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Effect of alkaloid-rich extract from Eggplant (*Solanum kumba*) fruit peels on manganese-induced neurodegeneration in fruit fly (*Drosophila melanogaster*) Model

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ABSTRACT: Eggplant fruits commonly called garden egg in Nigeria have been found to be rich in phytochemicals with various therapeutic properties, however little is known about the alkaloid-rich constitutes. This study sought to identify and investigate the effect of alkaloid-rich extract obtained from eggplant fruit peels and its biological activities on manganese-induced ($MgCl_2$) toxicity in fruit fly (*Drosophila melanogaster*). Fruit flies were induced with ($MgCl_2$) and were subsequently treated, homogenized and the acetylcholinesterase (AChE) enzyme activity, *in-vivo* reactive oxygen species and *in-vitro* antioxidants such as ferric reducing antioxidant property (FRAP) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTs*) were determined, while the gas chromatography coupled with mass spectra (GC-MS) was used to analyse the alkaloid constitutes in the peels. The results reveal that the alkaloid-rich extract (0.05-0.10mg/ml) diet had high antioxidant property which reduced the reactive oxygen species level generated in the fruit flies and also increased the life span of the fruit flies with 50% reduction in mortality rate with an increased locomotors performance. The alkaloid-rich diet also modulated the activity of AChE enzyme in all the treated flies. The GC-MS showed alkaloids with anti-oxidative, anti-inflammatory and cholinergic inhibitor activities. Conclusively, this study suggests that the eggplant fruit peels diet could reduce neuronal stress.

Keywords: Eggplant fruit peels, alkaloid, *Drosophila melanogaster*, acetylcholinesterase, GC-MS chromatography

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Introduction

Neurodegenerative diseases (NDDs) are traditionally defined as disorders with selective loss of neurons and distinct involvement of functional systems defining clinical presentation. Neurodegenerative diseases affect the central nervous system causing progressive nervous system dysfunction. These debilitating and incurable conditions are characterized by loss of neuronal cell functions and are often associated with atrophy of the affected nervous system structures. An important subset of neurodegenerative disease concerns dementias associated with aging. [1]. Alzheimer's disease (AD) is the most common clinically recognized dementia in aging populations, and 43% of people 85 or older are thought to suffer from Alzheimer's in the United States [1] Parkinson's disease (PD), another common nervous system disorder associated with the elderly, affects 1-3% of the population over 60. United Nations population projections estimate a world population of 400 million people 80 years of age or older by the year 2050 [2]. Given the financial, societal and personal impact of the burden of these neurodegenerative diseases, determining the cause, prevention and treatment have become a major focus for basic and clinical research.

Management of neurodegenerative conditions, especially Alzheimer's like disease (AD) has been with the use of drugs such as tacrine, donepezil and rivastigmine which are cholinesterase inhibitors consequently increasing the brain's acetylcholine. This is because in AD conditions there are elevated cholinesterase activity [3]. Several studies have shown that monoamine oxidase (MAO) inhibitors could countervail some of these processes through various neuroprotective mechanisms, such as interaction with the mitochondrial outer membrane, as well as the up-regulation of anti-apoptotic proteins and neurotropic factors [4, 5].

In recent years the use of these synthetic drugs have declined drastically due to their adverse side effects and much prevalence/importance have been given to the traditional ways of treating these neurodegenerative conditions. Study has shown that plant-based products could be used in the management of neurodegenerative diseases [6] Thus, recent efforts are focused on plant products. Phytochemicals have been reportedly serves as natural cholinesterase inhibitors with little or no side effects, found useful as a dietary intervention in the management of neurodegenerative disorders [7]. Human beings have used plants for the treatment of diverse ailments for thousands of years [8, 9). Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day lives. These medicines are relatively safer and cheaper than synthetic or modern medicine [10]. Thus, the quest for natural plant products rich in antioxidant is gaining much importance in neurotoxicity management and in the treatment. However, one of the major concerns of researchers is in the reduction in the number of higher laboratory animals for research and testing due to ethical issues. Therefore, the fruit fly *Drosophila melanogaster*, has been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for promoting the 3Rs (reduction, refinement and replacement) of laboratory animal usage in toxicity studies [11]. Drosophila possesses systems which control nutrient uptake, storage and metabolism and these systems have been reported to be analogous to those of humans [12, 13].

Drosophila melanogaster is known for its high sensitivity to toxic substances and is being considered as a useful model for toxicity studies as well as evaluating the biological action of pharmacological agents [14] Drosophila has been previously demonstrated to be a useful model for elucidating the mechanisms underlying manganese-induced neurotoxicity [15]. Alkaloids are plant-originated nitrogen-containing compounds [16]. Some cholinesterase inhibitors (galantamine and rivastigmine) commonly used to treat neurodegenerative-related diseases are alkaloid origin [17] highlighting the importance of alkaloid-rich plants as an alternative source for the management of neurodegenerative diseases. Eggplant (*Solanum spp*) is an edible fruits vegetable with different species that include wild relatives as well as primitive cultivars and landraces of the important vegetable crop [18]. The African eggplant fruit play a central role in the tradition and culture of people in sub-Saharan Africa [19]. It is offered as a gift in traditional ceremonies, such as marriage, child naming and other social occasions as a sign of blessing and fruitfulness [19]. It is an important to fruit-crop found useful in traditional folklore medicine as it is used to treat certain ailments. It has proven beneficial to patients suffering from anemia because of its rich iron content. Rich meal of garden eggs is used to induce lactation in freshly delivered women [20]. Frequent consumption of garden eggs reduces intraocular pressure (glaucoma) and convergence insufficiency [21]. In addition, it prevents heart diseases and blood pressure [22].

Phytochemical screening of the whole eggplant fruit and relatives of this genus have revealed the presence of steroidal glycoalkaloids, tannins, sesquiterpenoids and other essential bioactive compounds as highlighted in [19, 22-24] studies, but there is little or no information on the indigenous eggplant fruit peels activities in biological systems as literature reported that the colour part of a fruit has the highest phytochemicals, therefore for those who only consume the outer part known as the flesh or peel there is still need to investigate further if it can still be beneficial to them because previous studies on eggplant fruits have shown that high consumption of the fruit due to its rich dietary fiber might have induced constipation in some subject [18,19] as such it could be the reason why some avoid the consumption of the whole eggplant fruit. Furthermore, recent study on indigenous eggplant whole fruit polyphenol extract and diet have revealed that it could manage neuronal issue in diabetic model. So, this study was designed to investigate the effect of alkaloid-rich extract from eggplant fruit peels (*Solanum kumba*) on acetylcholinesterase enzyme linked to Alzheimer's disease (AD) like symptoms using fruit fly *Drosophila melanogaster* as a model.

Materials and Methods

Sample collection and identification

Eggplant fruits were obtained from a local farm in Akure, Ondo State, Nigeria during the month of April, 2018. Authentication of the sample was carried out at the Department of Forestry and Wood Technology Federal University of Technology, Akure, Nigeria with a Voucher number IFE-17718 while the green skin was peeled from the whole fruit for the experiment.

Drosophilia melanogaster Stock Culture

Wild type *D. melanogaster* (Oregon strain) stock culture was obtained from Department of Biochemistry, University of Ibadan, Oyo State. The flies were maintained and reared on normal diet made up of corn meal medium containing 1% w/v brewer's yeast and 0.08% v/w nipagin at constant temperature and humidity (25 ± 1oC; 60% relative humidity respectively) under 12 h dark/light cycle conditions. All the experiments were carried out with the same *D. melanogaster* strain.

Reagents

Chemical reagents such as acetylthiocholine iodide, sulphanilamide, reduced glutathione, n-n-diethyl-para-phenylenediamine (DEPPD), ferrous sulphate, semicarbazide were procured from Sigma Al-drich Co. (St Louis, Missouri, USA). Trichloroacetic acid (TCA) and sodium acetate was sourced from Sigma Al-drich, Chemie GmbH (Steinheim, Germany), hydrogen peroxide, methanol, acetic acid, hydrochloric acid, aluminium chloride, potassium acetate, sodium dodecyl sulphate, Iron (II) sulphate, potassium ferrycyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England). Ascorbic acid and starch were products of Merck (Darmstadt, Germany). Except stated otherwise, all other chemicals and reagents were of analytical grades and the water was glass distilled.

Instruments

Test tubes, beaker, water bath, centrifuge machine, weighing balance, spectrophotometer, cuvettes, conical flask, measuring cylinder, standard flask, micro pipette, amber bottles, centrifuge tubes, universal bottles, microplates, microplate readers.

Methods

Extraction of alkaloid

The plant material was air-dried for one week; ground mechanically using an electric blender to powder which was then used for analysis. The alkaloid constituent was then determined using the method of [25].

Stock solution preparation

From 50g of the sample 4.58g of the alkaloid extract was gotten. This alkaloid extract was dissolved with 3ml Dimethyl Sulphoxide (DMSO) after which 60mls of water was added. Thereafter, the solution was poured into a stock bottle and stored in the refrigerator for subsequent analysis.

Preparation of Drosophila food using Agar method

The diet preparation was done for toxicological studies on *D. melanogaster* according to the method of [26].

Experimental Design

The flies (both gender, 3–5 days old) were divided into 7 groups containing 60 flies each. Group I was placed on normal diet alone while group II – VII, were placed on basal diet containing; *Solanum* *kumba* (SM) alkaloid extract of 0.1% and 0.05% inclusion in the diet (equivalent weight replacement) as shown.

Groups:
I Basal Diet
II Basal Diet + manganese chloride ($Mgcl_2$)
III Basal Diet + Manganese chloride ($Mgcl_2$) + 0.1% SM
IV Basal Diet + Manganese chloride ($Mgcl_2$) + 0.05% SM

The flies were exposed to these treatments for 5 days and the vials containing flies were maintained at room temperature. All experiments were carried out in triplicate (each experimental group was carried out in five independent vials) [27].

Survival Study

A study was conducted to assess the effect of alkaloid extract inclusion on survival rate of flies after five days of exposure. Flies both genders, of 3–5 days old were divided into four groups containing 60 flies each. Each group was exposed to different alkaloid extract inclusion (0.1 and 0.05%). The flies were observed daily for the incidence of mortality and the survival rate were determined by counting the number of dead flies for the first five days. The data were subsequently analysed and plotted as cumulative mortality and percentage survival after the treatment period [27, 28].

Measurement of Locomotors Performance (Negative Geotaxis)

The negative geotaxis assay was used to evaluate the locomotors performance of flies [29]. In brief, after the treatment period of five days, the flies from each group were briefly immobilized in ice and transferred into a clean tube (11 cm in length 3.5 cm in diameter) labelled accordingly. The flies were initially allowed to recover from immobilization for 10 mins and thereafter were tapped at the bottom of the tubes. Observations were made for total number flies that crossed the 6 cm line within a period of 6 s and recorded. The results are expressed as percentage of flies that escaped beyond a minimum distance of 6 cm in 6 s during three independent experiments.

Preparation of Tissue Homogenate

The flies were immobilized in ice and homogenized in 0.1 M phosphate buffer, pH 7.4. The resulting homogenates were centrifuged at 10,000 X g, at 40C for 10 mins in a Kenxin refrigerated centrifuge Model KX3400C (KENXIN Intl. Co., Hong Kong). Subsequently, the supernatant was separated from the pellet into labelled Eppendorf tubes and used for the various biochemical assays.

***In-vitro* biochemical assays**

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging ability

The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS*) scavenging ability of the fruits was determined according to the method described by [30]. The Trolox equivalent antioxidant capacity (TEAC) was subsequently calculated using trolox as the standard.

Determination of reducing property (FRAP)

The reducing property of the extracts was determined by assessing the ability of the extract to reduce FeCl3 solution as described by [31]. The absorbance was measured at 700 nm in the JENWAY UV-Visible spectrophotometer. Then, the ferric reducing antioxidant property was subsequently calculated as ascorbic acid equivalent.

Lipid Peroxidation and thiobarbibutric Acid reactions

The lipid peroxidation assay was carried out using the modified method of [32]. TBARS (thiobarbituric acid reactive species) produced was measured at 532 nm using spectrophotometer. Malondialdehyde (MDA) produced was calculated and reported as percentage of control.

***In-vivo* biochemical assays**

Determination of Total Protein

Total Protein content of fly homogenates were measured by the Coomassie blue method according to [33] using bovine serum albumin (BSA) as standard.

Reactive Oxygen Species (ROS) level

ROS level in the whole fly tissue homogenates was estimated as H_2O_2 equivalent according to by the method of [34] with slight modifications. The absorbance was measured at 505 nm using a spectrophotometer. ROS levels was estimated from an H_2O_2 standard calibration curve and expressed as Unit/mg protein, where 1 unit = 1 mg H_20_2/L.

Acetylcholinesterase (AChE) Activity Assay

Acetylcholinesterase activity was assayed according to the method of [35]. The AChE activity was thereafter calculated and expressed as mmolAChE/min/mg protein.

Data Analysis

The results of replicate readings will be pooled and expressed as mean ± SEM values. One-way Analysis of Variance (ANOVA) will be used to analyze the results followed by Turkey column comparison test, with levels of significance accepted at $p<0.05$, $p<0.01$ and $p<0.001$. All statistical analysis was carried out using the software Graph pad PRISM (V.5.0) [36].

Results

Table 1 reveals the *in vitro* antioxidant property of alkaloid extract of eggplant (*Solanum kumba*) using radical species 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging ability (ABTS), also ferric reducing antioxidant property (FRAP).

Fig 1: *Solanum kumba* fruits.

Table 1: Antioxidant activity of alkaloid-rich extract from eggplant fruit peels (Solanum *kumba*)

Sample	**ABTS (mmolTEAC/μM)**	**FRAP (μgAAE/g)**
Eggplant peel	0.65±0.06	32.51±2.53

Values represent means of triplicate reading.

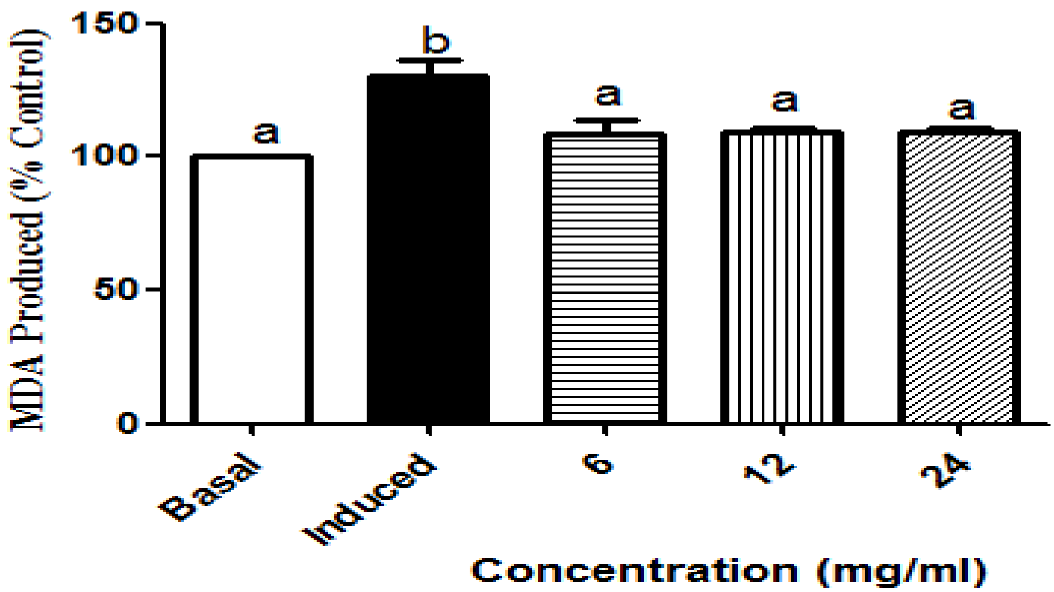

Fig 2: Effect of dietary inclusion of alkaloid-rich extract from eggplant fruit peels on the MDA produced (% control) in *D. melanogaster* at different concentration. Bars represent mean ± SEM. Bars with different letter shows significantly different (P<0.05).

KEYS

GROUPS

I Basal

II Induced

III Basal Diet + Eggplant 0.5mg/ml +$MnCl_2$

IV Basal Diet + Eggplant 1mg/ml+ $MnCl_2$

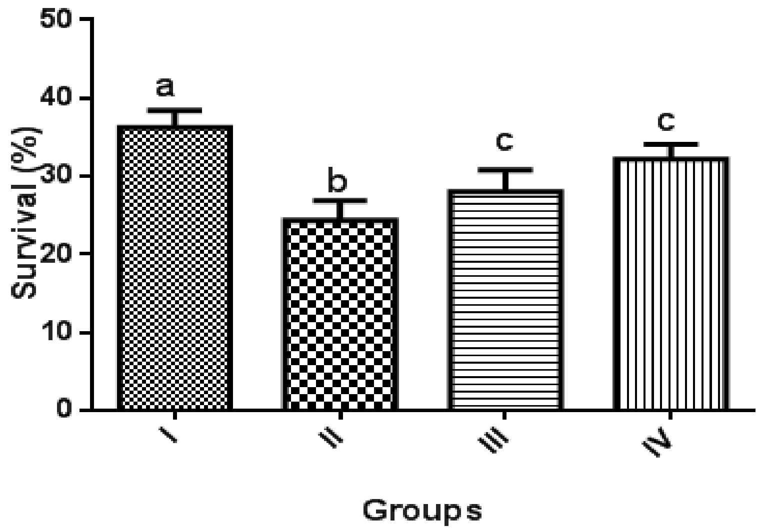

FIG 3: Day 7 Survival Rate (%) of *D. melanogaster* fed with supplemented alkaloid-rich extract of eggplant fruit peels. Bars represent mean ± SEM. Bar with the same letter are not significantly different ($P < 0.05$).
KEYS
GROUPS
I Basal Diet
II Basal Diet + $MnCl_2$
III Basal Diet + Eggplant 0.5mg/ml + $MnCl_2$
IV Basal Diet + Eggplant 1mg/ml+ $MnCl_2$

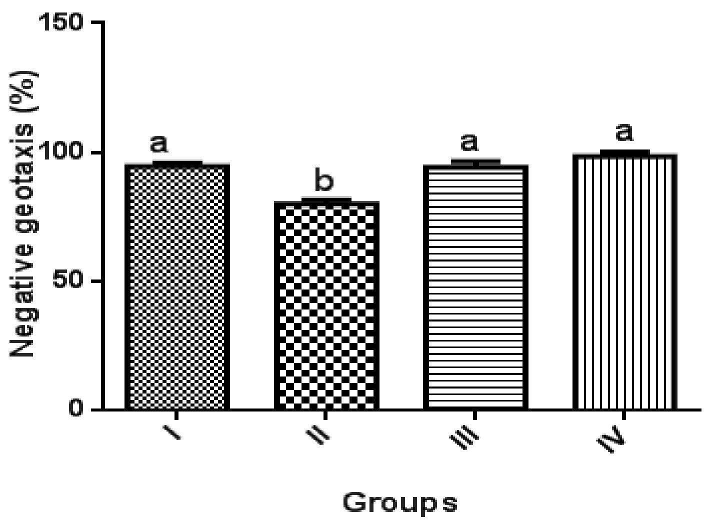

FIG 4: Effect of dietary inclusion of alkaloid-rich extract from eggplant fruit peels on the percentage climbing activity in 6cm in 6 seconds in *D. melanogaster*. Bars represent mean ± SEM. Bar with the same letter are not significantly different ($P < 0.05$).
KEYS
GROUPS
I Basal Diet
II Basal Diet + $MnCl_2$
III Basal Diet + Eggplant 0.5mg/ml + $MnCl_2$
IV Basal Diet + Eggplant 1mg/ml+ $MnCl_2$

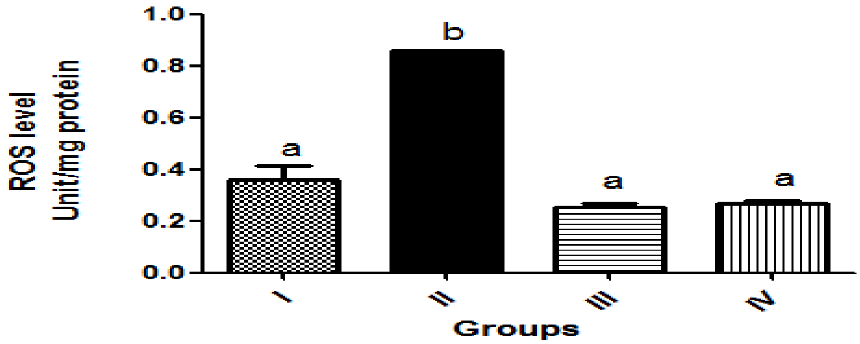

FIG 5: Effect of dietary inclusion of alkaloid-rich extract of eggplant fruit peels on the ROS activity in *D. melanogaster*. Bars represent mean ± SEM. Bars with different significantly different (P<0.05).
KEYS
GROUPS
I Basal Diet
II Basal Diet + $MnCl_2$
III Basal Diet + Eggplant 0.5mg/ml + $MnCl_2$
IV Basal Diet + Eggplant 1mg/ml+ $MnCl_2$

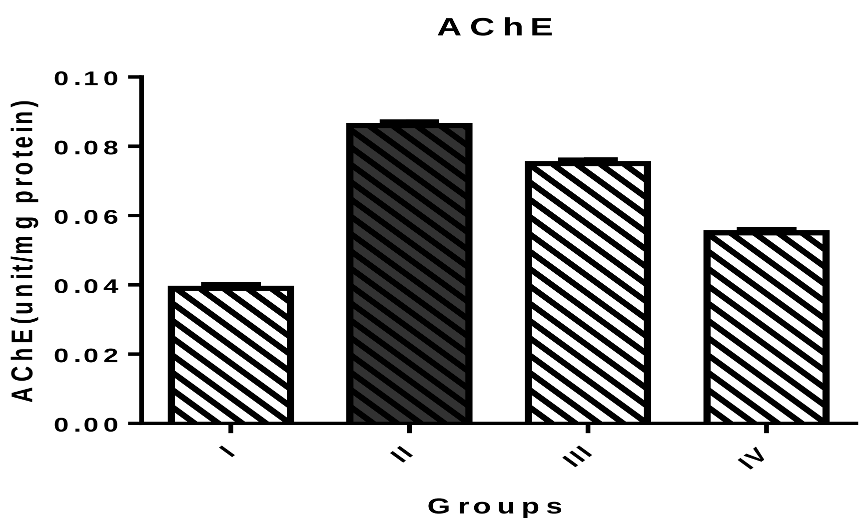

FIG 6: Effect of dietary inclusion of alkaloid-rich extract of eggplant fruit peels on the Acetylcholinesterase activity (µmolAcsch/h/mg/protein) in *D. melanogaster*. Bars represent mean ± SEM with significantly difference (p<0.05)
KEYS
GROUPS
I Basal Diet
II Basal Diet + $MnCl_2$
III Basal Diet + Eggplant 0.5mg/ml + $MnCl_2$
IV Basal Diet + Eggplant 1mg/ml+ $MnCl_2$

Table 2. Alkaloid identify in the eggplant peel using GC-MS chromatography

Constituents	Retention Time	%
1,2-Ethanediamine, N,N'-dimethyl-	8.20	0.43
1,3-Bis(2-chloroethyl)urea	11.96	4.53
1-Pentanol,4-amino	12.93	4.68
1,2,5-Oxadiazol-3-carboxamide	24.05	2.72
Octadenamide-9- (Oleamide)	32.23	2.22
% Total		14.58

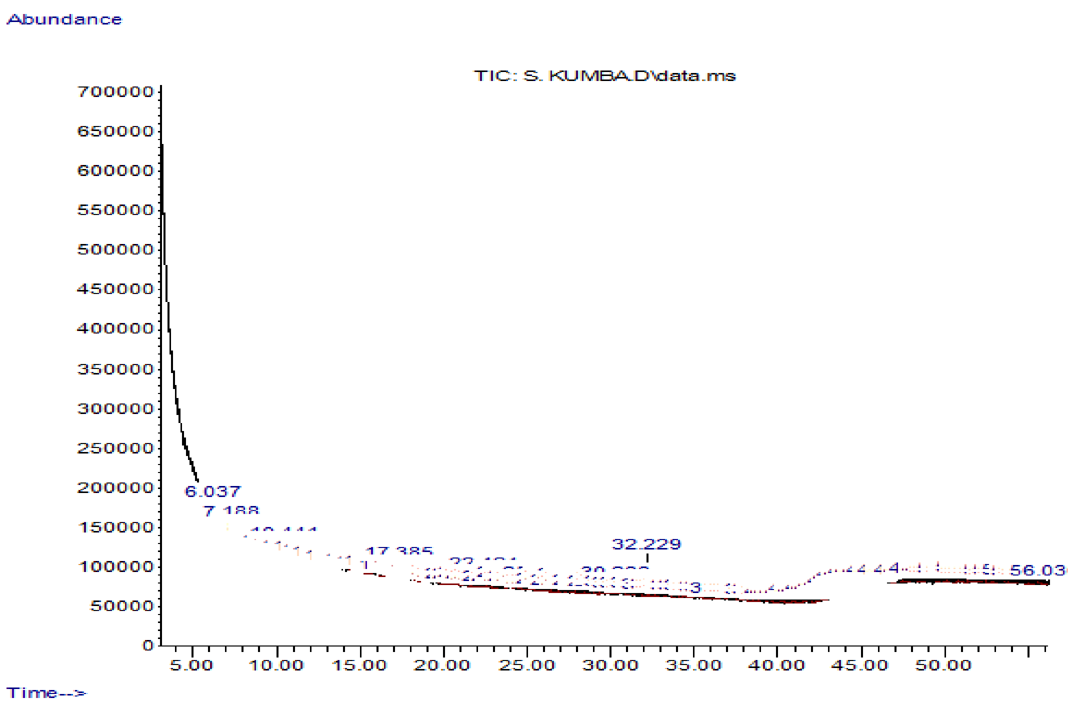

Fig 7. Chromatogram of the alkaloid characterized from the eggplant fruit

Table 1 reveals the antioxidant property of the eggplant fruit peels which was able to reduced radical species formed. Figure 2 shows the effect of dietary inclusion of alkaloid-rich eggplant fruit peels extract on the lipid peroxidation activity in *D. melanogaster*. It was observed that there was no significant difference ($P<0.05$) in the treated groups when compared with the basal group however the induced group with pro-oxidant sodium-nitroprusside (SNP) have an increased lipid peroxides generated when compared to the basal group and the eggplant fruit peels treated groups. Figure 3 shows the effect of alkaloid-rich extract of eggplant fruit peels on the life span of *D. melanogaster*. Dietary inclusion of $MnCl_2$ reduced the life span of *Drosophila melanogaster* significantly ($p>0.05$) when we compared control flies and the treated groups with eggplant fruit peels diet checking by the number of days required to reach 50% mortality which clearly revealed that the alkaloid-rich extract might have attenuated manganese induced neurotoxicity which could have led to neurodegenerative state, however on day 7 survival of *D. melanogaster* revealed significantly ($p<0.05$) difference in the percentage survival of the flies induced with $MnCl_2$ and induced with eggplant peels extract inclusion. While Fig 4 shows the effect of dietary inclusion of eggplant fruit peels extract on locomotors (climbing movement) known as negative geotaxis in *D. melanogaster*, the flies fed with diet induced with $MnCl_2$ had the same climbing rate in comparison to the control group but significantly low in the induced group without any treatment thus, the treatment enhanced the locomotors activity. Fig 5 shows the effect of dietary inclusion of the eggplant fruit peels extract on the Reactive Oxygen Species (ROS) level. This revealed that flies fed with eggplant fruit peels extract diet had a reduced Reactive Oxygen Species (ROS) level ($P< 0.05$) when compared with the untreated groups. Fig 6 shows the effect of dietary inclusion of eggplant fruit peels extract on acetylcholinesterase (AChE) enzyme in *D. melanogaster*, flies induced with manganese chloride had a significantly ($p<0.05$) increased in acetylcholinesterase enzyme activity when compared with the control and the eggplant fruit peels treated groups, it was also observed that the diet significantly ($p<0.05$) decreased the AChE level to the positive control level. While Table 2 and Fig 7 show the quantified alkaloid presence in the eggplant fruit and their peaks in the chromatogram which reveals that Octadenamide-9- (Oleamide) was the most abundant alkaloid which have been found to various therapeutic ability including cholinergic inhibitor.

Discussion

Diet has been implicated to play a vital role in ageing processes [37]. Dietary phenolic such as phenolic acids and flavonoids are beneficial for longevity by reducing oxidative stress, regulating signal transduction and gene expression [37]. Several reports have confirmed that these phytochemicals are more abundant in the skin, peel of fruits and vegetables because these ‘chemicals” have been found to serve as protection and self-defense from insect and other predators. [39]. *Drosophila melanogaster* (fruit fly) has been recently used as an alternative model for screening of therapeutic agents for the treatment of degenerative diseases using Manganese-induced toxicity as a model for induced neurodegeneration [15, 38, 28] pathology. The positive health effects of phenolic phytochemicals are linked to their ability to counter the negative effects of reactive oxygen species generated during cellular energy metabolism [39]. Flavonoids are also known as a class of widely distributed phytochemicals with antioxidant activities. Phytochemical investigation of eggplant (*Solanum kumba*) and relatives of this genus [18] has revealed presence of high levels of steroidal glycoalkaloids, tannins, sesquiterpenoids and other essential bioactive compounds as stated[19, 23, 24]. The results of this study reveals that eggplant fruit peels have anti-oxidative property from the *in vitro* antioxidant indices, the presence of the phytochemicals in it could have mopped up the peroxides form during neurodegenerative state. A growing body of evidence suggests that the *D. melanogaster* exposure to Manganese (Mn) might led to locomotors dysfunction, oxidative stress and mortality [40, 41]. Although little is known about the precise mechanism of action of Mn in *D. melanogaster* but, its involvement in dopaminergic (Daergic) neurodegeneration and oxidative stress is well supported by previous studies [40, 41] which reported that Mn can induce the loss of Daergic neurons, increase reactive species, increase MDA, nitric oxide, and decrease the activity of antioxidant enzymes [40,41]. Also, the notion that Mn might led to both the oxidation of dopamine and also the mitochondrial dysfunction are the two potential mechanisms by which Mn-induced oxidative stress was documented [42, 43,44].

This present study demonstrated that dietary exposure to manganese at 30 mmol per kg caused a significantly ($p<0.05$) increase in the cumulative number of dead flies and consequently, a significant reduction in the percentage of life flies following 7 days of the treatment regimen. This observation could be attributed to the cytotoxic effect of manganese (Mn) which has been previously reported. However, dietary supplementation of eggplant (*Solanum kumba*) fruit peels significantly prevented the manganese mediated toxicity by decreasing the mortality and consequently increased the (%) of the life flies to which the result followed the same trend with the flies locomotor ability as well as increase in their climbing performance. Acetylcholinesterase (AChE) is a serine protease that hydrolyses acetylcholine, a neurotransmitter which regulates motor function and locomotion [45] an increase in its activity in the flies could have led to neurodegeneration in the fly which invariably resulted to dead flies. Dietary supplementation of eggplant fruit peels alkaloid–rich extract could have been associated with the decreased in AChE enzyme activity and also an increased climbing performance in flies, thus confirmed the protective role of eggplant fruit diet in *Drosophila melanogaster* induced manganese neurotoxicity.

Plant-based alkaloid extract from eggplant fruit decreases the level of acetylcholine in the acetylcholinergic receptors in the brain of the flies which could have prevented the flies in developing the neurodegenerative disorders as a result of the toxicant. The observed activity is basically due to the anti-oxidant, anti-amyloid, anticholinergic agent, adenosine receptors agonists, cholinesterase inhibitor, MAO inhibitor, α-synuclein aggregation inhibitor, nicotine and dopaminergic agonist properties in the eggplant fruit peels [46]

Conclusion

This study shows that alkaloid-rich eggplant fruit peels could protect and/or prevent manganese induced oxidative stress. More so, supplementation of the indigenous eggplant fruit diet could be associated with neuroprotection as observed with AChE enzyme activity. Therefore eggplant fruit whole and its peels could be a promising functional food candidate against neuronal stress and disorders thus, the need for its regular consumption as an alternative to synthesized drugs.

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