Subchronic exposure to Kafura; its neurotoxic potentials in young adult female Wistar rats

Anoka A. Njan, Mary O. Ologe, Olufunke E. Olorundare, Saheed O. Afolabi, Benjamin C. Ejimkonye, Solomon O. Olaoye, Chloe O. Fatiguna, Olugbenga Akinola, Anthonia Sojec, Ozlem Nazan Erdogan, Nnaemeka Asogwa, Olugbenga E. Iwalewa

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ABSTRACT

Background: Kafura pelebe (camphor) \(\text{C}_{10}\text{H}_{16}\text{O}\) is a chemical substance used mostly amongst the Yoruba ethnic group in Western Nigeria to treat infantile colic during early childhood. This study assess the neurotoxic potentials of Kafura following sub-chronic exposure in female albino Wistar rats.

Methods: Twenty-eight female rats (mean weight of 130 g) were randomly selected and assigned into four (4) groups. Control, received 1ml coconut oil while the treatment groups received 79, 158 and 237 mg/kg b.wt (dose p.o) of Kafura for the period of 14 days. On day fifteen, animals were dissected and the brain organ excised for the homogenate and histopathologic assay, blood samples were also collected for haematological analysis. Morris Water Maze experiment for reference memory was also carried out to ascertain effect of Kafura in the Central Nervous system (CNS).

Results: A trend toward decreased body-weight gain and increase brain weight was observed in Kafura-treated rats but was statistically not significant, compared to control. The biochemical assessment of the antioxidant status of brains of Kafura-treated rats showed significant \((p < 0.05)\) increase in activities of some anti-oxidant enzymes (Superoxide dismutase (SOD), Glutathione peroxide (GPx), and Catalase (CAT)). There was increase in acetylcholinesterase (AChE), Malondialdehyde (MDA), and Total protein activities in the brain of treated rats compared to control. Alterations of the haematological parameters were observed, with the plasma granulocytes, lymphocytes, and haemoglobin (HGB), showing significant decrease in the treated rats compared to control. The water maze test showed a marked increase in spatial learning and memory time (seconds) in kafura-treated rats, compared to control and across treated groups.

Conclusions: The present study provides indication that kafura Pelebe shows apparent neurotoxicity in experimental animals. Incessant exposure in humans though may lead to development of some central nervous system defects.

1. Introduction

Notwithstanding the immense progress in health care delivery in Nigeria, even at primary health care level, an estimated 75% of Nigerian population still consider Complementary and Alternative Medicine (CAM) as better alternative drugs [1]. Kafura pelebe (camphor) is a major component in present-day home remedies for a wide variety of symptoms, made naturally from the bark of the camphor \(\text{Cinnamomum camphora} \) trees, which can also be produced synthetically from vinyl chloride and cyclopentadiene, passing through the intermediate dehydrobormorbornyl chloride [2]. Kafura is sold as consumer and household products in markets and has become a common ingredient in most herbal concoctions used for its carminative properties [3]. In Nigeria, it is currently used mostly amongst the Yoruba ethnic group in Western Nigeria.
region to treat infantile colic during early childhood, the synthetic chemical is bought from the open market, put in a bottle of water and allowed to dissolve for weeks before it is used by mothers. There are reports of its use as nasal decongestant and cough suppressant [4] as well as antipruritic and counterirritant agent [5]. It is also used as inhalants in form of camphorated oil, at combination of 19% or 20% camphor in a carrier oil, for the household management of colds [6] Xu and his colleagues reported antinociceptive role of camphor through inhibitory effect on Transient receptor potential cation, subfamily A, member 1 (TRPA1) channel [7]. It increase activity of cytochrome P450, cytochrome b5, aryl-hydrocarbon hydroxylase and glutathione S-transferase, considerably elevating the level of reduced glutathione in the liver in mice at high concentration [8]. In the past, camphor had been used in progressive dosages to impel convulsion attacks in psychiatric patients [9]. Camphor induced diverse kinds of toxic and behavioural effects such as body jerks and hunched posture [10], convulsions and piloerection [9].

The human brain is very susceptible to injury caused by toxic agents, the developing brain being much more vulnerable [12]. The common neurodevelopmental disorders such as learning disabilities, sensory deficits, poor motor and social skills, epilepsy, among other often affects the nervous system, causing developmental disability in one out of every six children [13, 14] with Sub-Saharan Africa having more prevalence of these disorders [15]. Several cases of poisoning due to unintentional consumption of camphor in humans, especially children, have been reported with symptoms ranging from confusion, irritability, stomach upset, and seizures; most of which are CNS related [11, 16]. Unfortunately, the neurotoxic properties of Kafura have not been mostly characterised. Therefore, this work was designed to evaluate the neurotoxic potential of Kafura in the brain of rats following oral sub-chronic exposure.

2. Materials and methods

2.1. Drug and chemicals

Kafura Pelembe (Camphor) Elephant brand Ltd, China, coconut oil (Packed in UK by KTC edibles Ltd). Normal saline, Per Formaldehyde (PFA) 4%, Sucrose 0.25M, Rat Catalase (CAT) ELISA Kit MBS701713, Rat Glutathione Peroxidase ELISA Kit MBS744364, Malondialdehyde (MDA) ELISA Kit MBS9389391 (MyBiosource.com Company, San Diego, CA, USA), Rat Superoxide Dismutase (SOD) ELISA Kit KT-60703 (Kamiya Biomedical Company, Seattle, WA, USA), nitric oxide assay kit, Abcam®, USA.

2.2. Experimental animals

Five weeks old mice weighing between 20 – 26 g and seven weeks young female albino Wistar rats 130 ± 2 g were use in this experiment. The animals were obtained from a private animal farm at Ogbomosho, Oyo state and housed in polycarbonate rodent cages (170 mm [W] × 294 mm [D] × 176 mm [H]) at the animal house facility of the Faculty of Basic Medical Sciences, University of Ilorin. The indoor environment was maintained at 25 ± 1 °C and 50% humidity. Mice were also housed in the same facility with similar environmental condition. Animals were continuously supplied tap water and food throughout the experiment, and were kept under a 12 -h light-dark cycle. Efforts were made to reduce the quantity of animals used and their distress.

Animal handling were in agreement with the procedures of the Institutional Animal Care and Use Committee of the University of Ilorin, and the study procedure was accepted by the ethical committee of University of Ilorin, Nigeria, with the number UERC/ASN/2019/1545.

2.3. Acute toxicity study

Acute toxicity study was carried out using Lorkes method [17]. 12 female Swiss albino mice (20-26 g) were selected to be used in the study. Nine animals were spread into three groups of 3 animals each and were administered 10, 100 and 1000 mg/kg bodyweight doses of kafura intraperitoneally respectively. They were carefully observed for the first 4 h for signs of toxicity and mortality. No mortality and noticeable signs of toxicity were observed at doses of 10 mg/kg and 100 mg/kg. However, i.p. administration of 1000 mg/kg produced 100% mortality. LD₅₀ was, therefore, calculated using phase 1 as LD₅₀ = √(D₀xD₁₀₀) where D₀ = Highest dose that gave no mortality, D₁₀₀ = Lowest dose that produced mortality.

2.4. Sub-chronic exposure

Rats were divided into four groups of five animals each and administered orally doses of 79, 158, and 237 mg/kg body weight (b.wt) of Kafura Pelembe while the Control received 1 ml of coconut oil daily for 14 days. Doses were modify as per the most recently documented body weight and given at about the same time every day. All rats were sacrificed 24 h following the last treatment. The initial and final weights of the rats were documented before exposure to Kafura treatments and at the end of the experiment. The relative body weight was calculated and expressed as gram percentage (%).

Two rats from each of the four groups were subjected to transcranial perfusion with normal saline followed by 4% paraformaldehyde (PFA). The brain tissues were then removed, rinsed in 0.25 M sucrose and post fixed in 4% PFA until further processing. The cerebral cortex and the hippocampus from each group were processed for paraffin wax embedment. Tissues were cut to produce 5 μm sections, and slides were stained with haematoxylin and eosin (H&E) [18]. Slides were examined by a pathologist under a light microscope for histopathological study. The remaining five rats were also sacrificed and the brains excised, placed in 30% sucrose and stored at 4 °C for biochemical assay. All biochemical assays were performed by a third-party blinded from the whole experiment.

2.5. Morris Water Maze

Cognitive function of rats was evaluated using Morris Water Maze test method [19]. The test device was a circular water tank (180 cm width and 60 cm high) of dark grey plastic that was partly filled with water. Full cream milk (liquid) was used to make the water cloudy. The pool was separated into four equal quadrants, labelled A–B–C–D. A platform (12.5 cm in diameter and 38 cm high) was placed in one of the four maze quadrants (the target quadrant) and immersed 2.0 cm beneath the water. The platform stayed in the same quadrant throughout the experiment. The rats needed to find the platform using only distal spatial extra-maze cues accessible in the testing room. The cues were sustained all through the testing. The rats received four successive day-to-day training trials for 6 days beginning from the 7th day of drug administration, with each trial lasting for maximum of 60 s and a trial break of almost 90 s. Each rat had to swim until it mounted onto the platform immersed underneath the water. After mounting onto the platform, each rat stayed there for 30 s before the starting the next trial. The escape platform was kept in the same spot relative to the distal cues. If any rat fails to reach the escape platform within the maximal allowable time of 60 s, it was directed to the platform and allowed there for 30 s before subsequent trial. The time to climb the platform (escape latency in seconds) was measured. The mean escape latency was calculated and recorded a day after the training session.
2.6. Assessment of haematological parameters

Animals were weighed and sacrificed on day fifteen and blood samples collected via cardiac puncture for biochemical and haematological analyses. The samples for haematology were placed in bottles containing anticoagulant, ethylene diamine tetra-acetic acid (EDTA). We carry out haematological analyses using an automated haematology analyser (Pentra-XL 80, Horiba ABX, USA). Parameters assessed include total and differential leukocyte (WBC), erythrocyte (RBC), Haemoglobin (Hgb), Haematocrit (HCT), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), red distribution width (RDW), mean platelet volume (MPV) and platelet distribution width (PDW).

2.7. Biochemical assays

Brains from control and Kafura-treated rats were homogenized, and the homogenate was centrifuged at 3000 rpm for 15 min at 4 °C. We assessed acetylcholinesterase activity (AchE) and antioxidant status with the supernatant.

2.7.1. Determination of antioxidant enzyme status

Activity of catalase (CAT), Glutathione Peroxidase (GPx) and Malondialdehyde (MDA) were estimated using commercially available Rat Catalase (CAT) ELISA Kit MBS701713, Rat Glutathione Peroxidase ELISA Kit MBS744364 and Malondialdehyde (MDA) ELISA Kit MBS9389391 respectively from (MyBiosource.com Company, San Diego, CA, USA) with the aid of a SpectraMax plate reader (Molecular Devices, CA, USA) as stated in the manufacturer's manual. While Superoxide Dismutase (SOD) activity was assessed using commercially available Rat Superoxide Dismutase (SOD) activity was assessed using commercially available Rat Superoxide Dismutase (SOD) ELISA Kit KT-60703 from (Kamiya Biomedical Company, Seattle, WA, USA) and brain nitric oxide (NO) level was determined using nitric oxide assay kit (Abcam®) with the aid of a SpectraMax plate reader (Molecular Devices, CA, USA) as stated in the manufacturer's guidebook.

2.7.2. Determination of brain acetylcholinesterase activity

Acetylcholinesterase activity was evaluated by the method [20]. The incubation mixture (1 ml) contained 50 mM Tris-HCl, pH 8, 240 mM sucrose and 120 mM NaCl. The protein concentration of the incubation mixture (1 ml) contained 50 mM Tris-HCl, pH 8, 240 mM sucrose and 120 mM NaCl. The reaction was initiated after addition of 0.03 ml of 3,5,5o-dithionitrobenzoic acid (DTNB) and 0.05 ml of acetylthiocholine iodide which was used as substrate. The final concentrations of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction followed spectrophotometrically by the increase of absorbance (OD) at 412 nm.

2.8. Statistical analysis

We stated every data as means ± standard error of mean (mean ± SEM). Statistical group analysis was performed with GraphPad (version 6) statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups and means were compared using Tukey’s multiple comparisons test. Values of p ≤ 0.05 were considered significant.

3. Results

3.1. Acute toxicity test of Kafura in mice

The animals showed significant signs of toxicity after the administration of Kafura at dose level of 1000 mg/kg as shown in Table 1A and Table 1B. Breathing abnormalities and slow movement of the treated mice were seen, convolution and mortality were recorded at this dose. LD₅₀ was calculated to be 316.23 mg/kg.

3.2. Body-weight gain and brain-weight in Kafura-treated rats

The body-weight gain and the brain weight of the experimental animals in control and Kafura-treated group are shown in Figures 1 and 2A respectively. There was a decrease in the percentage body weight gain in the animals treated with Kafura (237 mg/kg), but not statistically different, compared to control. The brain weight of the Kafura-treated rats was noted to have increased slightly when compared to control.

3.3. Escape latency in Kafura-treated rats

The water maze test showed a statistically significant increase in escape latency (seconds) in kafura-treated rats, compared to control and across treated groups (Figure 2B).

3.4. Haematological parameters of Kafura-treated rats

The effects of oral administration of kafura on the haematological parameters are shown (Table 2). The levels of RBC, HGB, HCT, Lymphocytes, MID (Indicates the combined value of the other types of white blood cells not classified as lymphocytes or granulocytes), and percentage Granulocyte, showed a statistically significant decrease (P < 0.05) in the kafura-treated groups, compared to control. However, changes occurred with the other parameters such as WBC, platelet, lymphocyte, MCV, MCH, MCHC but were not statistically significant (P > 0.05).

3.5. Antioxidant level in brain of rats

The antioxidant levels in brains of rats were shown in Figures 3 and 4. The data indicated that kafura exposure caused a significant (P < 0.05) increase in activities of antioxidant enzymes (GPx, CAT, and SOD), whereas it caused a significant decrease in activity of MDA level at 79 mg/kg but significant increase at 158 and 237 mg/kg.

Levels of NO showed no significant changes in the kafura-treated group, compared to control (Figure 4C). The level of AchE in the brain

| Signs | Observation | Dose (mg/kg) |
|-------|-------------|--------------|
| Condition of the fur | Normal | 1000 |
| Skin | Normal | 1000 |
| Subcutaneous swelling | Nil | 1000 |
| Abdominal distension | Nil | 1000 |
| Colour and consistency of faeces | Normal | 1000 |
| Eyes dullness | Nil | 1000 |
| Eyes opacities | Nil | 1000 |
| Breathing abnormalities | † Tachypnea (respiratory rate) | 1000 |
| Gait | Body weakness, slow movement, hind limb paralysis | 1000 |
| Convulsion | Yes | 1000 |
| Death | Yes | 1000 |

LD₅₀ = 316.23 mg/kg.
showed a statistically significant increase in the *Kafura*-treated group, compared to control (Figure 4B).

### 3.6. Histopathological study

There were no changes in the histomorphometry of the hippocampal tissue of the tests group compared to control. All sections from the hippocampal tissue show preserved molecular (M) layer comprising neurons in glial matrix and preserved compact layers of the cornus ammonis (CA1-CA4) comprising layers of pyramidal cells. The dentate gyrus (DG) is composed of preserved compact granular cells. There are no degenerative changes seen (Figure 5A).

There were no changes in the histoarchitecture of the cerebral cortex in the test group compared to control. All sections from the cerebrum show preserved layers of the cortex. Neurons (N), oligodendrocytes (O) and astrocytes (A) were seen preserved. There are no degenerative changes seen (Figure 5B).

### 4. Blood assay result

| Table 2 |
|---|

Figure 1. Effect of *Kafura* on: (A) Body weight following 14 days treatment (n = 5), (B) Percentage body weight gain (g %) in adult female rats (n = 5). Data are expressed as mean ± standard error of mean (SEM) of 5 animals. *Values differ significantly from control and between groups (P < 0.05). (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

Figure 2. Effect of *Kafura* on: (A) Brain weight in adult female rats (n = 5), (B) Escape Latency (n = 5). Data are expressed as mean ± standard error of mean (SEM) of 5 animals. *Values differ significantly from control and between groups (P < 0.05). (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

Figure 3. Effect of *Kafura* on: (A) Glutathione Peroxidase (GPx) activity in adult female rats (n = 5), (B) Catalase (CAT) activity in adult female rats (n = 5), (C) Superoxide Dismutase (SOD) activity in adult female rats (n = 5). Data are expressed as mean ± standard error of mean (SEM) of 5 animals. *Values differ significantly from control and between groups (P < 0.05). (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).
in many cases to be associated with neurodegenerative diseases [21, 22].

Excess ROS such as hydrogen peroxide and hydroxyl radical, characterize oxidative stress, which leads to neuronal damage via free radical oxidation [26]. Lipid peroxidation is a chain reaction of various active cell damaging compounds, and can be evaluated by quantifying organ lysate malondialdehyde (MDA) levels. MDA is a carrier of HGB which is the iron-containing oxygen-transport metalloprotein [34]. HCT is the measure of RBCs as a percentage of the total blood volume [34].

Antioxidant enzyme levels are often increased in the presence of excess ROS [26]. Our study showed that the administration of *kafura* in rats caused significant increase in the levels of endogenous antioxidant enzymes such as Glutathione peroxide (Gpx), Catalase (CAT), and Superoxide dismutase (SOD) (Figure 3A, B, C) and this may be a response to a build-up of cell damaging radicals. The scavenging of superoxide radicals by SOD produces H2O2, thereby diminishing the toxic effects of these radicals. CAT subsequently catalyses the conversion of H2O2 to water and oxygen [25], while Gpx also uses reduced glutathione (GSH) and reduced NADPH as cofactors to reduce H2O2 to water [27]. Hence, the higher and lower concentrations of H2O2 are detoxified by CAT and Gpx respectively. Therefore, high levels of H2O2 upregulate CAT activity and down regulate the activity of SOD [28]. A subtle build-up of damaging radicals, manifesting as superficial stress in the brain of the *kafura*-treated rats might be responsible for the notable increase in these antioxidant enzymes levels induced by our test agent in this study.

The evaluation of the activity of acetylcholinesterase (AChE) is essential in confirming the neurotoxic effect of suspected neurotoxic agents. In this study, *Kafura* at dose of 237 mg/kg caused a significant increase in the activity of brain AChE (Figure 4B). Aside from its established role in hydrolyzing acetylcholine a major cholinergic neurotransmitter, AChE is an extensively studied enzyme of principal significance for synaptic integrity, neurodevelopment, and apoptosis [29]. Increase in AChE levels has been reported to cause impairment in learning ability and memory retrieval, as well as induction of morphological damage in the brain of rats exposed to hypobaric hypoxia (HHB) [30]. This neurotoxic effect of AChE may result either from cholinergic transmission disruption as a result of acetylcholine (Ach) depletion consequent upon increase activity of the enzyme and/or decrease cholinergic activity on non-neuronal cells (astrocytes and microglia) in the brains [31]. The Increased AChE observed in the highest dose in this study could have triggered disturbance of cholinergic activity in the brain of exposed rats.

Water escape task has been widely used to evaluate spatial reference memory of rats with the platform always positioned in the same place [32]. In our water escape task experiment, there was statistically significant increase in escape latency (Figure 2A) of the treated groups compared to control. Our test agent, therefore, triggered a neurotoxic effect on the experimental animals manifesting as impaired learning and memory.

Our study also showed a fall in red blood cells (RBC) count, haematocrit (HCT), and haemoglobin (HGB) content of the blood, which according to Criswell et al. [33] can be correlated to defective haematopoiesis with consequent precipitation of anaemia. Red blood cell is a carrier of HGB which is the iron-containing oxygen-transport metalloprotein [34]. HCT is the measure of RBCs as a percentage of the total blood volume.
volume of blood and these RBCs are essential for oxygen transport as well as nutrients distribution [35]. The statistically significant decrease in RBC, HCT, and HGB therefore suggests that kafura also caused peripheral toxicity in the exposed animals. Ugwuene et al. [36] reported that HGB is involved in the transport of oxygen to tissues of animals for oxidation of ingested food and release of energy needed for other body functions including transport of carbon dioxide out of the body. This could explain the marginal decrease in the percentage (%) body weight gain (Figure 1B) of kafura-treated rats. The decreased % body weight gain is an indicator of adverse side effect which may have induced appetite suppression with deleterious effect on the haematological parameters and general health status of the exposed animals.

The hippocampus and the cerebral cortex are populated specifically by neurons expressing glutamate receptors that are essential in learning and memory [37]. Cholinergic neurons expressing cholinergic receptors and which utilise acetylcholine as neurotransmitter also populate these regions of the brain and are very essential in cognitive functions. These receptors and enzymes are reported to be specific targets for chemicals with neurotoxic potential [38]. In this study, Kafura Pelbe did not alter the histopathological outlook of the hippocampus and the cerebral cortex

Figure 5. 5A: Representative stained sections of hippocampus of rat groups: (A) control, (B) 79 mg/kg, (C) 158 mg/kg, (D) 237 mg/kg, (E & F) Mortality at 158 mg/kg and 237 mg/kg respectively. H&E x40. 5B: Representative stained sections of cerebral cortex of rat groups: (A) control, (B) 79 mg/kg, (C) 158 mg/kg, (D) 237 mg/kg, (E & F) Mortality at 158 mg/kg and 237 mg/kg respectively. H&E x40.
in treated rats. We suspect that the short treatment period of the animals may not have been sufficiently long enough to allow the development of necrobiotic changes in neurons or irreversible neuronal changes to become manifest anatomically in this brain regions.

6. Conclusion

In conclusion, the present study provides indication that *Kafura Pelebe* shows apparent neurotoxicity in experimental animals. Incessant exposure in humans though, may lead to development of some central nervous system defects.

Declarations

Author contribution statement

Anoka A. Njan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mary O. Ologe: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sahed O. Afolabi, Benjamin C Ejikimkoye, Solomon O. Oloaye, Chloe O. Fatigun: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Olugbenga Akinola: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Anothia Soje: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Ozlem Nazan Erdogan: Contributed reagents, materials, analysis tools or data.

Nnaemeka Asogwa: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Olugbenga E. Iwalewa: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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