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Mechanism and Changes Associated with Edible Camphor Induced Neurotoxicity

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ABSTRACT

Background and Purpose: Camphor is an additive in certain remedies for the treatment of coryza, bronchitis, musculoskeletal pains and wounds. This study investigated the possible seizure-induction mechanism of edible camphor (EC) and the biochemical changes in brain and blood after its administration.

Methods: Seizure characteristics were evaluated in twenty-five male Wistar rats randomly distributed into five groups and given intraperitoneal injections of 0.5 mL coconut oil; 100 mg/kg pentylenetetrazol (PTZ); 1000 mg/kg EC; 5 mg/kg diazepam + 1000 mg/kg EC; and 250 mg/kg carbamazepine + 1000 mg/kg EC respectively. Oral acute toxicity was evaluated using 15 male rats assigned to 3 groups (n=5) and orally administered a single sub-convulsive dose (300 mg/kg) of EC and monitored for 3, 24, and 168 hours respectively. Fasting blood sugar, blood lipid parameters and brain weight were evaluated. In a 28-day toxicity evaluation, 300 mg/kg/day EC was administered to test and control groups of male rats (n=10) and brains were excised for evaluation of weight, oxidative, inflammatory and cell component changes.

Results: At 1000 mg/kg, EC had superior seizure-inducing potential, longer seizure latency but shorter duration than PTZ. Acute administration of EC increased blood sugar, decrease serum phospholipids and triglycerides. Daily treatment for 28 days increased brain weight, and lysosomal enzyme activities neuroinflammatory indicators (TNFα, IL-4, IL-8, IL-9 and IL-10) except IL-2. Treatment also resulted in the elevation of malondialdehyde (MDA) level but reduction in catalase (CAT) and reduced glutathione (GSH) levels decreased, decreased acetylcholinesterase activity. Photomicrographs of the cerebral cortex of treated animals showed degenerated axonal and dendritic projections as well as soma filled with neurofibrillary tangles.

Conclusions: EC neurotoxicity may be due to inhibition of GABAergic neurotransmission resulting in oxidative, inflammatory and eventual neurodegenerative damages.

INTRODUCTION

Edible camphor (EC) is a pleasant-smelling cyclic ketone of the hydroaromatic terpene group originally obtained by distillation of the bark chips of *Cinnamomum camphora* tree and synthesised from turpentine oil (Mann *et al.*, 1994). It is a major additive in remedies for the treatment of coryza, musculoskeletal pains, and wound (Enaibe *et al.,* 2008). It is also employed in herbal preparations for managing certain inflammation-related illnesses such as rheumatism, sprains, bronchitis (Lee *et al.,* 2006) as well as treatment of hemorrhoids and erectile dysfunction (Somade *et al.*, 2017). Some recipes of "paraga" (an alcoholic herbal mixture locally made in West Africa including Nigeria) contain edible camphor (Kehinde and Adegoke, 2012).

Exposure to EC in large quantities has been linked with toxic effects in human population. For example, its exposures via oral, cutaneous or nasal routes, have been associated with sudden collapse (Arena, 1979); liver, lung and brain damages; and death (Eldridge *et al.*, 2007). The symptoms associated with EC noxiousness include nausea, vomiting, headache, dizziness, convulsion, confusion, irritability, neuromuscular hyperactivity and depression (Martin *et al.,* 2004). Though many of these symptoms follow its exposure (Jankelowitz *et al.*, 2009; Peters *et al.*, 2011; Njan et al., 2020), convulsion remains the most commonly reported (Mathen *et al.*, 2018). Koppel *et al* (1988) reported the first case of persistent neurologic deficits following several episodes of tonic-clonic seizures in a 54-year-old woman who was accidentally orally exposed to EC.

Even though convulsion is recognized as the most commonly reported systemic toxic manifestation of EC, its seizure evoking mechanism remains poorly understood and the characteristics of camphor-induced convulsion are largely undocumented. This study therefore aimed to investigate the possible seizure-evoking mechanism of edible camphor, compare the characteristics with a standard laboratory convulsant agent, and determine possible biochemical changes in brain and serum following sub-chronic oral exposure in Wistar rats.

MATERIALS AND METHODS

Drugs, Chemicals and Kits

These include EC (Elephant brand Ltd, China), pentylenetetrazol (PTZ) (Sigma-Aldrich, USA), diazepam (DZP) (Swipha-Biogaran, Nigeria), and carbamazepine (CZP) (Novartis, UK). Coconut oil (Packed in UK by KTC Edibles Ltd) was used as vehicle of administration for all test drugs. Normal saline solution (Biomedical Ltd, Nigeria), 4% paraformaldehyde (PFA), 0.25 M sucrose, rat catalase (CAT) ELISA kit MBS701713, rat glutathione peroxidase (GPx) ELISA kit MBS744364, and malondialdehyde (MDA) ELISA kit MBS9389391 were all obtained from MyBiosource Company, San Diego, CA. USA. SpectraMax plate reader (Molecular Devices, CA, USA) was used for rat interleukin ELISA Kit KT-60703 (CUSABIO®) USA, acetylcholinesterase (AChE) kits (CUSABIO®, USA), and nitric acid assay kit (Abcam®, UK).

Experimental Animals

Seven weeks old male Wistar rats weighing 150 ± 3 g were use in this experiment. The animals were obtained from a private animal farm at Ogbomosho, Oyo State, Nigeria and were housed in polycarbonate rodent cages (170 mm [W] × 294 mm [L] × 176 mm [H]) at the animal house of the Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria. The indoor environment was maintained at 25 ± 1°C temperature, 50% humidity and 12/12 h light/dark cycle. Animals were allowed to acclimatize for a period of 2 weeks prior to commencement of the experiments and were given unhindered access to rat chow (Crown Flour Mill Nigeria Ltd) and potable water.

Ethical Considerations

The experimental protocols were reviewed and approved by the Institutional Animal Research Ethics Committee of the University of Ilorin with approval number: **UERC/ASN/2019/1545**. Strict compliance to institutional and the National Institute of Health (USA) guide for the care and use of laboratory animals (NIH Publications No.80-23) revised 1996 was observed throughout the experiment. Efforts were made to minimize animal sufferings and the overall number of animals used in this experiment.

Camphor-Induced Convulsion

Camphor-induced convulsion was compared to that of PTZ (a commonly used seizure-inducing agent in laboratory animals). Twenty-five male Wistar rats were randomly distributed into 5 groups of 5 animals each and were intraperitoneally administered with 0.5 mL of coconut oil (Group 1); 100 mg/kg of PTZ only (Group 2); 1000 mg/kg of EC only (Group 3); 5 mg/kg of DZP + 1000 mg/kg of EC (Group 4), and 2.5 mg/kg of CPZ + 1000 mg/kg of EC (Group 5) respectively. The 1000 mg/kg dose of EC was determined from a pilot study. Doses of PTZ (Velãoå, 2006), DZP (Vito *et al.,* 2014) and CPZ (Nsimba, 2009) as previously used were adopted in this study. Seizure induction or lack of it was observed in the animals and seizure characteristics such as latency, duration, frequency, and mortality were recorded. Latency refers to the time (in minutes) between administration of convulsant agent and the onset of convulsion; duration connotes recovery time (in seconds) for one episode of seizure; frequency defines the number of seizures within

15 min in the same animal while mortality defines death of an animal within 60 min of seizure induction.

Acute Toxicity Study

Using the method of Ali *et al.* (2012), 15 male Wistar rats were randomly assigned to 3 experimental groups of 5 animals each. Experimental groups had oral administration of 300 mg/kg EC stat and were monitored against respective controls (that had 0.5ml of vehicle orally) for 24, 72 and 168 hours respectively. Animals were weighed and sacrificed by cervical dislocation at the end of each study period. Blood was obtained by cardiac puncture for fasting blood sugar and clotted sample centrifuged to harvest serum for evaluation of cholesterol and triglycerides. Whole brain tissues were excised and weighed.

Sub-acute Neurotoxicity

Twenty Wistar rats were randomly distributed into control and test groups of 10 animals each. Animals in the control group were orally administered 0.5 mL of coconut oil while those in the test group had 300 mg/kg of EC for 28 days. All animals were weighed at the beginning then every 7 days and sacrificed on day 29 by cervical dislocation. Brains of the rats were excised, weighed and prepared for biochemical and histological analysis as described later. Brains for histology were fixed in 4% paraformaldehyde (PFA). Brain homogenate (10% w/v) was prepared in 0.25 M sucrose using a Teflon homogenizer (Genetix Biotech, India). The homogenate was centrifuged at 4℃ and 4000 rpm for 10 min. The supernatant was aliquoted into plain bottles and stored frozen until used.

Measurement of Body and Organ Weights

The animals were weighed before and after each experiment and weights were recorded as $\alpha =$ initial weight, $\beta =$ final weight, $\lambda =$ brain weight. Percentage body weight gain, ω was determined as:

$$\omega = \frac{\beta - \alpha}{\alpha} \times 100$$

Relative brain weight, ϖ was determined as:

$$\bar{\omega} = \frac{\lambda}{\beta} \times 100$$

Determination of Brain Oxidative Stress Status and Quantification of Inflammation Markers

Activities of catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) and malondialdehyde (MDA) levels were evaluated using commercially available rat kits while brain nitric oxide (NO) level was determined using nitric acid assay kit with the aid of SpectraMax plate reader following manufacturer's instruction. Cytokine levels (TNF-α, IL-2, IL-3 and IL-4, IL-6, IL-8, IL-9, IL-10) were quantified using rat interleukin ELISA kits.

Brain Lysosomal Enzyme Activity

RNAse, DNAse and Acid phosphatase activities were determined using their respective ELISA kits according to the manufacturers' instructions while paraoxanase-1 (PON-1) activity was determined by mass spectrophotometry.

Histopathology Analysis

Brain sections fixed in 4% PFA were washed in 10 mmol/L phosphate buffer (pH 7.4) at 4℃ for 12 h. After serial alcohol dehydration, brains were embedded in paraffin wax and cut into sections of 5 µm thickness with the use of a microtome. The sections were fixed to glass slides and subsequently serially rehydrated and stained with hematoxylin-eosin dye. Prepared sections were examined under a light microscope (Olympus TX 21, USA) at ×40 magnification. Some slides were further processed for silver staining and examined at ×400 magnification for evidence of neuronal degeneration under a Nikon light microscope. Photomicrographs were taken with a 5-megapixel microscope camera (Amscope, China) during histology examinations.

Statistical Presentation and Analysis

Data are presented in tables and bar charts as mean ± standard error of mean (S.E.M.). Data were analyzed using Student's t-test or ANOVA with Tukey's post hoc test (GraphPad Prism version 6.01) to determine significance between groups. $P<0.05$ was considered statistically significant.

RESULTS

Seizure Characteristics of EC Compared to PTZ

At 1000 mg/kg, EC evoked seizure in more numbers of experimental animals, with longer seizure latency, shorter seizure duration and produced lesser mortality than PTZ at 100 mg/kg (Table 1).

Acute Effect of EC on Blood Biochemical Parameters

We observed a statistically significant ($P<0.05$) increase in the FBS 3 h post treatment with EC. This change, however, normalized within 24 h. There was a statistically significant decrease in triglycerides levels on day 7. Serum cholesterol levels was unchanged in the tested animals compared to control (Figure 1).

Acute Effect of EC on Body and Brain Weights

There were no changes in body weight but there was a marginal non-statistically significant increase in brain weight of treated animals compared to control on day 7 (Figure 2).

Table 1: Characteristics of edible camphor (EC) and pentylenetetrazol (PTZ) -induced seizure in male Wistar rats

Treatment	Convulsed	Onset (min)	Duration (s)	Dead
PTZ (100 mg/kg)	3/5	1.2 ± 0.1	62.3 ± 1.3	3/5
EC (1000 mg/kg)	5/5	3.5 ± 0.4*	27.0 ± 1.4*	1/5
EC (1000 mg/kg) + DZP (5 mg/kg)	0/5	0	0	0/5
EC (1000 mg/kg) + CMZ (2.5 mg/kg)	2/5	8.4 ± 0.04**	31.0 ± 8.0*	0/5

*P<0.05, **P<0.01 vs control. For convulsion and mortality, numerator = number of animals affected; denominator = number of animals tested. Other data are given as Mean ± S.E.M. DZP = diazepam; CMZ = carbamazepine

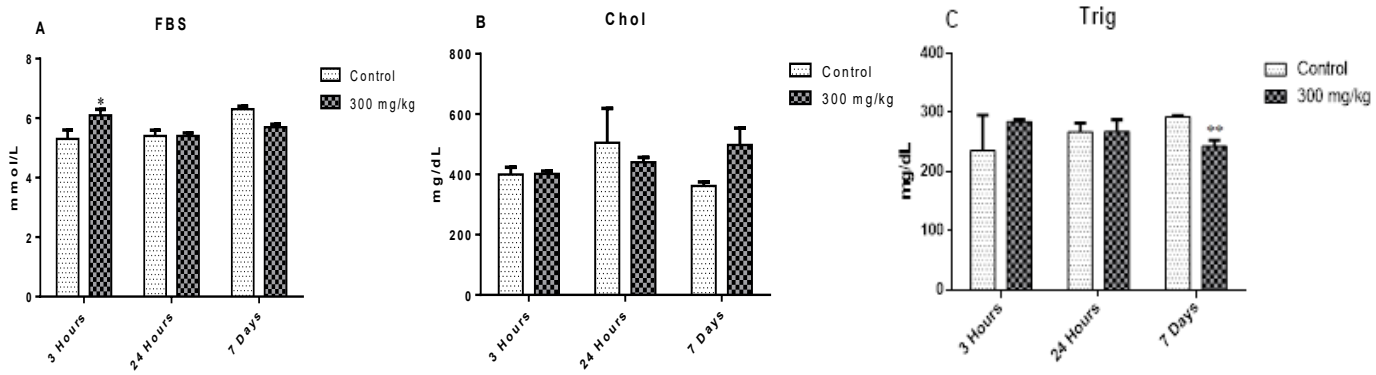

Figure 1: Effects of an acute oral treatment with EC on fasting blood Sugar FBS (A) serum cholesterol (B) and triglycerides (C) levels at 3, 24 and 168 hours in Wistar rats. * P<0.05 vs control, ** P<0.01 vs control. n=5

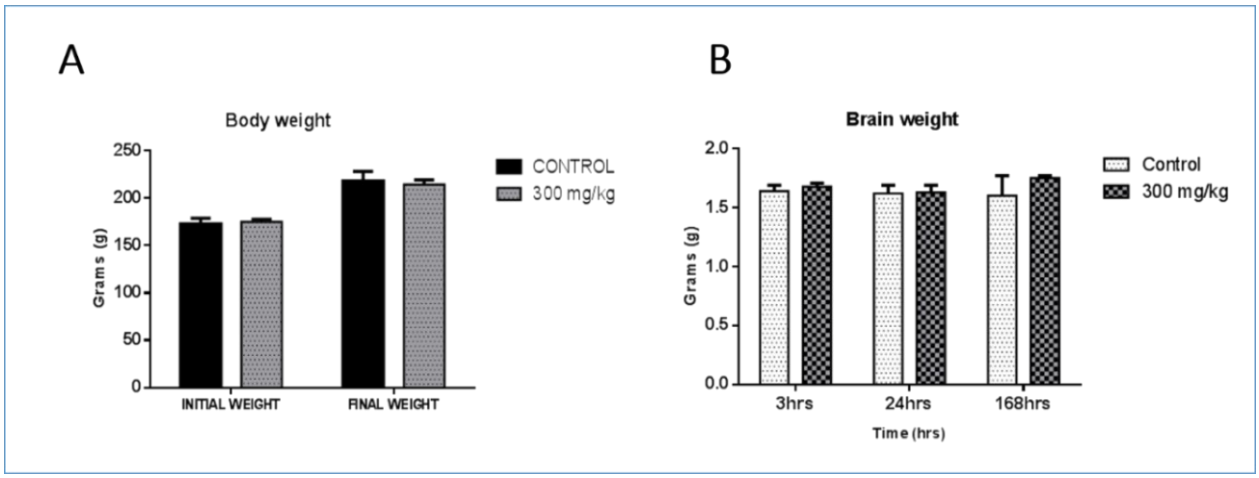

Figure 2: Effects of an acute oral treatment with EC on the body and brain weights in male Wistar rats. Values are not significantly different from control. n=5

Sub-acute Effect of EC on Wistar Rats

Effect on Body and Brain Weight Parameters

There were no significant changes in body weight of treated rats compared to control groups after 28 days (Figures 3A and B). There were also no significant changes in relative brain weights of treated rats compared to control groups after 28 days (Figure 3C).

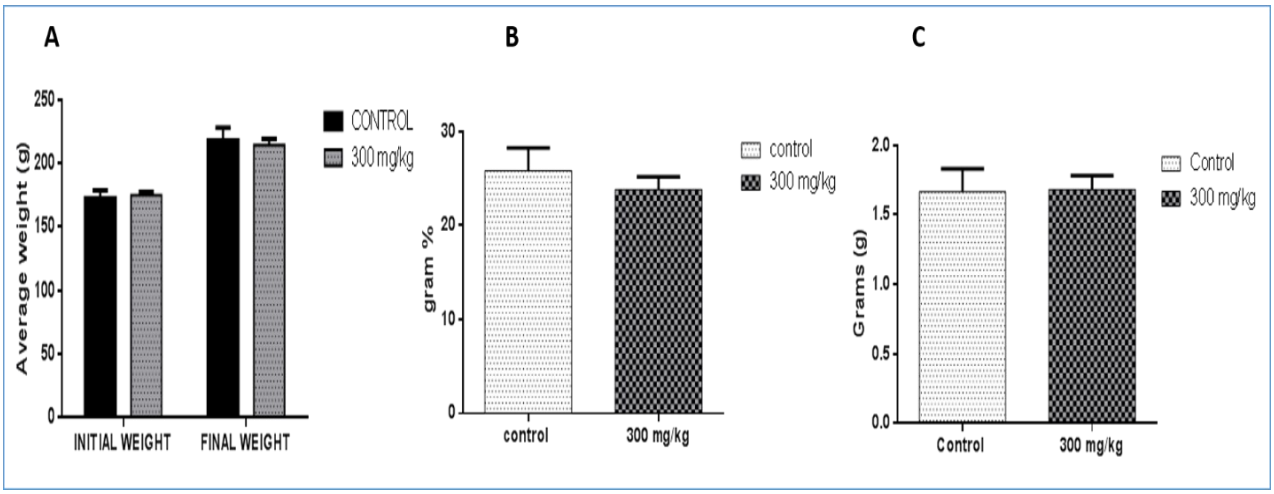

Figure 3: Effects of a sub-acute oral treatment with EC on the body and brain weights in male Wistar rats. Values are not significantly different from control. n=10

Effect on Oxidative Stress Parameters in Rat Brain

There was a non-statistically significant (P<0.05) increase in MDA levels in the treated group compared to control (Figure 4A). CAT and GPx levels were significantly decreased in the treated group compared to controls (Figure 4B and C). There was significant decrease (P<0.05) in reduced glutathione (GSH) levels and a non-statistical increase in NO activity in the brains of the treated animals versus control (Figures 5A and B).

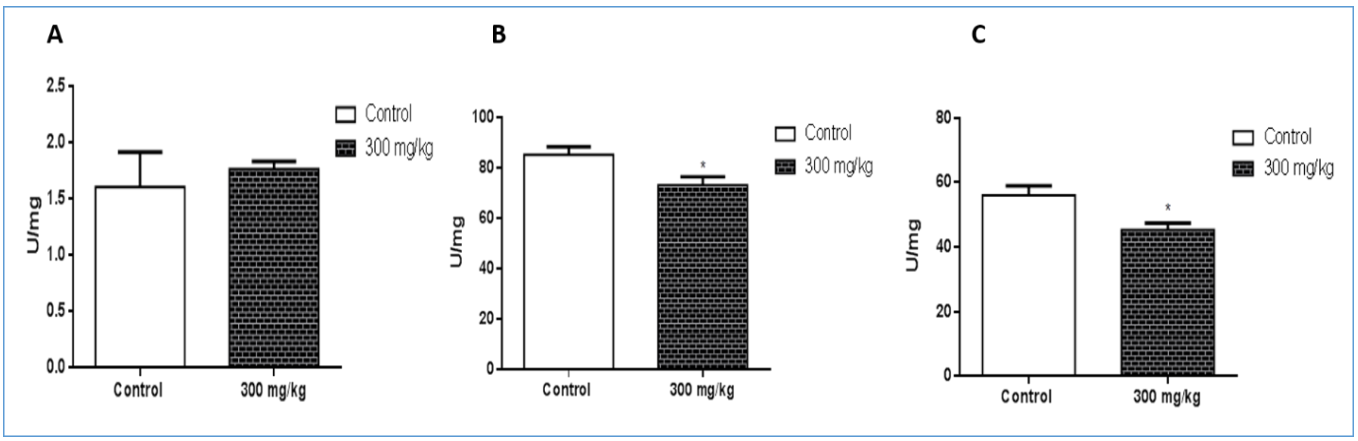

Figure 4: Effect of a sub-acute oral treatment with EC on MDA level (A), CAT (B) and GPx (C) activity in rat brain. *P<0.05 is significant vs control. n=5

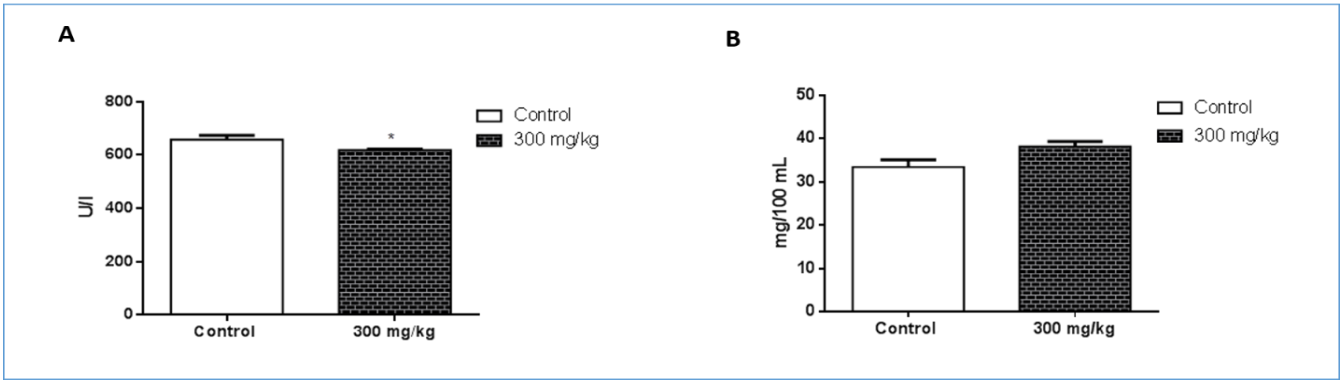

Figure 5: Effect of a sub-acute oral treatment with EC on GSH (A) and NO (B) levels in rat brain. *P<0.05 is significant vs control. n=5

Sub-chronic Effect of EC Treatment on Inflammation Markers in Rats Brain

There were significant increases in inflammatory cytokines TNF-α (P<0.05), IL-4 (P<0.05), IL-8 (P<0.001), and IL-9 (P<0.01) levels and a decreased in anti-inflammatory cytokine IL-2 (P<0.001) levels in treated group compared to control. Brain levels of IL-3, IL-6 and IL-10 were also non-statistically increased in treated rats compared to control (Figures 6, 7 and 8).

Sub-chronic Effect of EC on Lysosomal Enzyme Activities in the Brain

There was significant increase in the activities of total acid phosphatase (P<0.001), free acid phosphatase (P<0.01), RNAse (P<0.05), DNAse (P<0.01) and paraoxonase-1 (P<0.01) activities in the brains of treated rats compared to control (Table 2).

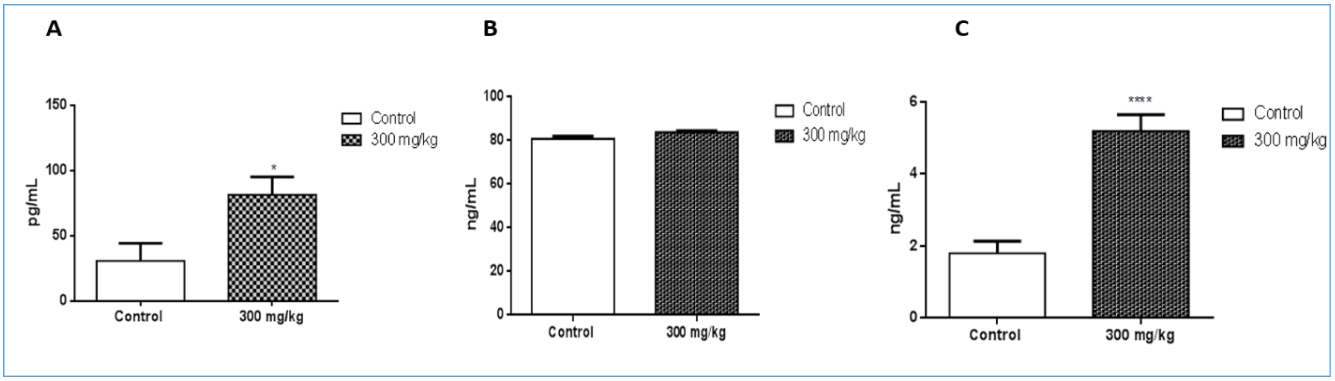

Figure 6: Effect of a sub-acute oral treatment with EC on TNF- α (A), IL-6 (B), and IL-8 (C) levels in rat brain. *P<0.05 is significant vs control. ***P<0.001 is significant vs control. * P<0.05 is significant vs control. n=5

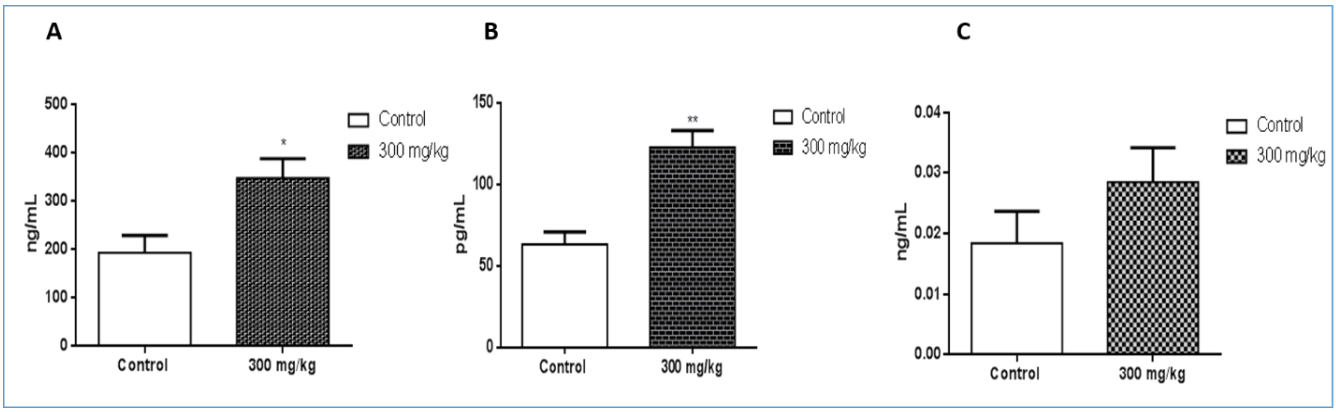

Figure 7: Effect of a Sub-acute oral treatment with EC on TNF- α (A), IL-6 (B), and IL-8 (C) levels in rat brain. *P<0.05 is significant vs control. **P<0.001 is significant vs control. *P<0.05 is significant vs control. n=5

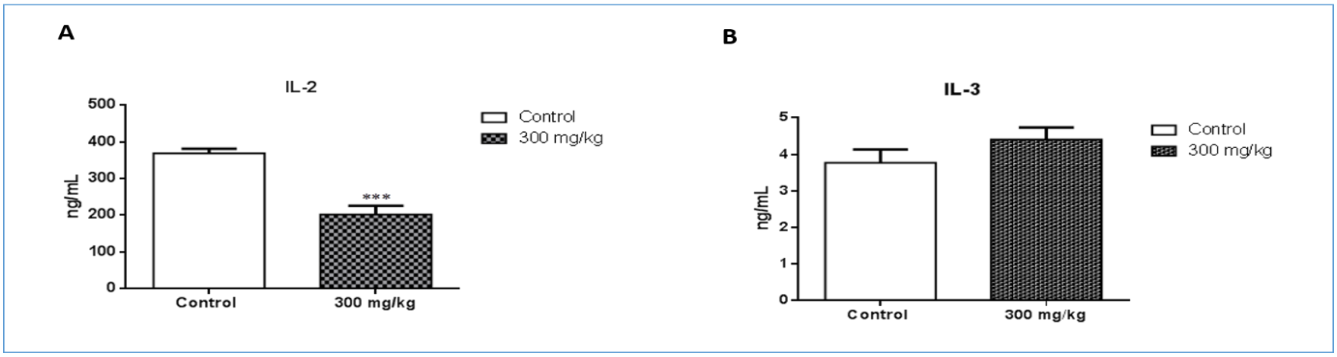

Figure 8: Effect of a Sub-acute oral treatment with EC on IL-2 (A), IL-3 (B). ***P<0.001 vs control. Control. n=5

Sub-acute Effect of EC Treatment on Cerebral Cortex Histology

(A) Hematoxylin-Eosin Stain

There were histopathological changes in the brain of treated rats compared to control. Photomicrograph of the cerebral cortex of control rats revealed suprachiasmatic nuclei presenting with sparsely distributed granule cells, interspersed with succinctly expressed stellate cells. Cellular density and staining intensity were typical of normal cortex histology Figure 9 (1A). Granule cells appear normal in distribution and well positioned in their eosinophilic neuropil. There was, however, overexpression of stellate cells Figure 9 (1B).

(B) Silver Stain

Figure 9 (2A and 2B) are representative photomicrograph showing a high-power magnification (×400) silver staining slides of the cerebral cortex of control and EC-treated animals respectively, of the external granular layer. Granule cells showed marked degenerated axonal and dendritic projections as well as soma filled with neurofibrillary tangles (red arrow heads) in the treated group. The control group showed normal characteristic morphology of neuronal projections with no intrasomal aggregates.

Table 2: Effects of a sub-acute oral treatment with EC on lysosomal enzyme activities in the brain of Male Wistar rats

Brain Homogenate Parameters	Control	300 mg/kg EC
RNAse (Units/mL)	3.4 ± 0.3	4.3 ± 0.5*
DNAse (Units/mL)	17.2 ± 4.4	25.2 ± 4.8**
T-ACP (U/L)	3.4 ± 1.2	20.9 ± 2.7***
Free ACP (U/L)	0.7 ± 0.2	2.7 ± 0.4**
Paraoxonase 1 (ng/mL)	7.4 ± 1.1	12.7 ± 0.8**

*P<0.05, **P<0.01, ***P<0.001 vs control. n=10.

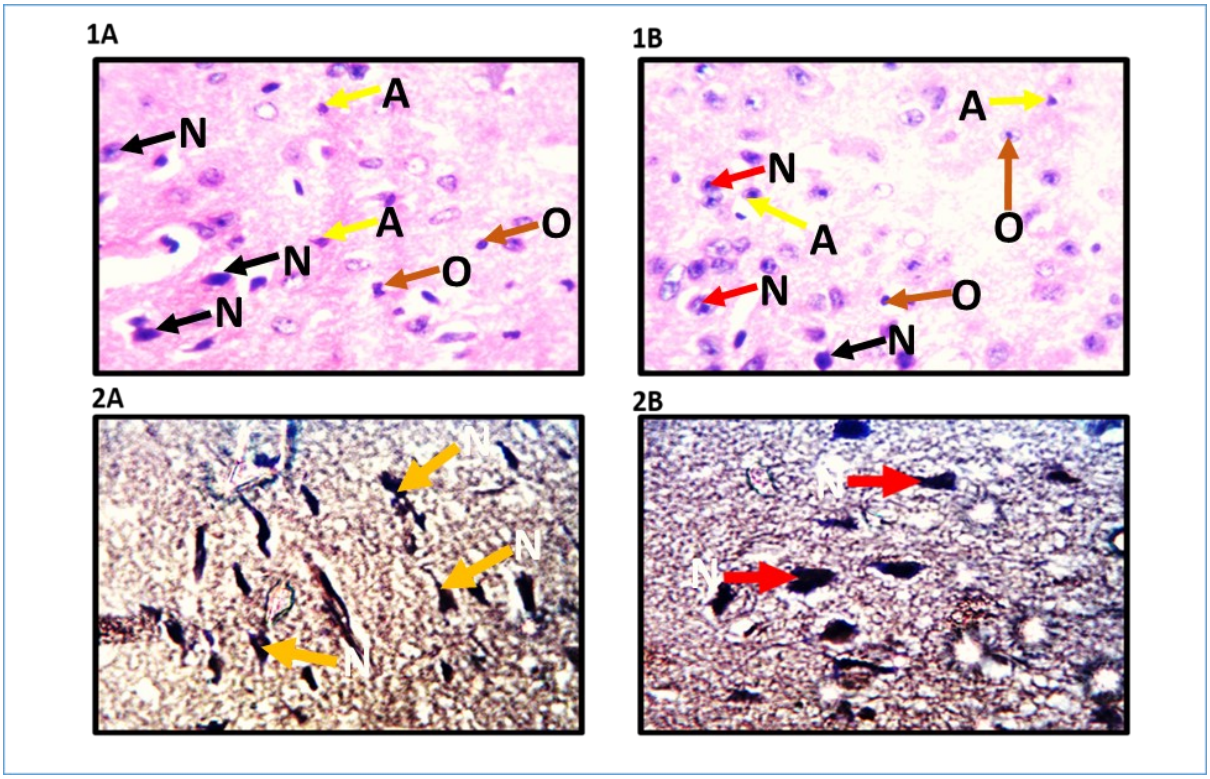

Figure 9: Photomicrographs Showing effects of a sub-acute oral treatment with EC on cerebral cortex of experimental rats, H and E staining (1A and 1B) and silver staining (2A and 2B). Neurons (N), Astrocytes (A), and Oligodendrocytes (O).

DISCUSSION

This study has revealed the possible involvement of GABAergic pathway in EC-induced convulsion. It has also shown that EC induces oxidative and inflammatory conditions as well as structural damages to the brains of exposed Wistar rats.

Convulsion, a sudden and transient involuntary movement of the body or a part thereof, occasionally resulting in unconsciousness with or without changes in bowel and/or bladder functions (WHO, 2018) results from disordered, synchronous and rhythmic aggregated neuronal discharges within a specific region of the brain (James, 2001). It occurs occasionally unprovoked and at times following stimulation (WHO, 2018). It distinctly differs from epilepsy which is a chronic disease condition that is associated with multiple unprovoked convulsions (Wannang *et al.*, 2008). Though many literatures have associated camphor with convulsions, the mechanism(s) relating to its pathophysiology and the seizure characteristics remained largely unknown or at best poorly understood.

PTZ, a standard laboratory chemical agent used in seizure induction in animals (Balakrishnan *et al.*, 1999) is considered a non-competitive GABA receptor antagonist since it blocks the picrotoxin-sensitive site of the $GABA_A$ receptor (De Sarro *et al.*, 1999; Guzmán *et al.*, 2003; Wasowski and Marder, 2012). Convulsions following its administration in laboratory animals is well characterized depending on the dose and route of administration (Zhao and Holmes, 2006). The PTZ animal model of generalized myoclonic seizures has extensively been used for the assessment of anti-epileptic drugs (Pagonopoulou *et al.,* 1993). The inhibition of seizures induced by PTZ in laboratory animals is a major protocol used to characterize a prospective anticonvulsant remedy (De Almeida *et al.,* 2011). Hence, we compared the seizure characteristics of intraperitoneally injected PTZ with that of EC in Wistar rats. EC at 1000 mg/kg evoked convulsion in more animals with less mortality, longer latency but shorter duration than PTZ at 100 mg/kg (Table 2) suggesting its suitability as an alternative to PTZ in seizure induction experiments in animals.

Benzodiazepines, such as diazepam (DZP), inhibit the effects of PTZ by potentiating GABA-mediated inhibition in the brain (Riss *et al.*, 2008) while carbamazepine (CZP) only relatively diminishes PTZ-induced convulsions by preventing repetitive and/or sustained firing of neuronal action potential via preferential binding to voltage-gated sodium channels in their inactive conformation in neurons (Rogawski *et al.*, 2016). This means that GABA neurotransmission-related convulsions are almost always completely defused by DZP and to a lesser extent by CZP.

In the present study, pre-administration of DZP completely inhibited EC-induced convulsions while CBZ only partially attenuated it in the experimental animals. This suggests a direct antagonistic effect of EC on GABAergic neurotransmission and partial or only secondary effect on sodium channel inhibition in the brains of the animals. Similar findings were reported in studies by Krall *et al.* (1978) where DZP pre-treatment blunted PTZ-induced seizures and by Wolfgang. (2011) in rodent models of PTZ-induced seizures with prior exposure to CZP.

Hyperglycemia is reportedly linked with GABA dysmetabolism and reduced GABA intra-islet transmission in the pancreas (Wan *et al.*, 2015), it is therefore not surprising that EC at 300 mg/kg induced transient marked elevation of blood sugar levels in the exposed animals shortly post-exposure. The normalization of this derangement within 24 hours and beyond is also suggestive of a short duration disturbance of GABA intra-islet transmission within the pancreas of the animals. The single EC administration may explain the indifference in the serum triglycerides and cholesterol of the animals tested.

Oxidative stress results when the steady-state equilibrium of pro-oxidants and antioxidants favors the path of the former, thereby creating an enabling environment for oxidative damages (Stamler *et al.*, 1992) including fatty acid and protein oxidation, lipid peroxidation, carbohydrate and nucleic acid damages (Maes *et al.*, 2011).

The central nervous system (CNS) particularly has an unusual metabolic behavior with respect to oxygen demand, consuming roughly 20% of all inhaled oxygen at rest, despite accounting for only about 2% of total body weight (Silver and Erecinska, 1998). This indicates that significant metabolism of oxygen occurs in the CNS despite its low capacity for antioxidant defenses, rendering the CNS highly susceptible to oxidative damage (Halliwell, 1996; Estevez *et al.*, 2011). Insults of any kind to the CNS may increase its reactive oxygen species (ROS) or/and reactive nitrogen species (RNS) pool thereby overstretching its innate antioxidant system and consequent oxidative damage leading to CNS disorders such as Parkinson's disease, stroke, dementia, and epilepsy (Kong and Lin, 2010). To preclude these catastrophe, excessive ROS are degraded into harmless molecules by antioxidant enzymes including SOD, CAT and GPx as well as endogenous nonenzymatic antioxidants such as GSH (Baierle *et al.*, 2015) and exogenously derived non-enzymatic antioxidants such as nutritional vitamins C, A and E; uric acid, melatonin and carotenoids (Vasdev *et al.*, 2011).

CAT and GPx are the two most important free radical scavenging enzymes in the brain (da Silva and Goncalves, 2010). The activities of these enzymes were significantly reduced in the brains of the EC exposed animals (Figure 4B & 4C) suggesting great overwhelm of these enzymatic antioxidant mechanisms and a reduction in the antioxidant capacity of the organs. This finding agrees with the observation of Somade *et al.* (2017) who reported significant reduction in CAT levels in liver and kidney homogenates of camphor-treated rats. GSH, a nonenzymatic antioxidant, plays major role in mediating oxidative stress in the CNS (Arena *et al.*, 2019) by complementing the enzymatic antioxidant mechanism. We observed significant reduction of GSH in the brains of the exposed rats compared to control (Figure 5A) implying further overwhelm of the antioxidant status of the rat brains and a worsening decline in the overall antioxidant capacity of the brains of the exposed animals. Again, this finding agrees with the observation of Somade *et al.* (2017) who reported significant reduction in GSH level in the lungs of EC exposed animals.

Alteration in redox mechanism in favor of a decline in antioxidant capacity inversely supports excessive pooling of ROS or RNS leading to lipid peroxidation with consequent damage to biomolecules, cellular organelles and retardation of cellular function (Somade *et al.*, 2016). MDA, a typical product of lipid peroxidation directly reflects the severity of oxidative stress and its concentration in tissues or organs indirectly mirrors the degree of cellular damage in such tissues/organs, and so serves as an excellent biomarker or endpoint of oxidative stress in the CNS (Arena *et al.*, 2019). We observed that oral administration of EC led to increase MDA levels in the brains of the exposed rats (Figure 4A), this increase could portend loss of neuronal fatty acids. Somade *et al.* (2016) reported an increase in MDA levels in the liver and kidney homogenates of animals exposed to camphor at a dose of 6 g/kg body weight. Our finding however, contradicts that of Fu *et al.* (2016) who reported markedly reduced MDA levels in rats fed with the oil from natural seed of *C. camphora*. This may be as a result of the natural state of the oil used in this latter study or a possibility that the oil contained some other phytochemicals.

Brain inflammation and oxidative stress (OS) are associated occurrences commonly found in many neurological syndromes and numerous studies agree with the reality of a self-sustaining cycle between OS and brain inflammation (Castellani *et al.*, 2014; Fischer and Maier, 2015). Stressful conditions of the CNS provoke release of damage-associated molecular patterns (DAMPs) from neurons leading to subsequent activation of neuroglial cells. Microglia releases tumor necrosis factor alpha (TNFα) in bouts resulting in the initiation of acute

inflammatory processes (Jarvinen *et al.*, 2008) with intention to prevent further damages by the intruding noxious agent/chemical. In our sub-acute study, we observed a significant increase in the TNFα levels in the brain homogenates of exposed animals compared to control (Figure 6A) suggesting an ongoing inflammatory response in their brains. Recruited immune cells as well as the resident microglia release other inflammatory cytokines and chemokines towards aiding inflammation that eventually leads to tissue damage. A related phenomenon may have occurred in the brains of the EC exposed animals in our study, this is evident by the increased levels of pro-inflammatory cytokines IL-8 (Figure 6C) in the brain lysate of the exposed animals compared to control. Interleukins 4, 9 and 10 produced in response to tissue insults acts to suppress immune response following such affronts and support damage resolution and tissue regeneration. We observed increased level of these cytokines in the brain homogenate of the exposed rats compared to control (Figure 7A, B and C) implying a negative feedback response to the ongoing neuronal damage and an effort towards halting further damage to the brain. We however, observed a significant decrease in the brain level of IL-2, another immunomodulatory cytokine produced by neurons and astrocytes in the central nervous system of both rodents and humans (Chun-Lei Jiang and Lu., 1998). This may be due to neural damage caused by the prolonged exposure to EC with resultant diminished IL-2 release.

Lysosomal enzymes remove accumulating cellular debris following inflammation and/or oxidative damage to cells (Acharya *et al.*, 2005). Total and free acid phosphatase (TACP, Free ACP) levels were elevated in the brain lysate of EC exposed animals compared to control (Table 3), indicating increased activity of these enzymes for debris clearance in the brains of the animals. This is similar to the observation by Suzuki *et al.* (1998) in animals with neurodegeneration following exposure to aluminum. Stressful conditions lead to damage of genetic materials within cells and has been observed in neurodegenerative illness such as Alzheimer's, Huntington's, and Parkinson's diseases (Hedge *et al.*, 2011, Merlo *et al.*, 2016). This often results in increased activity of DNases and RNases which help in mopping up damaged genetic materials in injured cells (Bernardi, 1971). In this experiment we confirmed increase activity of these enzymes in the brains of the EC exposed rats signifying an intrinsic response to damages to genetic materials in the brain cells. This finding is similar to that observed by Acharya *et al.*, (2005) in a picrotoxin-induced neurotoxicity study and Khandkar *et al.* (1996) in a paracetamol-induced hepatotoxicity study.

Paraoxonase- 1 (PON-1), in very small quantities in the brain is released during stressful and inflammatory conditions of the CNS because of its antioxidant and anti-inflammatory roles (Litvinov *et al.,* 2012). The significant increase of this enzyme in the brain lysate of the exposed animals compared to control signifies an increase in its activity and as such, agrees with the of oxidative and inflammatory damages in the brains of these animals (Table 3).

Damaged proteins and genetic materials from oxidative stress accumulate as intracellular aggregates and/or extracellular plagues within the brain as a mark of many neurodegenerative diseases (Samina, 2017). These neurotoxic aggregates include misfolded tau and amyloid-β proteins, Lewy bodies, and mutant Huntington's proteins found in brains sections sufferers of Alzheimer's, Parkinson's and Huntington's disease respectively (Samina,2017).

The presence of neurofibrillary tangles as well as axonal and dendritic projections degeneration in the cortical and hippocampal sections of the brains of the experimental animals, therefore suggest an ongoing neurodegenerative process (Figures 9, 2A and 2B).

CONCLUSION

We conclude that EC neurotoxicity is as a result of GABAergic neurotransmission inhibition, resulting in oxidative, inflammatory and eventual degenerative damages to neurons.

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CONFLICTS OF INTEREST

All authors declare no conflict of interest with regards to undertaking and publishing of this work.

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AUTHORS' CONTRIBUTIONS

AAN and OEO conceptualized the study; UO, OI and SOO carried out the experiments; EN CIU and BE carried out the analysis; and AAN and OEO supervised the study. All authors read the manuscript and approved its submission.

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