Kolaviron, isolated from *Garcinia kola*, inhibits acetylcholinesterase activities in the hippocampus and striatum of wistar rats

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KEY WORDS

Kolaviron
Acetylcholinesterase inhibitors
Acetylcholine
Histochemistry
Brain

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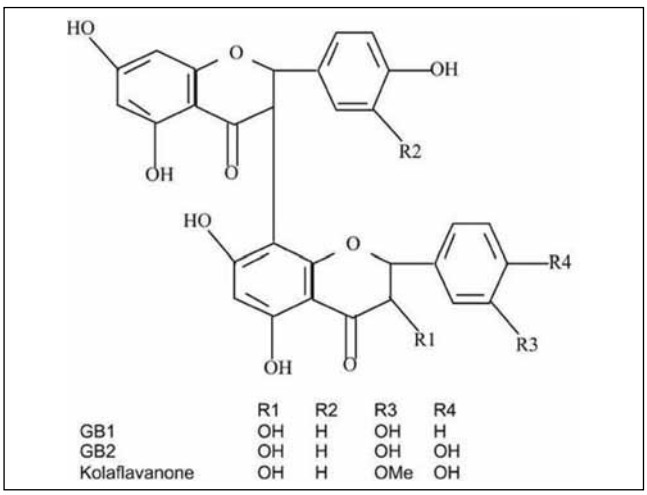
ABSTRACT

Background: Kolaviron, isolated from seeds of *Garcinia kola*, have been shown to possess wide pharmacological properties. **Purpose:** The present study examined the effect of kolaviron on acetylcholinesterase activities in the hippocampus and striatum of adult Wistar rats. **Methods:** In this study, histological and histochemical methods were used to investigate the effects of kolaviron on the histology of the hippocampus and striatum and on acetylcholinesterase activities in these brain regions. **Results:** We showed that kolaviron produced no neurodegenerative changes in the hippocampus and striatum. Kolaviron did not significantly alter ($p<0.05$) neuronal density in these brain regions. Kolaviron significantly reduced ($p<0.05$) acetylcholinesterase staining intensity, suggesting a likely inhibiting effect on this enzyme. **Conclusion:** To the best of our knowledge, this study provides the first evidence that kolaviron could act as an acetylcholinesterase inhibitor. Kolaviron may be developed as a herbal-based natural product with therapeutic potential in the management of neurodegenerative disorders associated with disturbed cholinergic neurotransmitter systems.

doi : 10.5214/ans.0972.7531.200203

Introduction

Kolaviron is the major component isolated from the seeds of *Garcinia kola* and contains biflavanones (GB1, GB2 and kolaflavanone).[1,2] *Garcinia kola* Heckel (family *Guittiferae*)[3] is a herb grown in Nigeria and has a striking astringent, bitter and resinous taste. It is popularly called Bitter Kola in Nigeria. Local Nigerian names include; Orogbo in Yoruba, Ugolu in Ibo and Akan in Urhobo. It is used in folklore remedies for the treatment of several ailments such as liver disorders, hepatitis, diarrhoea, laryngitis, bronchitis and gonorrhoea. Flavonoids, oleoresins, tannins, saponins, alkaloids, cardiac glycosides are amongst the phytochemical substances that have been isolated from *G. kola*.[1] The pharmacodynamics behind *G. kola* action is based on kolaviron (Figure 1).[4–6] Kolaviron, the biflavonoid complex in *G. kola*, is responsible for the strong antioxidant properties of *G. kola* which limits the oxidative conversion of amino acid by reactive oxygen species to other damaging fatty acid products.[4] It has been reported to prevent hepatotoxicity mediated by several toxins.[2,7,8] It exhibit strong antioxidant activities both in vivo and in vitro experimental models.[9]



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Fig. 1: Chemical structure of kolaviron.

Acetylcholinesterase (AChE) plays a crucial role in cholinergic neurotransmitter systems. It is responsible for terminating the nerve impulses at cholinergic and neuromuscular synapses by splitting the neurotransmitter acetylcholine (ACh) into choline and acetate.[10,11] ACh is dynamic neurotransmitter, acting both in the central and peripheral nervous system. ACh is known to classically excite hippocampal pyramidal neurons by acting as a powerful modulator of synaptic transmission at both GABAergic and glutamatergic synapses through a broad range of muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs).[12] ACh exerts powerful modulatory effects in the striatum which has been recognized as one of the brain areas with the highest concentration of markers of cholinergic neurotransmitter systems. ACh also acts via a variety of mAChRs and nAChRs in the striatum, where it affects the activity of striatal neurons both directly and through modulation of glutamate release from corticostriate terminals and of dopamine release from nigrostriatal terminals.[13] As a result of its powerful modulatory activities on striatal and hippocampal neurons, ACh plays an important role in movement, learning and memory.[14]

In view of foregoing, ACh and AChE activities are pivotal factors in the development of drugs for the management of neurodegenerative disorders. Since there is a global advocacy for increase in use of herbal sources in therapeutic management of various diseases including neurodegenerative disorders, the present study has attempted to localize histochemically the activity of AChE in the hippocampus and striatum following kolaviron treatment, as well study the effect of kolaviron on the histology of the hippocampus and striatum in adult Wistar rats.

Methods

Animal management

Twenty adult male albino Wister rats weighing between 150 g-200 g were used for this study. The animals were housed in

the animal holding of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife. The animals were fed with standard rat pellet and given water liberally. Animal were housed in clean plastic cages under natural light and dark cycle and at room temperature. All animals were handled in accordance with the guidelines for animal research as detailed in the Guidelines for the Care and Use of Laboratory Animals by the National Research Council of the National Academy of Sciences, 2011.[15]

Kolaviron extraction and Animal treatment

Kolaviron was isolated from *G. kola* as previously described.[1] In brief, powdered dried seeds of *G. kola* were extracted with n-hexane, in a Soxlet extractor. The defatted, dried marc was repacked and then extracted with methanol in a Soxlet extractor. The extract was concentrated and diluted to twice its volume in distilled water and partitioned with chloroform. The concentrated chloroform fraction gave a yellow-brown solid known as kolaviron. Animals were randomly divided into four groups (A, B, C, and D) of five rats each. Group B, C and D were experimental groups and were given 200, 400, and 800 mg/kg body weight of kolaviron daily for 4 weeks. Kolaviron was dissolved in cornoil (Sigma USA), and given orally using an intragastric tube. Group A served as control and were given vehicle for extract (corn oil) for 4 weeks. At the end of administration, animals were sacrificed by cervical dislocation and the brain excised. A mid-sagittal cut of the brain was made. Some of the brain tissues were fixed in 10% formal saline for histological studies and others in cold 10% formol calcium for 48 hours and used for histochemical studies. Tissues for histological studies were processed for routine paraffin wax embedding, sectioned on a rotary microtome at 6 μm thickness, and stained using haematoxylin and eosin (H&E) method described by Drury and Wallington, 1980.[16]

Histochemical demonstration of AChE

Serial sections of 10 μm thickness were obtained on a cryostat. Sections were processed for AChE demonstration by using acetylthiocholine iodide as substrate (as previously described by Felipe and Lake, 1983),[17] in a solution containing cuprous and ferric sulphate (as modified by Ogundele et al, 2012).[18]

Working solutions of the incubating medium were prepared a clean room under standard laboratory conditions. 5 mg of acetylthiocholine iodide (Sigma, USA), was weighed using a sensitive weighing balance. 6.5 ml of 0.1 M acetate buffer (pH.6.0) was prepared by dissolving 0.605 g of acetic acid in 100 ml of distilled water and the pH adjusted using sodium hydroxide. 0.1 M sodium citrate prepared by dissolving 2.94 g of sodium citrate in 100 ml of water. 30 mM cuprous sulphate prepared as 0.58 g of salt in 100 ml of purified water. 5 mM potassium ferricyanide prepared by dissolving 0.165 gm of salt in 100 ml of purified water. To prepare the incubating medium, 5 mg of acetylthiocholine iodide was added to 6 ml of acetate buffer in a glass conical flask and the following reagents were added in this order; 0.5 ml of 0.1 M sodium citrate, 1 ml of 30 mM cuprous sulphate and 1 ml of 5 mM potassium ferricyanide. The mixture was continually stirred with a magnetic stirrer. The incubating medium was applied to sections and incubated in an oven at 37^0C for 20 minutes, rinsed in distilled water and counter stained in haematoxylin, cleared and mounted in DPX. Areas of AChE are seen as brown or red coloured under a light microscope. All reagents used are of analytical grade.

Photomicrography, Histomorphometry, Image analysis and Statistical analysis

Sagittal stained sections were viewed under a Leica DM750 digital light microscope, and with the aid of an atlas of the rat brain,[19] the striatum and hippocampus were located and observed. Digital photomicrographs were taken by an attached Leica ICC50 camera. Photomicrographs of H&E stained section were imported onto on to OpenOffice.org™ (OOo-dev 3.4.0) software for histomorphometric neuronal count. Image Analysis and Processing for Java (Image J), a public domain software sponsored by the National Institute of Health (USA), was used to analyze and quantify AChE staining intensity. Imported RGB images are converted to grayscale images on Image J. The software quantifies staining intensity by measuring the pixel value of each pixel in grayscale images following threshold of areas of staining activity and converting the pixel value to brightness value or gray value, in a scale of 0 to 255 from less brighter (that is more intensity) to more brighter (that is less intensity). Also percentage area of AChE activity was also measured. Data were expressed as mean $\pm$ SEM, analyzed using One-way ANOVA, and followed by Student Newman-Keuls (SNK) test for multiple comparisons. GraphPad Prism 5 (Version 5.03, GraphPad Inc.) was the statistical package to be used for data analysis. Significant difference was set at $p<0.05$.

Results

Effect of kolaviron on hippocampus and striatum

In the present study, treatment with kolaviron at 200, 400 and 800 mg/kg body weight did not cause any histological alteration or neurodegenerative changes in the hippocampus and striatum. Photomicrographs of control and treated groups showed normal histology of the hippocampus, with numerous pyramidal shaped neurons in the CA3 (*Cornu Ammonis*) region of the hippocampus proper (Figure 2) and also normal

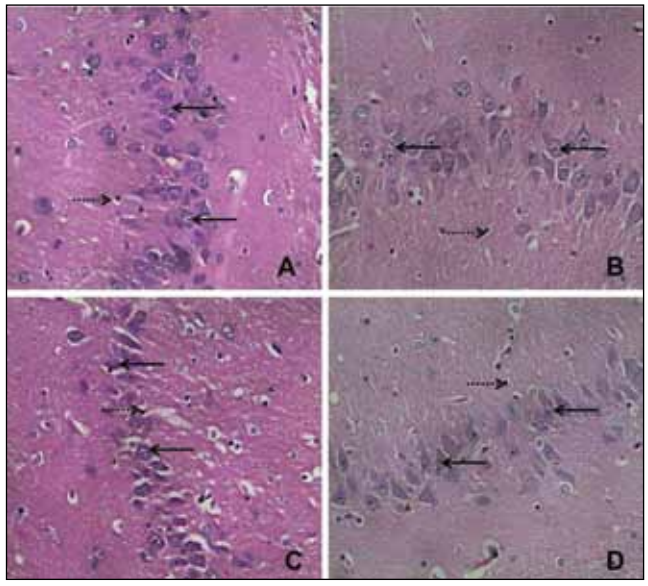


Fig. 2: Photomicrographs of CA3 region of the hippocampus. H&E $\times$400. Observe normal pyramidal neurons (arrows), with distinct blue staining and prominent deep blue nucleoli in Control (A) and Treated groups (B, C, and D). Also easily identified are numerous oligodendrocytes (dashed arrows) with classic "fried egg" appearance

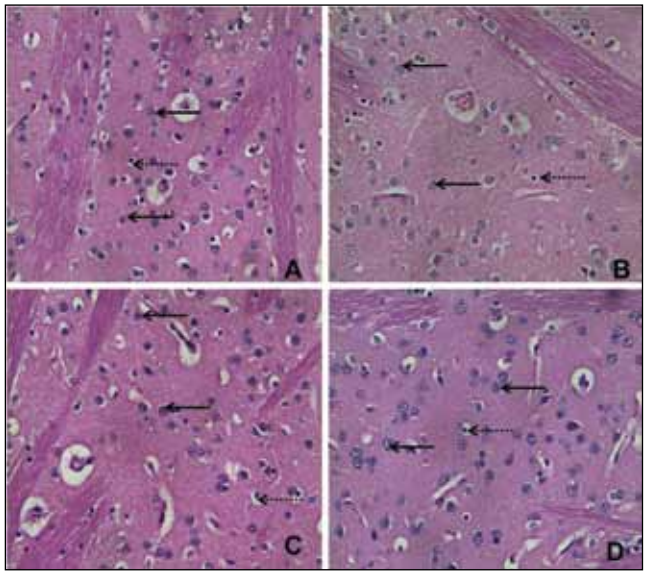


Fig. 3: Photomicrographs of the Caudate Putamen (Striatum). H&E ×400. Observe normal pyramidal neurons (arrows), with distinct blue staining and prominent deep blue nucleoli in Control (A) and Treated groups (B, C, and D). Also easily identified are numerous oligodendrocytes (dashed arrows) with classic "fried egg" appearance.

Table 1: Histomorphometric neuronal count in the hippocampus and striatum

	Hippocampal (CA3) neuronal count ($N/10^3 \mu m^2$)	Striatal (CPu) neuronal count ($N/10^3 \mu m^2$)
Group A	34.96 ± 1.90	93.17 ± 3.64
Group B	38.92 ± 1.37	83.35 ± 4.14
Group C	31.48 ± 1.69	89.43 ± 2.35
Group D	34.77 ± 1.59	94.80 ± 3.61

Values are mean ± SEM of data obtained. (n = 3) N – Numbers of neurons

histology of neurons within the striatum (CPu – Caudate Putamen) (Figure 3). Neurons exhibit distinct blue nuclei staining with prominent deeply stained nucleoli. Also numerous oligodendrocytes are clearly identified with their classic "fried egg" appearance as seen in non-perfused brain tissues. Histomorphometric neuronal count showed no significant difference ($p<0.05$) in neuronal density of CA3 hippocampal neurons and striatal neurons (Table 1).

Effects of kolaviron on hippocampal and striatal AChE activities

The present study has also shown that kolaviron reduced AChE staining intensity in hippocampus and striatum. As shown in Figure 4 and Figure 5, treatment with kolaviron at the various doses reduced the staining intensity AChE compared to control. Further analysis confirmed significant decrease ($p<0.01$) in staining intensity in kolaviron treated animals as shown by higher mean gray values compared to control groups in the hippocampus and striatum, though there was no significant

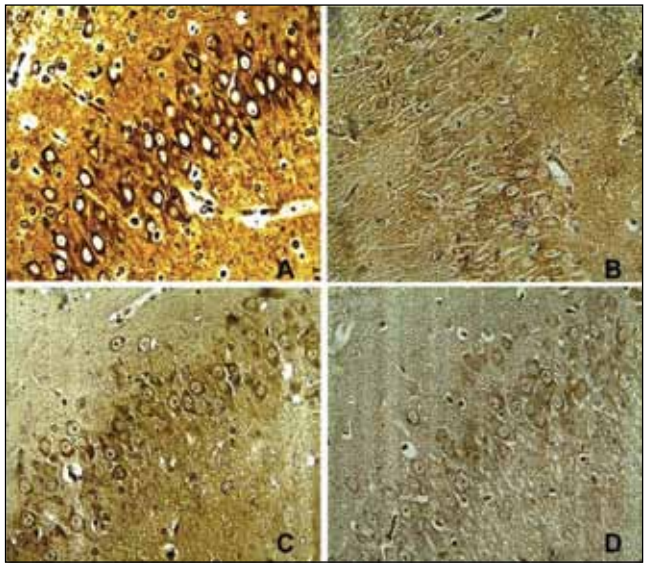


Fig. 4: Photomicrographs of CA3 region of the hippocampus for histochemical demonstration of AChE activities. ×400. Observe decreased staining intensity of Treated groups (B, C, and D) compared to Control (A).

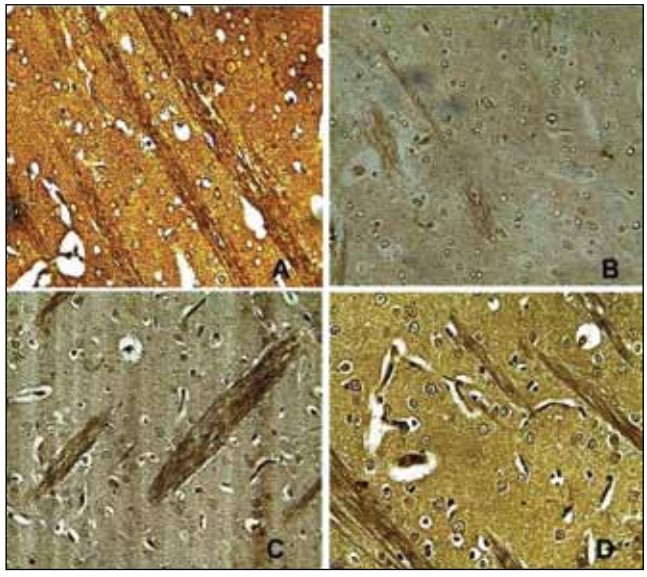


Fig. 5: Photomicrographs of the Caudate Putamen (Striatum) for histochemical demonstration of AChE activities. ×400. Observe decreased staining intensity of Treated groups (B, C, and D) compared to Control (A).

difference ($p<0.05$) in the percentage area of AChE activity. No significant difference ($p<0.05$) in staining intensity was observed between kolaviron treated groups (Table 2).

Discussion

We have previously reported that kolaviron could afford some protection to hippocampal neurons following methamphetamine-induced neurotoxicity.[1] Also kolaviron has been shown to protect neurons against gamma radiation induced oxidative

Table 2: Image analysis of AChE staining intensity in the hippocampus and striatum

	Hippocampus		Striatum	
	Mean Gray Value	%Area of AChE Staining	Mean Gray Value	%Area of AChE Staining
Group A	69.63 ± 4.36	52.88 ± 2.58	67.09 ± 1.65	54.88 ± 3.03
Group B	94.97 ± 2.43 α	55.72 ± 1.78	96.76 ± 6.83 α	48.50 ± 1.80
Group C	96.71 ± 2.90 α	52.92 ± 2.89	89.34 ± 4.53 α	48.16 ± 2.82
Group D	96.55 ± 1.76 α	51.70 ± 3.10	92.28 ± 3.35 α	54.36 ± 3.86

Values are mean ± SEM of data obtained. (n = 3).
α - p<0.01 compared to Control.

stress.[20] The present study indicates that kolaviron at various doses, does not cause neurodegenerative changes in the hippocampus and striatum.

The results from this study also suggest that kolaviron is a likely inhibitor of AChE activity, as indicated by reduced staining intensity of AChE. Dysregulated cholinergic neurotransmitter systems have also been implicated in the pathophysiology of a variety of neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and Schizophrenia.[13,14] Long-term treatment with acetylcholinesterase inhibitors is presently the main therapy for AD, and shows potential for other neurodegenerative disorders, which includes PD, HD and tardive dyskinesia. AChE inhibitors also show antipsychotic properties that have advanced the development of cholinomimetic therapy for schizophrenia.[14] A decrease in the activity of the cholinergic neurons is a common feature of AD. Currently, four of the five medication used in treatment of cognitive manifestations of AD are AChE inhibitors (donepezil, tacrine, galantamine and rivastigmine) with the other being an NMDA (N-methyl-D-aspartate) receptor antagonist (memantine). The AChE inhibitors are used to reduce the rate at which ACh is broken down, in order to increase the concentration of ACh in the brain and thus compensating for the loss of ACh caused by the death of cholinergic neurons.[21,22] These drugs are not without side effects with the most common side effects include nausea and vomiting, both of which are probably due to excessive cholinergic activity. Other less common side effects are bradycardia (decreased heart rate), reduced appetite and weight, elevated gastric acid production, and muscle cramps.[23] In view of the likely effects of kolaviron as AChE inhibitors, kolaviron could be developed as an alternative therapy probably with fewer side effects, in the management of AD and other neurodegenerative disorders. Kolaviron has already been shown to prevent oxidative damage to the brain following gamma irradiation.[10] Also we have shown that kolaviron improves impaired cognitive functions following methamphetamine challenge in adult rats.[1] Methamphetamine on the other hand has been shown to impair cognitive functions by altering brain ACh systems. Methamphetamine alters ACh receptors in adult rats and decreases choline acetyltransferase, the enzyme responsible for synthesizing ACh, in adult humans.[24] It is thus probable that the likely abilty of kolaviron to inhibit AChE activities in the brain of rats, thus reducing the rate at which ACh is broken down might be a mechanism by which kolaviron improves methamphetamine-impaired cognitive functions.

Conclusions

In conclusion, the key finding of this study was that kolaviron reduces AChE staining intensity in the hippocampus and striatum of adult rats, thus acting as a likely inhibitor of AChE. However, this calls for further studies attempting to corroborate these findings and elucidate the detailed mechanism of a possible kolvarion-mediated inhibition of AChE. Kolaviron may be used as a novel herbal-based natural product with therapeutic potential in the management of neurodegenerative disorders associated with dysregulated cholinergic neurotransmitter systems.

Acknowledgements

The authors acknowledge Dr. Nwoha PU of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, and Prof. Farombi EO, of the Department of Biochemistry, University of Ibadan, Nigeria, for valuable support and technical guidance.

List of Abbreviations

GB – Garcinia biflavonoid
AChE – Acetylcholinesterase.
ACh – Acetylcholine
mAChRs – Muscarinic acetylcholine receptors
nAChRs – Nicotinic acetylcholine receptors
CA – *Cornu Ammonis*
CPu – Caudate Putamen
NMDA – N-methyl-D-aspartate
PD – Parkinson's disease
AD – Alzheimer's disease
HD – Huntington's disease

This article complies with International Committee of Medical Journal editor's uniform requirements for manuscript.

Conflict of Interests: None, Source of funding: None
Received Date : 6 March 2013; Revised Date : 29 March 2013
Accepted Date : 22 April 2013

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