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HISTOLOGICAL STUDY ON THE PROTECTIVE ROLE OF *KHAYA SENEGELENSIS* ON AMITRIPTYLINE-INDUCED STRIATUM NEUROTOXICITY IN MALE WISTAR RATS

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Abstract

Khaya senegalesis(Ks)has been used in ethnopharmacology for the treatment of stroke. This study investigated the histological and protective role of Ks in amitriptyline-induced striatum neurotoxicity in albino Wistar rats. Thirty-nine rats weighing184-254g were randomly assigned to five treatment groups. The positive control (group A) received normal saline while groups B-E were given 4.2mg/kg amitriptyline for 3 day, to induce stroke. Group B rats were stroke induced but not treated. Rats in groups C, D and E received low (200mg/kg), medium (300mg/kg), and high (400mg/kg) doses of Ks respectively. The extracts were administered twice daily for 14 days. The rats were sacrificed using chloroform inhalation and brain collected and fixed in 10% buffer saline for histological analysis. Results revealed normal histoarchitecture of striatum with neurovascular unit, neuron, blood vessel, neuroglial cell in positive control and a degeneration in the striatum with characterized neurovascular unit, with mild lymphocytic infiltrates, moderate to severe degenerated neuron, capillaries proliferation, cellular hypertrophy, and perivascular oedema and astrocytosis in the negative control group. However, administration of low and medium doses of Ks shows minimal to moderate degeneration in the striatum; neurovascular unit, with less lymphocytic infiltrates, moderate degenerated neuron, with loss in nuclei, glial cells, with dense nuclei and glial cell, with mild tissue oedema, mild capillaries proliferation with perivascular oedema. Although administration of high dose of Ks shows normal orientation of striatum; neurovascular unit, neuron, glial cells, with dense nuclei, blood vessels. In conclusion, high dose of Ks at 400mg/kg is protective and ameliorate striatum neurotoxicity induce by amitriptyline.

Keywords: *Khaya senegenlensis*, stroke, amitriptyline, striatum, neurotoxicity

1.0 Introduction

Stroke remains a public health concern and brain injury resulting from stroke is a leading cause of death and long-term disability globally (Adeloye, 2014; Feigin *et al* 2017; Lozano, 2012; Murray, 2012).Stroke also known as cerebrovascular accident is a common neurological disorder or acute cerebral blood circulation disorder with a sudden onset that is caused by cerebral artery stenosis, occlusion or rupture, which may result from a variety of predisposing factors with clinical manifestations indicative of temporary or permanent brain dysfunction (Jauch *et al*., 2013). It is also a clinical syndrome, of presumed vascular origin, typified by rapidly developing signs of focal or global disturbance of cerebral functions lasting more than 24 hours or leading to death. It contributes significantly to the morbidity and mortality of medical admissions and epidemiological evidence suggest that fifteen million cases of stroke are recorded globally every year, with greater than one-third of these cases been fatal (Johnston, *et al*., 2009). Africa bears the greatest burden of hypertension (Global Health Observatory, 2017) which is the strongest and most common modifiable risk factor for stroke (Lackland, *et al.* 2014, Campbell *et al*., 2015) prominent among people of African descent showing disease burden even with younger age having worse outcomes (Owolabi *et al.,* 2009). In Nigeria, stroke is a major cause of hospital admissions, deaths and its incidence has been put at 26/100,000, thus accounting for 2.4% (Ogun, *et al.,* 2005). Studies have also shown that the risk of recurrent stroke is 26% within 5 years of a first stroke and 39% by 10 years (Mohan *et al.,* 2011). It is accepted that 85% of strokes are due to cerebral infarction, 10% due to primary haemorrhage and 5% due to subarachnoid haemorrhage (Mant, *et al.,* 2004),

Neuropathic pain, caused by lesion or dysfunction of the peripheral or central nervous system (Finnerup, 2015), substantially affects the quality of life and is associated with heavy individual and societal burden (Attal, *et al.* 2015). Available treatments with anticonvulsants, antidepressants, opioids, and lidocaine or capsaicin patches are only moderately effective and may induce poorly tolerated side effects that negatively impact compliance (Piano, 2014). Over the past decade, a substantial decrease of stroke mortality was achieved largely due to advancements of acute stroke management such as use of statins (Scheitz, *et al.,* 2016).Numerous peripheral and central pathways have been suggested as new therapeutic avenues (Yaksh, *et al.,* 2015). However, as a substantial proportion of stroke survivors suffer from long-term disability that imposes an enormous medical and societal burden Feigin,*et al.,* 2014, development of effective treatments to improve neurological function for stroke patients are of major importance.

The plant *Khaya senegalensis* (Desr.) belongs to the Juss Family called Meliaceae. It is widely distributed in the sub-Saharan savannah and has a round evergreen crown of dark shiny foliage, pinnate leaves with a characteristic round capsules that grows up to 40m high (Keay, *et al.,* 1998). The therapeutic value of *Khaya senegalensis* has

been recognized in different systems of traditional medicine for the treatment of various conditions. The decoction of the stem bark extract is commonly used for treating jaundice, dermatoses, malaria, fever, mucous diarrhea, and venereal diseases as well as for hookworm infection and a taeniacide remedy.

Amitriptyline hydrochloride, (also known as Elavil) is a drug with a tricyclic antidepressant and analgesic properties, which is widely used in the treatment of depression and neuropathic pain (Bryson, 1996). Although ingestion of Elavil of about 750mg or more by an adult may result in severe toxicity (Evans, 2000). In experimental animals this dosage (overdose) can be used to induce ischemic stroke, including weakness on one side of the body, blurred vision and lack of balancing. This work therefore investigates the neuroprotective role of a natural medicinal plant (Kahayasenegelsis) in ameliorating the effect occasioned by stroke using Wistar albino rats.

2.0 Materials and methods

Place and duration of study: This study was conducted at the Department of Anatomy and Forensic Anthropology, Faculty of Basic Medical Sciences, University of Cross River State, Okuku, Nigeria.

Reagents and chemicals: All reagents and chemicals used were of good analytical grades and quality.

2.1 Collection and identification of plant material:

Fresh matured bark of *Khaya senegalensis* was gotten from a Forest at Ukelle community of Yala LGA of Cross River State, Nigeria. The tree bark was identified and authenticated to be *Khaya senegalensis* in the Department of Botany, University of Lagos, Nigeria and stored in the herbarium.

2.2 Preparation of plant extracts:

Fresh bark of *Khaya senegalensis* was well cleansed and diced into smaller pieces using a sterile knife to aid the drying process and after which they were air dried at room temperature for a period of four weeks. The stem bark was then oven dried at 50c for 3hrs and thereafter homogenized to semi powder form using a manual machine mill. 244g of coarse powder of bark *Khaya senegalensis* was packed into a thimble and inserted to the Soxhlet extractor. The Soxhlet was inserted into the quick fit bottom flask containing solvent. The solution was left to concentrate using a rotary evaporator & the dried extract of *Khaya senegalensis* yielded 221g, was collected and preserved at 4℃ for further use.

2.3 Experimental animals

Thirty-nine adult healthy albino Wistar rats weighing between 184g – 254g were obtained from the animal house, department of Anatomy, University of Cross River State and acclimatized for four weeks before commencement of the experiment. The rats were housed in well-ventilated plastic cages under standard condition of temperature (28 ± 2^0C) and relative humidity (40±5%) with a 12-hour light dark cycle. The animals were maintained on rat chow and provided with water *ad libitum* throughout the duration of the experiment (Enyievi *et. al*, 2020). Hygiene was maintained by constant cleaning and removal of faeces and spilled feed from cages on a daily basis. The

administration regiment lasted for two weeks. At the end of the administration period, the animals were sacrificed and samples collected for analysis.

2.4 Induction of ischemic stroke model

After one week of acclimatization, 100mg of amitriptyline hydrochloride was dissolved in 20ml of distilled water, 1.4ml per kg body weight of the solution was given to each animal in all the treatment groups orally for three days. Ischemic stroke was confirmed and symptom were visible two days later in amitriptyline-induced Wistar albino rats.

2.5 Experimental protocol

Thirty-nine adult healthy albino wistar rats weighing between 184g – 254g were obtained from the animal house, department of Anatomy, University of Cross River State and acclimatized for four weeks before commencement of the experiment. The rats were housed in well-ventilated plastic cages under standard condition of temperature (28±2^{0}C) and relative humidity (40±5%) with a 12-hours light dark cycle. The animals were maintained on rat chow and provided with water *ad libitum* throughout the duration of the experiment. Hygiene was maintained by constant cleaning and removal of faeces and spilled feed from cages on a daily basis. The administration regiment lasted for two weeks. At the end of the administration period, the animals were sacrificed and samples collected for analysis.

Groups	Treatment	Administration
1	Positive control	Normal control + rat chow and water ad libitum
2	Negative control	Stroke - Induced not treated rats
3	Low	AMT +200mg/kg *Khaya senegalenesis*
4	Medium	AMT + 300mg/kg *Khaya senegalenesis*
5	High	AMT + 400mg/kg *Khaya senegalenesis*

Fig 1. Table of group, treatment and administration

2.6 Collection of blood samples for analyses

At the end of two weeks' administration, all animals were fasted for twelve hours but had free access to water. They were anaesthetized under chloroform vapour and dissected. Blood sample was collected by cardiac puncture using 5ml sterile syringe into sample containers, thereafter centrifuge at 3500rpm for 10 minutes to remove blood cells and recover serum. The serum was pipetted using pasteurized pipette into another sample container, stored in a refrigerator at 4^{0}C until when it was subjected to biochemical analysis. Thereafter, the animal skull was opened to harvest the whole brain which was fixed in the perfusion fixative (10% formal saline). After overnight fixation, the brain and organs was taken for tissue processing.

2.6 Tissue processing for heamatoxylin and eosin

After the rats were sacrificed using suffocation by chloroform, Brain tissues were carefully harvested out of the rats, trimmed to remove any blood. The tissues were immediately fixed in neutral buffered formalin; after 72 hours, 2-3mm in thickness were dissected out and post fixed in another freshly prepared neutral buffered formalin and then transferred to a graded series of alcohol.

On day 1, they were placed in 70% alcohol for 7 hours, then transferred to 90% alcohol and left in 100% overnight.

On day 2, the tissues were passed through three changes of absolute alcohol for an hour each then cleared in xylene.

Once cleared, the tissues were impregnated using molten paraffin wax in the oven at 58°C. Three changes of molten paraffin wax at one-hour intervals were made, after which the tissues were embedded in wax and blocked out. Prior to embedding, it was ensured that the mounted sections to be cut by the rotary microtome were orientated perpendicularly. Serial sections of 5µm in thickness from a solid block tissue were cut using a microtome machine. The cut section was fixed on clean albuminized slides to prevent sections washing off the slides and later stained with Haematoxylin and Eosin staining techniques, after which they were passed through ascending grade of alcohol, cleared in xylene and mount using a cover slip in DPX mountant, allowed to dry at room temperature and observed under the digital light microscope.

Tissues fixed in 10% neutral buffered formalin were processed for paraffin wax embedding. This is the most commonly used embedding medium in both normal and pathological histology. Cutting qualities are good, the blocks are durable and their storage presents no special problems. Tissue processing was performed using standard routine methods. All tissues are dehydrated through ascending grades of ethanol by immersion as follows:

70% alcohol - - - 30 mins
90% alcohol - - -- 30 mins
95% alcohol - - -- 30 mins
Absolute alcohol I - -- 30 mins
Absolute alcohol II - -- 30 mins

Dehydrated tissues are cleared in xylene as follows:
1:1 absolute alcohol and xylene- 30 mins
Xylene I - - -- 30 mins
Xylene II - - -- 30 mins

The tissues are infiltrated in three changes of molten paraffin wax at 56^0C as follows:
Paraffin wax I - - -- 40 mins
Paraffin wax II- - -- 40 mins
Paraffin wax III- - -- 40 mins

Tissues were then embedded in paraffin wax using stainless steel embedding moulds smeared with glycerine so that Paraffin blocked tissues can be separated from the mould after embedding. Paraffin blocked tissues are trimmed and mounted on wooden blocks for sectioning on a rotary microtome. Sections of 5 µm wereobtained on a rotary microtome. The sections are spread in warm bath, and collected on clean glass slides

smeared with egg albumen. The slides are then dried on a drying plate at a temperature of 40^{0}C overnight to enhance adherence, and stored in slide racks until ready for staining.

2.7 Haematoxylin and Eosin (H&E) staining procedure for routine histology

Paraffin wax is poorly permeable to stains, so sections are de-waxed in two changes of xylene for three minutes each. Xylene is again removed because it is not miscible with aqueous solutions and low grades of alcohol. Sections were passed in absolute alcohol in two changes of two minutes each. To avoid the possibility of diffusion current causing damage and perhaps detachment of sections, sections were rehydrated through 95%, 90%, 70% and 50% ethanol for about two minutes each and then brought to water. Sections of 5 µm were used.

2.8 Reagents required

I. Erlich's Hematoxylin

Hematoxylin	1 g
95% alcohol	100 mls
Distilled water	100 mls
Glycerol	100 mls
Potassium alum	3 g
Glacial acetic acid	10 mls

II. Differentiator (1% HCl in 70% Alcohol)

Conc. HCl	1 ml
70% alcohol	99 mls

III. Eosin Y Solution (CSHL Protocols 2014)

1. Prepare eosin Y stock solution. Add 2.0 g of water-soluble eosin Y to 40 mL of double-distilled H2O, and mix until dissolved. Then add 160 mL of 95% ethanol, and mix. Store at room temperature.
2. Prepare eosin Y working solution. Add 200 mL of eosin Y stock solution to 600 mL of 80% ethanol and mix well. While working in a fume hood, add 4 mL of glacial acetic acid and mix well. Store covered at room temperature.

2.9 Procedure

Procedure of H and E as described by Drury and Wallington (1980) was adopted as follows:

a. Sections were dewaxed in xylene for 2 changes of 2 minutes each.
b. Rehydrated in descending grades of alcohol; absolute alcohol, 95%, 90%, 70%, and 50% ethanol for 2 minutes
c. Rinsed in distilled water.
d. Stained in haematoxylin for 10-15 minutes.
e. Washed well in running tap water for 2-3 minutes and examined microscopically to confirm sufficient degree of staining.
f. Excess stain was removed or differentiated in 1% acid in 70% alcohol for a few seconds as the acid breaks the mordant-dye linkages.
g. The sections were washed in running tap water for 2-5 minutes.
h. Sections are dipped in Ammonia water (1-2 dips)
i. Place slides in 80% Alcohol for 1 minute
j. Rinsed in tap water for 1 minute
k. Stained in 1% aqueous eosin for about 3-5 minutes.
l. Surplus stain was rinsed off in running tap water and examined with a microscope.

m. Dehydrated rapidly in ascending grades of ethanol. The overstating with eosin was removed in low grades of ethanol.

n. Mounted in Distrene Plasticizer Xylene (DPX) using clean glass coverslips.

3.0 Results and Observations

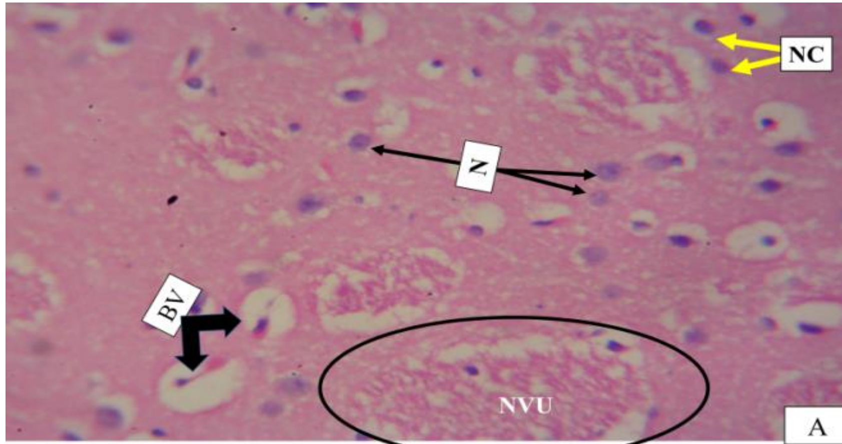

Fig 1: A photomicrograph of a section in the striatum, group A (positive control). Showing a normal histoarchitecture of striatum with neurovascular unit (NVU), neuron (N), blood vessel (BV), Neuroglial cell (NC). (H&E. X400).

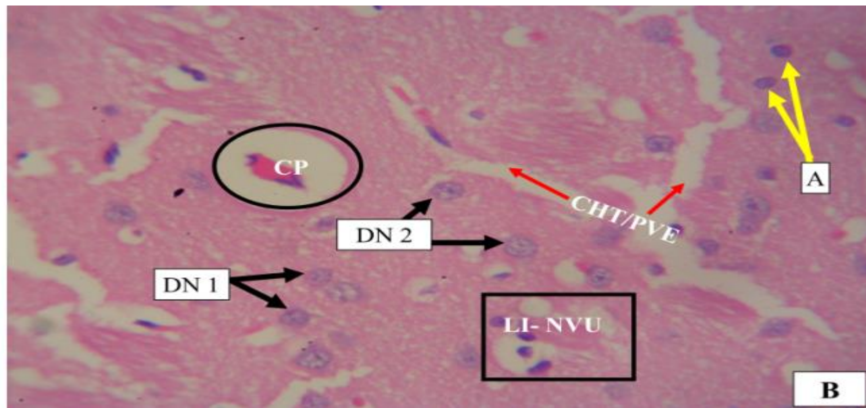

Fig.2: A photomicrograph of a section in the striatum of AMT treated rats, group B (negative control, AMT 4.2mg/kg). Showing a showing degeneration in the striatum with evidence with neurovascular unit (NVU) with mild lymphocytic infiltrates, moderate degenerated neuron (DN1) to severe degenerated neuron (DN2), capillaries proliferation (CP), cellular hypertrophy (CHT) and perivascular oedema and astrocytosis (H&E. X400).

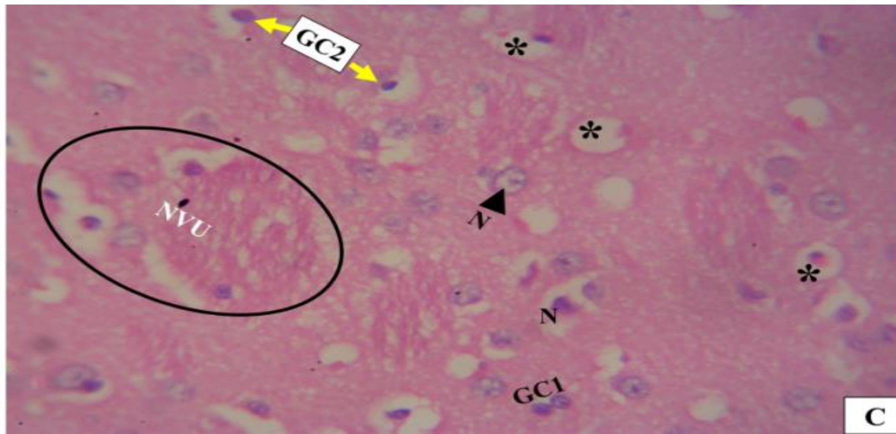

Fig.3: A photomicrograph of a section in the striatum of AMT and KS treated rats, group C (KS; 200mg/kg). Showing minimal degeneration in the striatum; neurovascular unit (NVU) with less lymphocytic infiltrates, moderate degenerated neuron (N) with loss in nuclei, glial cells (GC) with dense nuclei and glial cell (GC2) with mild tissue oedema, mild capillaries proliferation (*) with perivascular oedema. (H&E. X400).

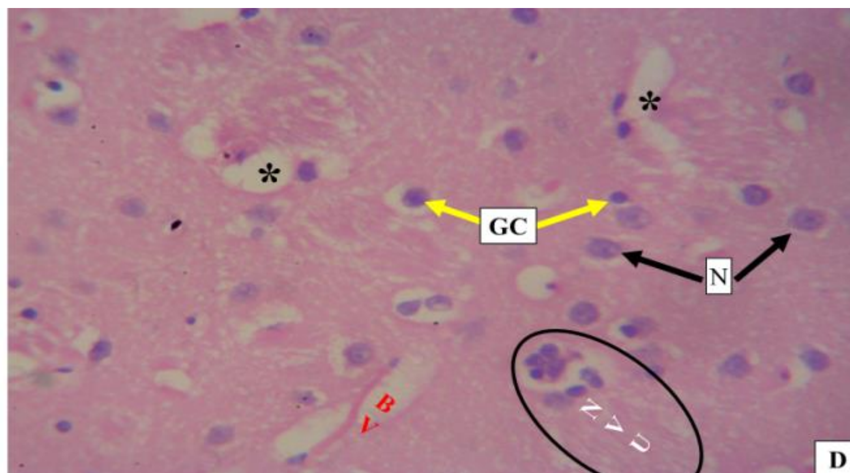

Fig. 4: A photomicrograph of a section in the striatum of AMT and KS treated rats, group D (KS; 300mg/kg). Showing moderate neurorepair of the striatum; neurovascular unit (NVU) with less cluster glial cells normal orientation of neuron (N), glial cells (GC) with dense nuclei, capillaries proliferation (*) with perivascular oedema. (H&E. X400).

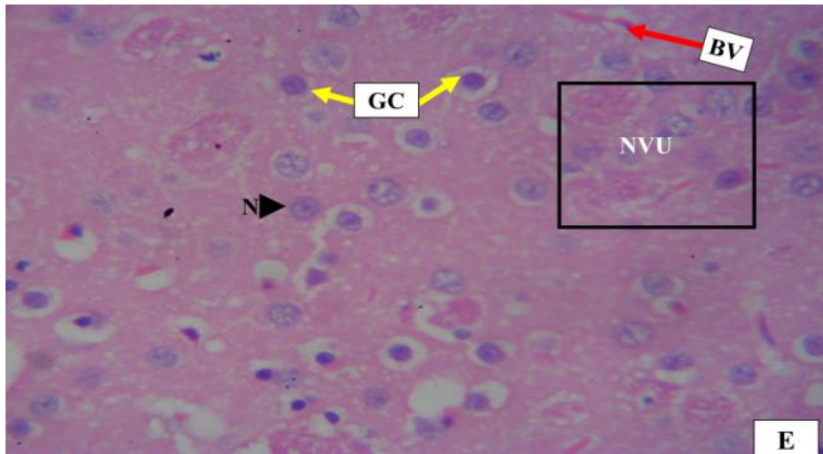

Fig 5: A photomicrograph of a section in the striatum of AMT and KS treated rats, group D (KS; 400mg/kg). Showing normal orientation of striatum; neurovascular unit (NVU), neuron (N), glial cells (GC) With dense nuclei, blood vessels (BV). (H&E. X400).

3.1 Neuro-histological Results

In fig. 1 A section striatum, revealed the normal histoarchitecture of striatum with neurovascular unit, neuron, blood vessel, Neuroglial cell.

Fig .2 AMT treated rats, the result showed degeneration in the striatum with characterized neurovascular unit, with mild lymphocytic infiltrates, moderate degenerated neuron (1) to severe degenerated neuron (2), capillaries proliferation, cellular hypertrophy, and perivascular oedema and astrocytosis.

In fig 3. Is a result showing the photomicrograph of a section in the striatum of AMT and KS treated rats. It Showed a minimal degeneration in the striatum; neurovascular unit, with less lymphocytic infiltrates moderate degenerated neuron, with loss in nuclei, glial cells, with dense nuclei and glial cell, with mild tissue oedema, mild capillaries proliferation with perivascular oedema.

Fig 4 A section in the striatum of AMT and KS treated rats Showed a moderate neurorepair of the striatum; neurovascular unit with less cluster glial cells, normal orientation of neuron, glial cells with dense nuclei, capillaries proliferation with perivascular oedema.

Fig 5 A section in the striatum of AMT and KS treated rats, showing normal orientation of striatum; neurovascular unit, neuron, glial cells, with dense nuclei, blood vessels.

4.0 Discussion

This work investigated the effect of crude extract of *K.senegalesis on* histological and protective role of amitriptyline-induced striatum neurotoxicity in Wistar albino rats. Amitriptyline induction was found to cause brain capillary injuries and increased perfusion deficits in the ischemic penumbra, thus resulting to brain proliferation between 12-24 hours. This finding is in consonance with the report which examined the effect of

amitriptyline therapy on cerebrospinal fluid and found out that predominant features were immunomodulation with a reduction in pro-inflammatory pathways of neuronal-glia communications and evidence of a neurotrophic effect (Royds *et al.,* (2021).

Results from this study clearly show that administration of KS to the treatment groups of the experimental animals for a period of two weeks resulted in reversal of some of the parameters occasioned by amitriptyline-induced striatum neurotoxicity. This observed protective effect could be attributed to the numerous active ingredients of the plant as reported by Abdallah, *et al.,* 2016; Katawa, *et al*., 2018; Koudoro, *et al.,* 2018. The bitter-tasting bark of the plant which was used in this study shows high value in traditional medicine, and thus agrees with the findings of Arbonnier, 2004, which suggested that the bark decoctions or macerations of KS are widely used in treatment of several diseases.

Although, observations of the histological section in positive control (group 1) animals which were treated with only distilled water showed normal pyramidal cells, neurovascular units, neurons, blood vessels, neuroglial cells and granule cells (figure3). However, observation of the negative control (group 2) showed a significance difference of a prefrontal cortex degeneration with evidence of astrocytosis, lymphocytes infiltrates, vacuolated neutrophils, pyramidal cell with irregular shape and surrounded by pericellular hallos, perivascular edema, perivascular cuffing and of cause a shrunken granule cell. Findings from this study conforms to that obtained by Li *et al.,*2014. Their study concluded that histology provides a snapshot of the ischemic brain at that given time thus making Imaging advantageous in profiling of ischemic damage over time within individual animals. The study further concluded that outcome measures of histological ischemic damage showed the progression of damage from an ischemic infarct to fluid filled cavity (edema). Another study conducted on post-stroke environment using animal model also find out that, there may be detrimental effects on both behaviour and histology, as social isolation has been associated with increased histological damage and mortality, together with delayed recovery, and enhanced neuro-inflammatory response, oxidative stress and oedema (Venna*, et al., 2012*), thus further justifying the findings from this study.

Consequently, standard dose (group 4) of KS administration showed a difference in normal orientation of neurovascular unit, pyramidal cell neuroglial cells, neutrophils vacuolated, and granule cells as compared to group 3 animals' models as well as the control groups. However, animals in group 5 were treated with high dose of the extract, were observed to have normal histoarchitecture of prefrontal cortex with normal orientation of neurovascular unit, pyramidal cell, neuroglial cells and granule cells with increase neutrophils and vacuolated cortical cells. The observed difference in this study was very similar to the findings of Rewell *et al*, 2017, whose study concluded that reduction in infarct size is an indication that a therapy is neuroprotective, and will improve outcome for patients, therefore suggesting that as time progressed after stroke onset, tissue

structures and the type and extent of damage differs significantly.

Considering that administration of *khaya senegalensis* extract reversed some parameters occasioned by brain damage in almost all the treated groups administered with low, standard and high doses, it is therefore suggestive that extract might be appropriate for modulation of the amytrpline-induced neurotoxicity and thus, prevent progression of stroke and other neurotoxic effects. Therefore, histological study on the protective role of *Khaya senegalesis* on amitriptyline-induced striatum neurotoxicity in Wistar albino rats warrants further study.

5.0 Conclusion

The results of this study revealed that khaya senegalensis was capable of reversing some of the effect following amitriptyline-induced striatum neurotoxicityin Wistar albino rats, thus suggesting that the plant can be used to improve stroke management outcomes. This thus implies that KS can provide amongst other things, a natural, safer and cost-effective alternative, to the conventional stroke drugs in the developing countries including Nigeria.

5.0 Significance Statement

This study is intended to contribute to the body of knowledge, thus validated the ethnopharmacological use KS on the treatment of stroke. It also provides a baseline data for future researches since it elucidates the active principles responsible for the anti-stroke activities of*Khaya senegalesis.*

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Competing Interest: The authors have declared that there is no competing interest exists.

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