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Research Article

Effect of Bitter Kola Seed Supplemented Diet on Ageing, Cholinesterase Activities and Redox Status in *Drosophila melanogaster* Model

Ogunsuyi O.B.[a, b*], Oluokun O.O.[b], Özek G.[c], Göger F. [c,d,e], *Oboh G.[b]

[a] *Federal University of Technology, Department of Biomedical Technology, P.M.B. 704, Akure, Nigeria*
[b] *Federal University of Technology, Department of Biochemistry, P.M.B. 704, Akure, Nigeria*
[c] *Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, 26470, Eskişehir, Turkey*
[d] *Anadolu University, Medicinal Plant, Drug and Scientific Research Center (AUBIBAM), 26470, Eskişehir, Turkey*
[e] *Yunus Emre Vocational School, Department of Pharmacy, 26470, Eskişehir, Turkey****

ABSTRACT

This research evaluated the effect of bitter kola (*Garcinia kola*) seed on ageing and ageing-related biochemical modulations such as critical neural enzyme activities and oxidative stress markers in *Drosophila melanogaster*. Experimental flies were fed diet supplemented with Bitter kola (BK) throughout their life span. Treated flies were also homogenised 10-days post-treatment and assayed for the activities of cholinesterase and catalase, reactive oxygen species (ROS), and thiobarbituric acid reactive substances (TBARS) contents. An LC/MS/MS phenolic analysis, as well as reducing property of BK seed was also determined. Results showed that flies raised on diet supplemented with BK seed exhibited significantly improved lifespan, locomotor performance and amelioration of impaired biochemical markers of ageing when compared with control. BK seed also modulated activities of cholinesterase. The LC/MS/MS analysis revealed abundance of Garcinia bioflavonoids. Therefore, BK seed could be considered a potential functional food candidate for reducing ageing and ageing-related biochemical impairments.

Keywords: *Bitter kola seed; Ageing; Drosophila melanogaster; ROS; Antioxidant.*

*Author for correspondence: *Email: goboh@futa.edu.ng; Tel: +234(0)7031388644*

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INTRODUCTION

Ageing, though considered as a multifactorial process, is the continuous buildup of changes over time that is linked with or results in an increased susceptibility to disease and death due to advancement in age (Harman, 1981). The major risk factor for some widespread diseases, like neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, is ageing and with the increase in ageing population posing a great threat to human health (Niccoli *et al*., 2012; Beard *et al*. 2015). The mitochondria are crucial in the free radicals theory of aging because it produces reactive oxygen species (ROS) and is also targeted by the ROS. Elevated level of oxidative stress can lead to lipid, protein and DNA oxidation which affect the mitochondrial function. When the level of ROS generated is above the pathological threshold, it results in apoptosis (programmed cell death) (Grimm and Eckert, 2017). In the determination of longevity, significant evidence has been established indicating the production of ROS and the subsequent response to oxidative stress as key factors (Long *et al*., 2014).

Alzheimer's disease (AD) is characterized by disturbances in cognitive functions including memory, decision-making, attention, planning, spatial orientation and language (Klafki *et al*., 2006). Contrary to general belief, statistics have shown that the black race have more incidences of AD relative to Caucasians (Froehlich *et al*., 2001); Recent reports have revealed that over thirty five million people in the world are living with some kind of dementia. The number is expected to increase to 65.7 million by 2030 and 115.4 million by 2050 (Prince *et al*., 2013). Interestingly, low prevalence of dementia in older persons was reported for the sub-Saharan Africa. However, according to reports from Alzheimer's Disease International, the prevalence of dementia in sub-Saharan Africa's growth rate is projected from 2.13 million people in 2015 to 3.48 million by 2030, and 7.62 million in 2050 (Guerchet *et al*., 2017).

Drosophila melanogaster has been widely used as models to study biochemical and molecular mechanisms underlying human diseases. The model has revealed a significant similarity in neurotoxicity between flies and humans. Different strains of flies used for extended lifespan experiment

show an increase in resistance to oxidative stress which is related to improved antioxidant enzymes' activity (Dudas *et al*., 1995; Harshman *et al*., 2000). Consequently, this is evidence that such fly model is a useful tool for investigating biomolecular mechanisms of age-related neurotoxicity, lifespan and screen potential therapeutic candidates.

Bitter kola (*Garcinia kola*) seed is a major food in sub-Saharan Africa with enormous medicinal properties. The seed offers many bioactive phytochemicals including alkaloids, phenolics, and bioflavonoids. Major works have been limited to kolaviron, the major bioflavonoid in BK seed, reporting among others its neuroprotective properties in experimentally induced neurotoxicity (Ijomone *et al*., 2012; Olajide *et al*., 2016; Farombi *et al*., 2018). However, in this study, we consider the general consumption pattern of BK seed rather than its extracts or fractions to understand how it can be an available neuroprotective functional food and improve longevity in a model of *Drosophila melanogaster*.

MATERIALS AND METHODS

Collection and Preparation of Sample: Fresh bitter kola (BK) seeds were sourced from Akure metropolis, South West, Nigeria, in the month of April, 2018. The samples were washed under running tap water to remove the dirt. The seeds were chopped into pieces and then air-dried until a constant weight was achieved. Thereafter, the samples were pulverized using a stainless steel blender. The powdered samples were stored in air-tight containers and kept in the refrigerator for further analysis.

Chemicals and Reagents: Chemicals and reagents used for this study such as n-n-diethyl-para-phenylenediamine (DEPPD), Thiobarbituric acid, Folin-ciocalteau's reagent and Trichloroacetic acid (TCA) were sourced from Sigma Aldrich, Chemie GmbH (Steinheim, Germany). Acetic acid, hydrochloric acid, aluminium chloride, potassium acetate, sodium dodecyl sulphate, dichromate acetic acid, Iron (II) sulphate, potassium ferricyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England).

D. melanogaster Stock Culture: *Drosophila melanogaster* (W1118 strain) stock culture was obtained from the Drosophila research lab, Functional Food and Nutraceutical Unit, Federal University of Technology Akure, Nigeria. The flies were fed a corn meal-based diet with 1% w/v brewer's yeast and 0.08% v/w nipagin at 25±2°C under 12 h dark/light cycle conditions.

Experimental Design: Three to five days old flies (both gender) were divided into 3 groups containing 40 flies each. Group I was normal control flies placed on a normal d*iet al*one, while group II and III, were flies placed on a normal diet containing BK seed at 0.05 and 0.1% of diet (equivalent weight replacement) respectively. The flies were fed these diets for 10 days. All experiments were carried out in triplicate with each experimental group consisting of five vials.

Treatment

Survival and Lifespan Study: The effect of dietary inclusion of BK seed on survival and lifespan of flies was assessed. 3-5 days old flies (both gender) were divided into three groups containing 40 flies each. Group I was fed the basal diet while groups II and III were exposed to 0.05 and 0.1% dietary inclusions of BK seed respectively (the choice of these percentage inclusions were based on our earlier study (Oboh *et al*., 2018) which showed that 0.1% BK seed dietary inclusions was tolerable to flies and increased their survival rate). This was followed by daily observation for possible mortality as an index of the survival throughout their lifespan. The survival data were subsequently analysed and plotted as a Kaplan-Meier survival plot after the treatment period.

Behavioural studies

Measurement of Locomotor Performance by Negative Geotaxis Assay: The flies were analysed for their locomotor performance after the treatment period via the negative geotaxis assay (Klafki *et al*., 2006). The experimental apparatus consist of a labelled sterilized tube (11 cm in length 3.5 cm in diameter) into which flies were transferred after being immobilized on ice. This was followed by a period of 10 min recovery, after which the flies were tapped at the bottom of the tube and the number of flies that crossed the 6 cm line within 30 s was recorded. The experiment was carried out in triplicates and the results represent mean of percentage of flies that escaped beyond a minimum distance of 6 cm in 6s.

Bioassays

Preparation of Tissue Homogenate: Flies were immobilized on ice and homogenized in homogenizing buffer (0.1 M phosphate buffer, pH 7.4) as previously described by Oboh *et al*., (2018). The homogenates were used for the various biochemical assays.

Determination of Cholinesterase Activity: Cholinesterase (AChE and BChE) activities were assayed according to the method of Ellman as previously reported (Oboh *et al*., 2018). The reaction mixture was made up of 50 µL of distilled water, 50 µL of 0.1M potassium phosphate buffer (pH 7.4), 30 µL of 10 mM 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), 15 µL of tissue homogenate, and 30 µL of 8 mM acetylthiocholine and butrylthiocholine iodide as substrates. Thereafter, reaction was monitored for 5 minutes at 412 nm in a spectrophotometer. The Cholinesterase activity was thereafter calculated and expressed as µmolAcSch/h/mg protein.

Determination of ROS levels (DCF assay): The method of Perez-Severiano *et al*. (2004) was used as recently described (Abolaji *et al*., 2017) to determine the level of ROS in the flies. Briefly, the reaction mixture was made up of 200 µL of 0.1 M potassium phosphate buffer (pH 7.4), 80 µL of distilled water, 10 µL of 200 µM of 2′,7′-dichlorofluorescein diacetate (DCFD) (final concentration of 5 µM), and 10 µL of sample (1:10 dilution) in a quartz cuvette (300–700 µL). Thereafter, fluorescence emission of DCF from the oxidation of DCFH was monitored for 10 min at 30 s intervals at 480 (excitation) and 530 nm (emission) wavelengths in a Spectramax spectroflourimeter (Molecular Devices, USA). The rate of

DCF formation was thereafter expressed as a percentage of the control.

Determination of thiobarbituric acid reactive substances: The thiobarbituric acid reactive substances were quantified as markers of lipid peroxidation end product using an established protocol as previously reported (Adedara *et al*., 2016) with slight modifications. In brief, 0.05 ml of tissue homogenate was added to 0.15 ml of 8.1% Sodium dodecyl sulfate (SDS), 0.25 ml HCL/ acetic acid (pH= 3.4) and 0.25 ml of Thiobarbituric acid (TBA) and the mixture was incubated at 100^0C for 1 hour. The resulting thiobarbituric acid reactive substances (TBARS) were quantified at 532 nm in a spectrophotometer and calculated as MDA equivalent.

Determination of Total Protein: Protein content of the homogenate was measured by the Coomassie blue method as previously described (Oboh *et al*., 2018).

LC-MS/MS Analysis: An ultrasonic system with working frequency fixed at 20 kHz (Elmasonic S 100 H, Germany) was used for extraction of phenolic compounds from the plant material samples (Vilkhu *et al*., 2008). Briefly, the dried and powdered plant material (1-4 g) was mixed with methanol in a 50 mL amber glass jar and the suspension was exposed to acoustic waves for 1 hour. The temperature (25 ± 2°C) was controlled by continuously circulating cold water using an external water bath. After the ultrasound accelerated extraction process, the supernatant was recovered and filtrated. The solvent was removed from the extracts under vacuum condition (Vacuum Pump, V-100, BÜCHI) using rotary evaporator (Hei-VAP, Heidolph). The dried extracts were stored in amber vials at +4°C before phytochemical analysis. LC-MS/MS analysis were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC- MS/MS instrument equipped with an ESI ion source was used in the negative ionization mode. Separations were performed on an ODS 150 x 4,6 mm, i.d., 3 µm particle sizes, octadecyl silica gel analytical column operating at 40ºC at a flow rate of 0.5 ml/min.

Statistical Analysis: The results were pooled and expressed as mean ± standard deviation (SD). Statistical significance was analysed with 2-way ANOVA coupled with Bonferoni post hoc test and level of significance set at $p<0.05$. All statistical analysis was carried out using Graph pad PRISM (V.5.0).

RESULTS

Effect of dietary inclusions of bitter kola (BK) seed on life span of D. melanogaster: Result on the assessment of the effect of dietary inclusions of bitter kola (BK) seed (0.05% and 0.1%) on life span (Fig. 1) of D. melanogaster revealed that there was no significant difference in the percentage survival of the flies to inclusions of BK seed, compared to control flies. The life span of flies was reduced by 40% which was restored by 12.7% in flies fed diet supplemented with 0.05% BK seed, while flies fed 0.1% BK seed exhibited no significant change in their life span.

Effect of dietary inclusions of bitter kola (BK) seed on locomotor performance of D. melanogaster: Presented in Fig. 2 is the negative geotaxis assay results of flies fed diet supplemented with BK seed (0.05 and 0.1%) as a measure of their locomotor performance. It was observed that the control flies exhibited significantly lower ($p<0.05$) climbing ability at day 10 compared day 5 which was significantly ameliorated in flies fed diet supplemented with BK seed.

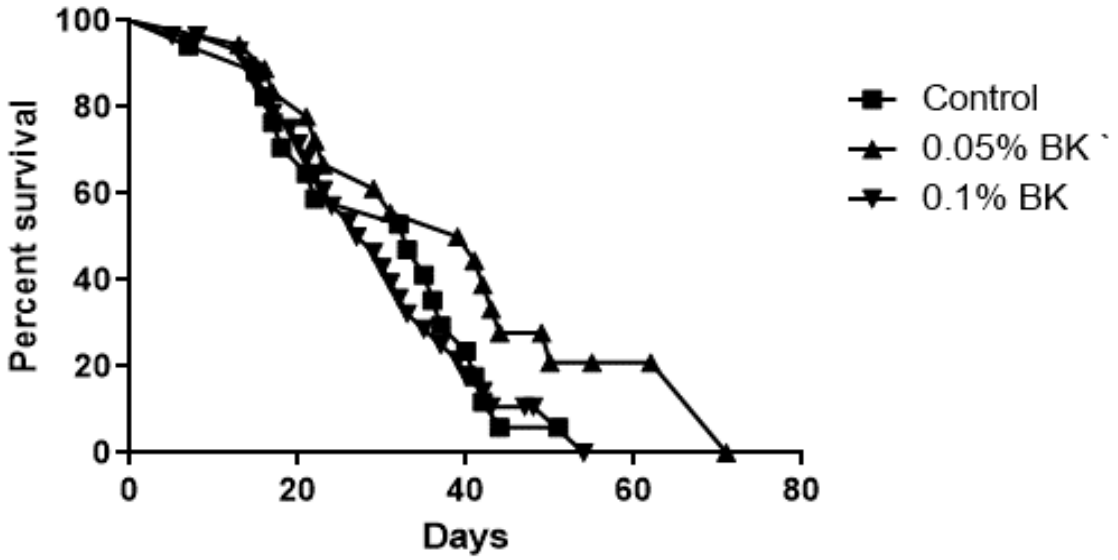

Figure 1:
Effect of Dietary Inclusions of Bitter Kola (BK) Seed on Life span of *D. melanogaster* Values represent mean ± SD**.**

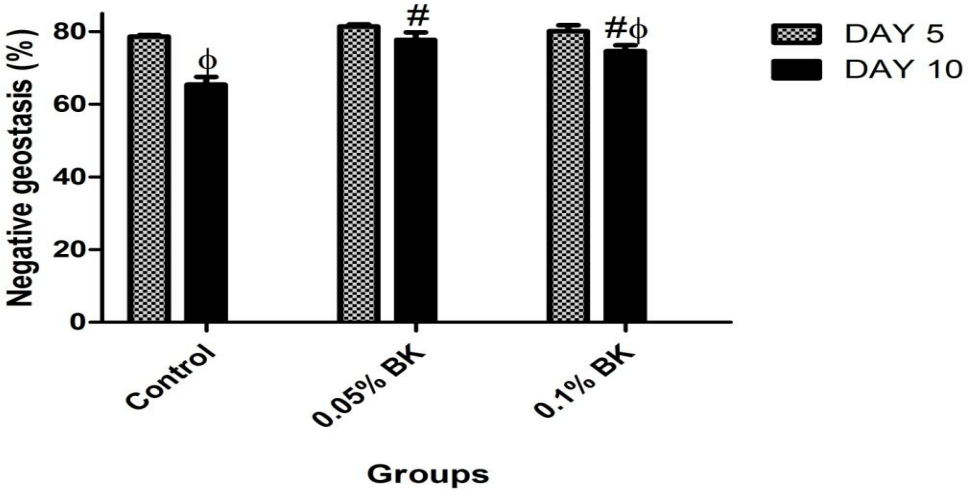

Figure 2:
Effect of Dietary Inclusions of Bitter Kola (BK) seed on Negative Geotactic ability of *D. melanogaster* of *D. melanogaster*. Values represent mean ± SD. # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10

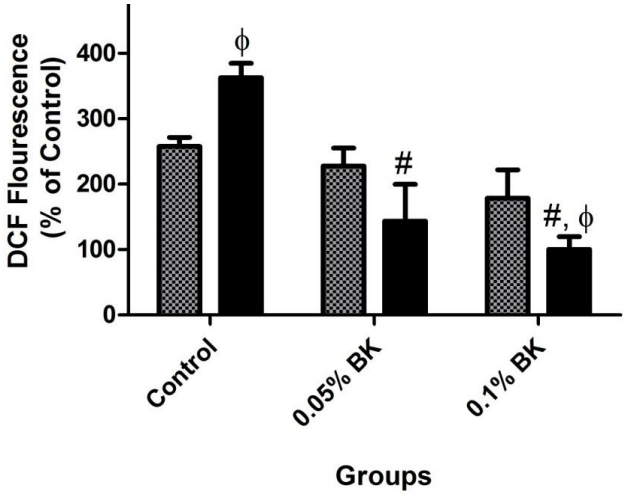

Figure 3:
Effect of Dietary Inclusions of Bitter Kola (BK) seed on Reactive oxygen species (ROS) level of *D. melanogaster*. Values represent mean ± SD. * mean values are significantly different (P<0.05) compared to Control flies at Day 5; # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10

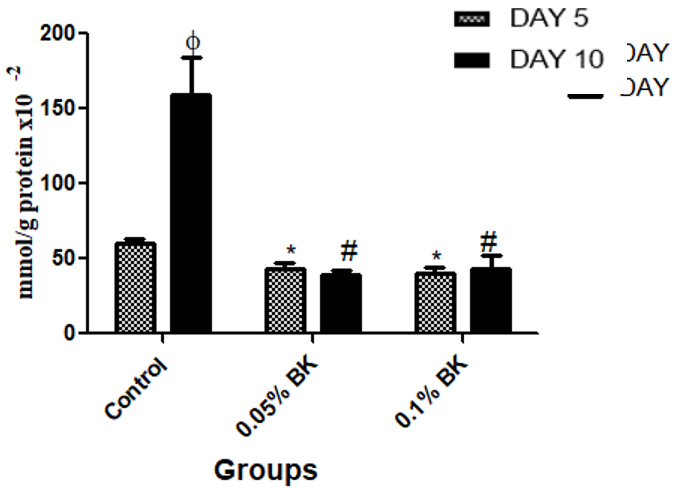

Figure 4:
Effect of Dietary Inclusions of Bitter Kola (BK) seed on thiobarbituric acid reactive substances (TBARS) content of *D. melanogaster*. Values represent mean ± SD. * mean values are significantly different (P<0.05) compared to Control flies at Day 5; # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10.

Effect of dietary inclusions of bitter kola (BK) seed on Catalase activity, ROS and TBAS levels of D. melanogaster: A significant increase in ROS (Fig. 3) and TBAS levels (Fig. 4) were observed in flies which progresses with age. However, the groups of flies exposed to BK seed dietary inclusions were able to exhibit ameliorated levels of both ROS and TBA. A significant increase in catalase activity was observed in the control flies, whereas there was a significant decrease in those fed diet supplemented with BK seed when compared with the control flies.

Effect of dietary inclusions of bitter kola (BK) seed on cholinesterase activities of D. melanogaster and LC/MS/MS characterization of BK seed: Fig. 6 and fig. 7 showed that the control flies exhibited significant age-dependent elevation in AChE and BChE activities at day 10 when compared with day 5. However, it was observed that flies fed diet supplemented with 0.05% and 0.1% BK seed exhibited significant reduction of both AChE and BChE activities, when compared with the control flies. Furthermore, the LC/MS/MS characterization of BK seed (table 1 and Fig. 8) revealed the presence of Garcinia bioflavonoids, kolaflavones and binaringenin..

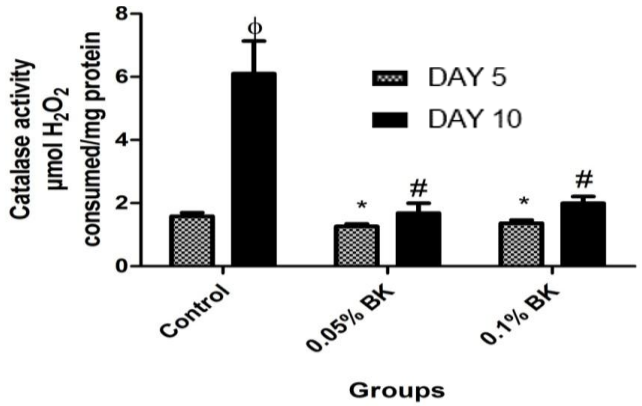

Figure 5:
Effect of Dietary Inclusions of Bitter Kola (BK) seed on catalase activity in *D. melanogaster*. Values represent mean ± SD. * mean values are significantly different (P<0.05) compared to Control flies at Day 5; # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10.

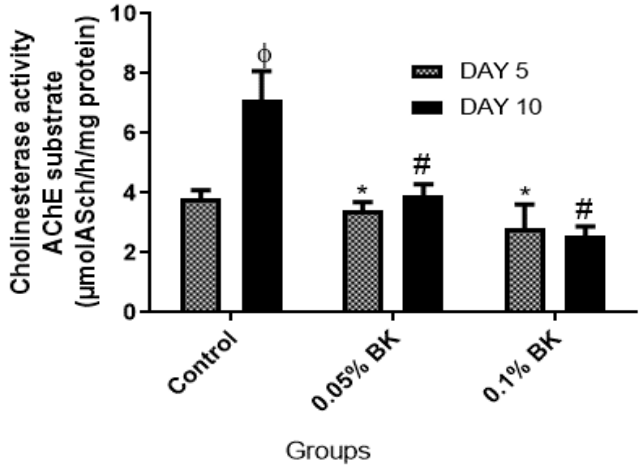

Figure 6
Effect of Dietary Inclusions of Bitter Kola (BK) seed on Cholinesterase (AChE Substrate) activity of *D. melanogaster*. Values represent mean ± SD. * mean values are significantly different (P<0.05) compared to Control flies at Day 5; # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10.

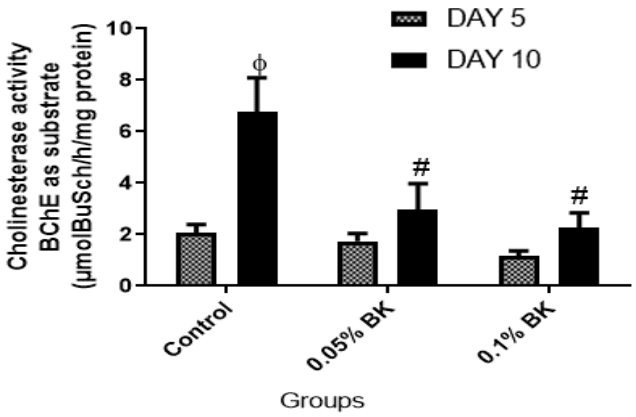

Figure 7:
Effect of Dietary Inclusions of Bitter Kola (BK) seed on Cholinesterase (BChE Substrate) activity of *D. melanogaster*. Values represent mean ± SD. * mean values are significantly different (P<0.05) compared to Control flies at Day 5; # mean values are significantly different (P<0.05) compared to Control flies at Day 10; ϕ mean values are significantly different (P<0.05) considering the interactions between Day 5 and 10.

Table 1.:
LC/MS/MS characterization of phenolics in Bitter kola seed

RT* min	$[M-H]^-$ *m/z*	Fragments	Identification
13.7	573	447	Garcinia biflavonoid 2
14.7	556	431,403, 295, 269, 125	Smilar to Kolaflavone
16.3	587	433, 295, 125	Kolaflavanone
17.1	557	431,295, 125	Garcinia biflavonoid 1
19.1	541	415, 151, 125	Binaringenin

*Retention time

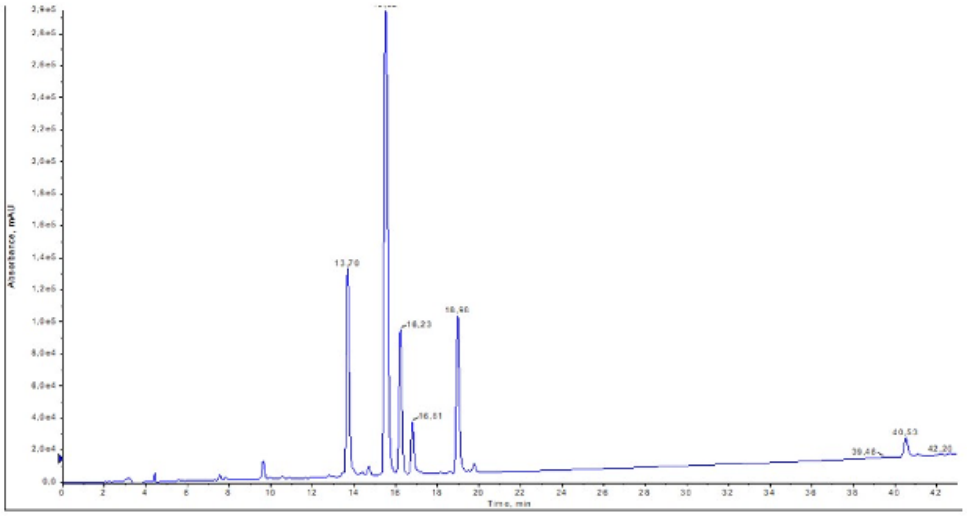

Figure 8:
LC/MS/MS phenolic characterization chromatogram for Bitter kola seed

DISCUSSION

The findings of this study suggest the neuroprotective properties of BK seed dietary supplementation due to its ability to significantly ameliorate the age-dependent decline in locomotor performance of flies. Furthermore, the ability of the dietary inclusion of BK seed at 0.05% to increase the life span of flies further suggests their neuroprotective properties and this could be associated with their constituent polyphenols with antioxidant and anticholinesterase properties. It has been shown that consumption of plants rich in polyphenols can prevent progression of ageing and its co-morbidities (Uysal *et al*., 2013). Reduced life span is a major index of Drosophila model of AD and could be mediated by multifactorial pathological events.

Oxidative stress is a major risk factor for aging, and is associated with several neurophatological conditions. In this present study, significant increase in ROS and TBARS level was observed in the control flies, and progresses with age. However, the groups of flies exposed to BK seed dietary inclusions were able to ameliorate these conditions. Ageing has been reported to be associated with increased ROS generation and TBARS production in Drosophila (Lennicke *et al*., 2020). Induction of oxidative stress during ageing could be linked to the excessive free radical generation and impairment of antioxidant enzymes in drosophila model (Cenini *et al*., 2019). Furthermore, the observed amelioration of elevated ROS and TBARS levels in BK supplemented diet fed flies indicate that BK seed exhibited antioxidant properties. This could be associated with antioxidant phytoconstituents present in BK seed, especially polyphenols. Previous studies have shown that BK seed has an abundance of bioactive phenolic compounds with antioxidant properties (Okwu, 2005; Okoko, 2009a; Adedara and Farombi, 2012). The significant increase in catalase activity observed in the control flies could be attributed to the increase in free radical generated (Fig. 3), and the flies exposed to BK seed dietary inclusions were able to ameliorate these conditions, hence, the reduction observed in the catalase activity, when compared with the control group.

Neurodegenerative and age-related diseases such as AD is characterized by gradual loss in brain cholinergic neurons which are critical to learning and memory functions (Oboh *et al*., 2017). Consequently, the use of cholinesterase inhibitors is common clinical interventions for AD. These drugs functions to prevent rapid breakdown of acetylcholine by inhibiting the activity of the degrading enzymes (Mayeux, 2001). The ability of BK seed dietary supplement to ameliorate increased cholinesterase activities using two substrates in the flies in this study could further support their therapeutic properties against age-related diseases and improve their lifespan. It is noteworthy that previous studies have established the anticholinesterase properties of kolaviron, a biflavonoid complex and a major constituent in BK in both cell-free and cell-based experimental systems (Ishola *et al*., 2017). Therefore, the observed amelioration in elevated cholinesterase activities in flies fed diet supplemented with BK seed could be associated with the constituent phytochemicals, especially the polyphenols. Furthermore, in accordance with previous studies (Okoko, 2009b; Ayepola *et al*., 2013), the LC/MS/MS characterization of BK seed revealed the presence some polyphenols which are Garcinia bioflavonoids, kolaflavones and binaringenin. The role of polyphenols as anti-ageing agents has been previously reported (Rolt *et al*., 2020). Indeed, phytochemicals with antioxidant properties are becoming rich sources for management of age-related neurodegenerative diseases such as AD (Calcul *et al*., 2012).

In conclusion, this study has shown that BK seed supplemented diet ameliorated age-associated impaired cholinergic enzyme activities using two substrates and improves antioxidant status in *Drosophila melanogaster*. This could contribute to their improved locomotor performance and increased lifespan especially at lower percentage inclusion. Thus, BK seed could hold promise as functional food for the management of ageing and ageing-related biochemical impairments; further studies at clinical trials are, however, recommended.

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