringa* treated flies. There was a percentage increase in mean survival time of the *Moringa* treated male flies by 12.3 ± 1.2, 15.5 ± 1.0 and 26.6 ± 0.4%, and of the females by 12.0 ± 1.1, 17.5 ± 0.8 and 24.3 ± 0.8% respectively relative to the concentrations of extract at 1, 2 and 5mg/ml respectively.

DISCUSSION

The climbing activity is a well-established biomarker of wellness and agility in *Drosophila melanogaster* (Kinghorn *et al.*, 2015). The effect of *M. oleifera* on healthspan was hereby evaluated by checking its effect on the climbing activity as age progressed. There was a significant effect of the extract on lifespan, climbing activity, and reproductive performance (Fig 2). The lifespan-extending effect of *Moringa oleifera* may be attributed to its rich nutritional and antioxidant properties and also its ability to increase oxidative stress resistance, this is peculiar to most anti-ageing compounds as reported in previous studies (Partridge *et al*., 2011, Im *et al*., 2016).
Our result is in line with previous findings in which *Moringa oleifera* extended lifespan in *C. elegans* through the oxidative stress resistance pathway. Other pathways contributing to extended lifespan in the *C. elegans* include activation of SKN, SIR-2.1 and DAF-16/FOXO transcriptional factor, and the inhibition of insulin/IGF signaling pathway (Im *et al.*, 2016).

Catalase is an endogenous primary antioxidant present in most organisms from worms, flies, rodents, mammals to humans, with the same modulatory effect (Dai *et al.*, 2014). There was a significant reduction in malondialdehyde production in fly homogenates which is a modulation of lipid peroxidation. This may be due to the presence of polyphenolic compounds, flavonoids, vitamins and minerals in the leaves extract which act as cofactors in antioxidant reaction pathways. These compounds were reported to boost the endogenous antioxidant defense system in organisms and improving lifespan-extending effects (Salehi *et al.*, 2020).

The brain is the most vulnerable organ when it comes to oxidative stress, due to its rich polyunsaturated fatty acids. Therefore, it is prone to lipid peroxidation reaction (Kinghorn *et al.*, 2015). To test the effect of *Moringa oleifera* on learning and memory as age progressed, flies were subjected to olfactory learning and memory assay with age. Our result revealed that the methanol leaves extract of *Moringa oleifera* (MEMO) showed a significant increase in learning and memory as age progressed. These findings are in line with a recent finding which reported the antioxidant boosting and memory-enhancing effect of *Moringa oleifera* (Jamari *et al.*, 2020).

Certain bioactive constituents in natural products tend to enhance memory through the activation of the brain cholinergic system (Pillay *et al.*, 2003; Elufioye *et al.*, 2010). These bioactive constituents are present in *Moringa oleifera* (Anwar *et al.*, 2007; Jamari *et al.*, 2020). Certain phytochemicals act as inhibitors of acetylcholinesterase, thereby, exerting a decrease on the enzyme activity, increasing the availability of the neurotransmitter, acetylcholine, which enhances learning and memory (Elufioye *et al.*, 2010). We reported the presence of potent antioxidants and phytochemicals in the present study (Table 1). These phytochemicals may be involved in neuroprotection and modulation of brain enzyme activities as reported of phytochemicals in previous studies (Hosamani and Muralidhara, 2009; Prasad, 2014).

The extract was able to inhibit the free radical chain reaction induced by the DPPH free radical in a concentration-dependent manner. The concentration at which 50% of the free radical was scavenged by the extract, that is, the IC_{50} value was 133.12µg/ml for *Moringa oleifera* with respect to Ascorbic acid which gave a value of 86.71 µg/ml. This in line with previous studies which showed the significant free radical scavenging effect of *Moringa* leaves extract (Shih *et al.*, 2011). The free radical scavenging activity of the ten fractions obtained from *Moringa* Ethylacetate fraction revealed F2 as the fraction with significant bioactivity. It gave the highest free radical scavenging activity against DPPH.

This suggests a significant antioxidant protection which was confirmed when challenging the flies with Paraquat to induce oxidative stress. Therefore, the *Moringa* Ethylacetate fraction, F2, could be the anti-ageing bioactive fraction of the extract.

In conclusion, we hereby suggest that *Moringa oleifera* leaves extract possesses anti-ageing bioactivity through its ability to enhance oxidative stress resistance. It was able to ameliorate the onset of age-related memory decline. The *Moringa* Ethylacetate fraction, F_2 is hereby reported to be the anti-ageing bioactive fraction of the crude methanolic extract of *Moringa oleifera*. This study endorses the efficacy of *Drosophila melanogaster* as a powerful model organism relevant to understand the anti-ageing effect of *Moringa oleifera* leaves extract. It will be important to further isolate and characterize through structural elucidation, the anti-ageing bioactive fraction of the leaves extract, as well as to extend the phenotypic analysis in the search for other interesting anti-ageing properties.

Acknowledgements

Biochemistry department, Faculty of Biomedical Sciences, the Postgraduate Studies and Research Directorate-Western-Campus, Institute of Biomedical Research, IBR, KIU-WC, Sayansi Research for Development in Africa, Trend in Africa as well as DrosAfrica are well appreciated.

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