ANTIOXIDANT MICRONUTRIENTS AND PHENOL FRACTION OF *PIPER GUINEENSE* EXTRACT EXHIBITS DIFFERENTIAL CD68 CEREBELLAR EXPRESSION ON AZT INDUCED- NEUROINFLAMMATION.

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Abstract

Background of the study: Exposure to HAART regimen especially Azidothymidine (AZT) therapy has neurotoxic adverse effects like neuroinflammation.
Aim: We assessed the role of Phenol extract of *P. guineense* leaf and antioxidants on the Azidothymidine challenge.
Material and Methods: Thirty-six adult Wistar rats were randomized into nine groups of 4 rats each. Azidothymidine (AZT) was administered to all groups except the control which received 0.1mL saline. Others received 100 mg/kg of AZT for 8 days, 100 mg/kg of AZT+100 mg/kg of *P. guineense*, 100 mg/kg of AZT+ 200 mg/kg of *P. guineense*, 100 mg/kg of AZT + 400 mg/kg of *P. guineense,* 100 mg/kg of AZT+ Zinc, 100 mg/kg of AZT+ 3mg/kg of Melatonin, 100 mg/kg of AZT+ 1000mg/kg Cellgivity, and 100 mg/kg of AZT+ 50 mg/kg of Selenium for 14 days respectively.Cerebellar amoeboid microglia expression was by identified by CD68 marker.
Result: AZT induces reactive microgliosis, and the *P. guineense* extract exhibited dose-dependent pleiotropic microglia retraction. The antioxidants: Zinc, melatonin, selenium and cellgevity deferentially mitigate the AZT effect by providing neuroprotection.The high dose of *P. guineense*, selenium, and cellgevity had a pronounced reversal effect on the microglia.
Conclusion: The effective dose of phenol extract *p.guineense* was beneficial in halting the neuroinflammatory effect of AZT in the cerebellum.
Keywords: *Neuroinflammation, anti-retroviral, Antioxidants, Piper guineense, Microglia, Micronutrients*.

Introduction

In the nervous system, neuroinflammation is a maladaptive reaction to tissue damage that involves the recruitment of immune cells and mediators, which are frequently produced to spur on neuro-regeneration[1,2,3]. Neuroinflammation is an Azidothymidine (AZT) adverse side effect. AZT is a nucleoside reverse transcriptase inhibitor associated with the adverse phenomenon in the brain that contributes to neuronal and glial damage in the management of HIV[4]. People on AZT and living with HIV have been found to have a variety of the neuronal damage over time [4,5,6,7,8]. Prominent among the damages, cerebellar dysfunction is characterized by granules cell and primary cerebellar atrophy [5, 6,7,9]. The disruption of the granular cells indicates a pathological mechanism which in turn impacts altered Purkinje cell activity[5,6,7].

In addition, components of nutritional vulnerability are affected by the AZT regimen and other retroviral therapy. As a result, it has long been known that nutritional deficits has co-morbidity with a number of illnesses, including HIV/AIDS. Therefore, micronutrients and antioxidants supplementations may improve the course of disease and the effectiveness of treatment by addressing deficiencies and immune system stress. Furthermore, inadequate diet might lead to increased toxicity or reduce the effectiveness of medications[10,11,12,13]. Physiological changes brought on by drugs are linked to micronutrient deficits. Micronutrient supplements, on the other hand, can postpone the onset of AIDS and increase survival rates. They also provide a low-cost method of reducing the negative effects of medication and enhancing treatment results[11,12,13].

Antioxidants are compounds that prevent oxidation from occurring and protect the organism from the damaging effects of free radicals. In order to maintain homeostasis, it is essential for scavenging excess reactive oxygen species (ROS). The immune system is strengthened by optimizing the intake of antioxidants, minerals, and other bioactive food ingredients[14]. Antioxidants, which are found in vitamins, minerals, and enzymes, guard and restore cells against the harm caused by free radicals that target proteins, fats, and DNA[14].

In the form of glutathione peroxidase, selenium is an essential vitamin that may synthesis DNA, repair oxidative damage to DNA, alter cellular antioxidant defense, and promote leukocyte adhesion[12.] When taken as a supplement with HIV medication, selenium lowers the risk of brain damage via inhibiting inflammatory cytokines and glutathione peroxidase's antioxidant properties[12]. The antioxidant has shown promise in reducing platelet aggregation, lipid peroxidation, and microglial responses[15,16].

On the other hand, zinc functions as an immunological modulator, antioxidant, ant-viral, and anti-inflammatory agent and is crucial for cell-mediated immunity[17,18]. The enzymes that help the antioxidant defense system function well depend on zinc as a co-factor [17]. Neurons produce zinc in a number of situations where microglial activation is evident[18,19, 20].

In addition to its important role in the circadian cycle, melatonin is a neurohormone with a wide range of biological properties, including anti-inflammatory, anti-apoptotic, and antioxidant properties[21]. Microglia, neurons, and astrocytes are home to melatonin receptors, which can easily cross the blood-brain barrier (BBB)[22]. Melatonin has been shown to have a neuroprotective impact against disorders of the central nervous system[23,24]. By controlling inflammation, apoptosis, or autophagy following brain damage, it helps to preserve cell survival and homeostasis[23,24].

Cellgevity® is a dietary supplement that is classified as an antioxidant. It specifically enhances glutathione and contains riboceine

(D-ribose L-cysteine), which is its active ingredient. Other significant ingredients include alpha lipoic acid, selenomethionine, turmeric root extract, broccoli seed extract, resveratrol, aloe extract, vitamin C, grape seed extract, quercetin, milk thistle seed extract, curcumin, cordyceps, etc. Because glutathione (GSH) may scavenge reactive oxygen species, it provides protection against damage caused by oxidative stress[25]. Glutathione is necessary for immunological system function, tissue growth and repair, and the amelioration of neuropathological changes in the hippocampal region[26.]

Herbal plants continue to be a source of promising treatments for a variety of illnesses. Piper guineense is referred to locally as Ata Iyere in Yoruba and Uziza in Igbo, and is also known as African black pepper, Ashanti pepper, and Benin pepper[27,28]. It has long been used to treat infections, inflammation, and infertility. Pharmacologically, it possesses well-known anti-inflammatory and antioxidant qualities[27,29]. Flavonoids derived from P. guineense also prevent HIV replication[15].

On a more positive note, this study examined the potential of P. guineense (Uziza) and particular antioxidants in reducing alterations in microglia related to neuroinflammation caused by AZT.

Materials and Methods

Plant: *P.guineense*

The plant material was procured from Ogbete market in Enugu State, Nigeria. The plant was identified by a curator in the Department of Plant Science and Biotechnology of the University of Nigeria, Nsukka, Nigeria and its authentic name was confirmed on www.plantlist.org[30]. The *Piper guineense* (Uziza) leaves were carefully selected to remove leaves with pathological features to ensure disease free collection. The leaves were then washed with distilled water and air-dried for seven days at room temperature. Thereafter, the *P. guineense* leaves were pulverized into a fine powder, using a milling machine. The 500 g of the powder was soaked in 80% methanol and left for 48 hours with continuous stirring. 10% of N-hexene was then added to the filtrate, and left in the open air to dry up. The final extract was then kept in the refrigerator.

Estimation of Total Phenolic and flavonoid contents

The method of Kupina et al.[31] was adopted in the estimation of total phenol in the extract. The total phenolic content of dry extracts was performed with a Folin-Ciocaltaeu assay. 1 ml of sample (1 mg/mL) was mixed with 1 ml of Folin Ciocalteu's phenol reagent. After 5 minutes,10 ml of 7% sodium carbonate solution was added to the mixture followed by the addition of 13 ml of deionized distilled water and mixed thoroughly. The mixture was kept in the dark for 90 minutes at 23^0C, after which the absorbance was read at 760 nm. The total phenolic content was determined from the extrapolation of the calibration curve which was made by preparing a Gallic acid solution. The estimation of the phenolic compounds was carried out in triplicate. The TPC was expressed as milligrams of Gallic acid equivalents (GAE)/g of dried sample.

The content of flavonoids in plant extracts was determined using the spectrophotometric method adopted from Dirar et al[32]. In a 96-well plate, 25 µl of standard or sample solution was added followed by 75 µl of ethanol. Afterwards, 5 µl of $AlCl_3$ (10% prepared in methanol) and 140 µl distilled water were added to the mixture. The plate was shaken for 30 mins prior to the measurement of the absorbance at 420 nm. All samples and standards were measured against a blank prepared concomitantly with the exception of $AlCl_3$. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Quercetin was used as a standard to construct the calibration curve. The TFC of the extract was determined using an equation obtained from the standard calibration curve and was expressed in terms

of Quercetin equivalent (mg of Quercetin/g of extract).

Drugs

Zinc and melatonin (Puritan Pride), and Cellgevity (Riboceine®) were purchased in bulk from a subsidiary of Max International, Selenium and AZT produced by (Aspen Pharmacare company in South Africa) was purchased from a registered pharmacy, Nigeria.

Animal Handling

Ethical approval was obtained from the Departmental of Ethics Committee on the Use of Laboratory Animals, Enugu State University of Science and Technology, Enugu State, Nigeria. All procedures were carried out following the National Academy of Science's Guide for Care and Use of laboratory animals[33]. Thirty-six (36) Wistar rats age 6-8 weeks, weighing 180-200 g were purchased from the animal husbandry of the College of Medicine, University of Nigeria, Enugu campus, Nigeria. The rats were acclimatized in the animal house of the College of Medicine, Parklane, ESUT for two weeks. The rats were housed in netted iron cages in groups of four, fed with the rat's chow (Growers mash, Nig, Ltd.), and provided water *ad libitum*. The laboratory conditions of temperature 32^0C, relative humidity of 60-70%, and 12hrs light-dark cycle were maintained. Thereafter, the animals were randomly divided into 9 groups of four animals each for the commencement of the study.

Experimental Design

Azidothymidine (AZT) was administered to all groups except the control which received 0.1mL saline. Others received 100 mg/kg of AZT for 8 days, 100 mg/kg of AZT+100 mg/kg of *P. guineense*, 100 mg/kg of AZT+ 200 mg/kg of *P. guineense*, 100 mg/kg of AZT + 400 mg/kg of *P. guineense*, 100 mg/kg of AZT+ Zinc, 100 mg/kg of AZT+ 3mg/kg of Melatonin, 100 mg/kg of AZT+ 1000mg/kg Cellgivity, and 100 mg/kg of AZT+ 50 mg/kg of Selenium for 14 days respectively.

Immunohistochemistry

For the analysis of phagocytic or amoeboid microglial morphology, the CD 68 anti-body marker for microglia was used. On the 15th day, all rats were anaesthetized with an overdose of thiopental sodium and transcardially perfused with 0.1 ml of phosphate-buffered saline (PBS, PH 7.4) at room temperature, followed by 4% paraformaldehyde. The brain was harvested and post-fixed in 10% neutral buffered formal saline for 72 hours: Thereafter, the cerebellum was dissected and manually processed for paraffin embedding. Paraffin-embedded blocks were sectioned at 10 µm thickness and further processed and deparaffinized. Immunoperoxidase was used to label CD68 antibodies for microglia. Endogenous peroxidase activity was blocked with pre-incubation in 0.3% H_2O_2. After washing, the sections were pre-incubated for 1 hour at room temperature in the appropriate normal serum before incubation in primary antibodies overnight at 4°C. The sections were then rinsed, incubated in secondary antibodies at 1:200 dilution for 2 hours at room temperature, and then reacted in Avidin-Biotin complex solution for 15 mins using 3-3-diaminobenzidine (DAB) as a chromogen. The sections were then mounted on slides, dried, dehydrated, cleared, and cover slipped. Thereafter, representative phtomicrographs of each group was captured using the Amscope 3.0 with a digital camera.

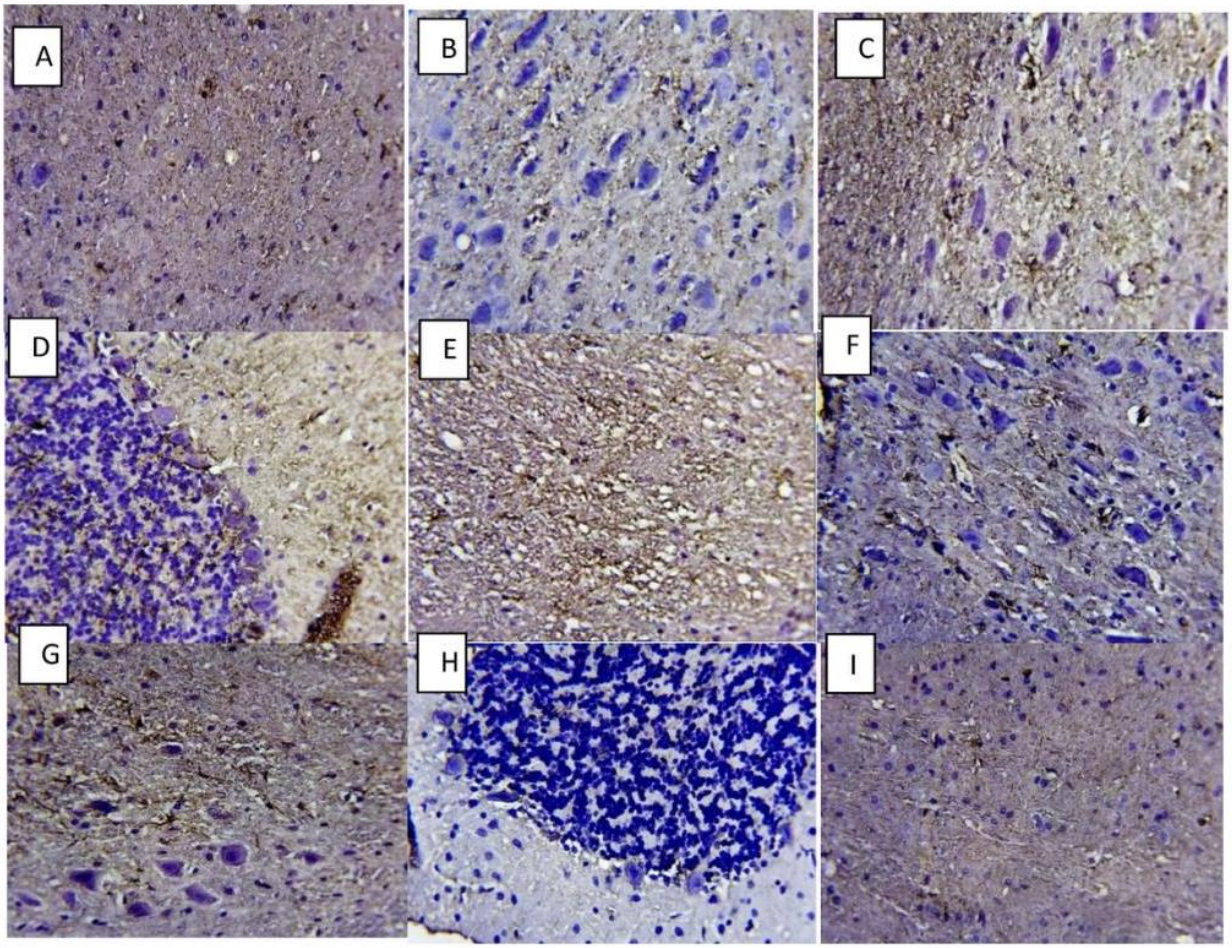

Figure I: CD68 immunostaining highlighted amoeboid microglia: A:normal saline, B :AZT only, C;AZT+100 mg/kg extract, D;AZT+200 mg/kg extract E;AZT+ 400 mg/kg extract, F; AZT+Zinc ,G: AZT +Melatonin, H: AZT + Cellgevity, H: AZT +Selenium. Anti-CD68. X 800.

Result and Discussion

Plant analysis revealed the concentration of total phenolic and flavonoid compounds as 31.99±4.21and 39.52±1.79 respectively. The CD68 is a granule protein and scavenger receptor found on macrophages. The macrophage of the brain is a microglial cell that plays a critical role in cross-talk responses during inflammation, hence its expression is associated with heightened neuroinflammation[34,35]. The over-activated microglial cells linked to neuroinflammation are a key indicator of neurological disorders. The over-activated microglia can disturb neuronal homoeostasis by releasing inflammatory mediators leading to neuronal

dysfunctions and death. Thus, inhibition of over-activated microglia might be an effective therapeutic approach for modulating neuroinflammation[34,35,36].

In a recent review, the close relationship between neuroinflammation and neurodegeneration has been linked to the dysregulation of glial-neuronal communications[21]. Under physiological conditions, microglia appear resting and ramified with extending processes as they constantly sensor the brain environment for any change or pathology that can cause them to respond in a variety of different ways[34]. Cell morphology alone does not fully indicate microglial function but examining a range of microglial markers aids a better understanding of the behaviour of these cells. for instance, Cerebellar microglia are a unique population of immunologically responsive cells that are normally inactive in the absence of CNS pathology[5]. However, this cell becomes acutely sensitive to assault in the CNS and remains a vital participant in the process of the resolution. They regulate the survival of Purkinje and Granular cells during pathological events like exposure to alcohol, toxin, infection, and inflammation[37]. A previous study has highlighted the differential behaviour of cerebellar microglia, including differential expression, immunological gene, rapid turnover, and sensitivity to haemostatic disruption in disease and injury[37].

It is clear in this study, the reactivity of microglia is revealed by the CD68 marker. The AZT group revealed positive reactivity to the CD68 marker with prominent microglia cells. This implies that AZT neurotoxic adverse drug effects are immune-mediated leading to apoptosis of Purkinje and granular cells. Studies support the fact that AZT is not actively transported across the blood-brain barrier but high levels of AZT accumulate in the cerebrospinal fluid, and subsequently diffuse into the overlying parenchyma[4,37,38]. This might explain the mechanism of AZT-induced microglia response (microgliosis) observed in our study. Additionally, due to the close anatomical proximity of the neurogenic niches to the ventricular system, it collaborates with the hypothesize that diffusion from CSF exposes neurons to relevant levels of AZT that are sufficient to perturb normal cell functions including microglia[4]. The low dose of *P.guineense* exhibited similar features of microglia reactivity as the AZT group. Comparatively, the *P.guineense* phenol extract exerted a dose-dependent effect on the reactivity of microglia. These findings imply the withdrawal of microglia following the neuroinflammation induced by AZT. This affirms the anti-inflammatory properties of *P.guineense* previously reported[27].

The zinc and melatonin revealed positive immunoreactivity to the CD68 marker but activated microglia were less compared to the medium and high doses of the *P. guineense*, cellgevity, and selenium. Melatonin is a well-known neuroprotective and anti-inflammatory agent[24]. The anti-inflammatory and antioxidant effects of melatonin are intertwined[22]. Neuropathological conditions are accompanied by low-grade inflammation, blood-brain barrier impairment, and alteration of sleep which affects melatonin secretion[39]. The deviation in melatonin secretion response positively to exogenous melatonin. Hence, exogenous melatonin administration reduces the damaging effects of neuroinflammation in rodents[39,40]. It is not surprising that melatonin alleviates the effect of AZT on microglia expression.

Growing evidence supports the anti-inflammatory role of melatonin in acute and chronic inflammation processes[21,23]. The main mechanisms of action attributed to melatonin are free radical scavenging, endogenous antioxidant enzyme stimulation, and improved efficiency of other antioxidants [21,23]. Our finding likely supports the latter mechanism: that is the effect of melatonin was secondary to the enhancement of other endogenous antioxidant activities. Zinc is an essential

mineral in health that acts as a cofactor for the synthesis and transcription factor in a view to maintain the antioxidant defense[41,42]. It stabilizes the membrane, and inhibits the enzyme nicotinamide adenine dinucleotide phosphate oxidase and prevents apoptosis[12].

Zinc regulates gene expression through transcription factor activity and is responsible for the activity of dozens of key enzymes in neuronal metabolism[43]. However, our result implies Zinc showed a weak anti-inflammatory effect on microglia activation which collaborates with other report[11].

On the contrary, selenium showed a prominent effect comparable with the high dose of *P. guineense,* and cellgevity. The selenium effect was attributed to its neuroprotection capability afforded by modulation of Ca^{+2} influx into ion channels and anti-inflammatory by abrogating microglia invasion, especially via biosynthetic stimulation of antioxidative seleno-protein in the brain[44]. Cellgevity is a recent dietary supplement that enhances natural glutathione levels in the human body and targets the removal of damaging toxins from the body[45]. The major ingredients of cellgevity are the new nutritional compound known as Riboceine which enables the body to produce an optimal amount of glutathione[45]. The microglia scavenging effect of cellgevity due to the high level of glutathione, as a potent antioxidant suggests a modulatory interplay between the antioxidant and anti-inflammatory pathways. These findings implicate oxidative stress as a contributor to the underlying neurotoxicity and antioxidant provides an access point for adjunctive therapies to complement AZT therapy and reduce neuroinflammation.

Conclusion

P. guineense extract exhibited dose-dependent pleiotropic, anti-inflammatory and antioxidant effects. However, the zinc, melatonin , selenium, and cellgevity deferentially mitigate the neuroinflammatory effect of AZT by providing neuroprotection either via anti-inflammatory or antioxidant mechanisms. Although the high dose of *P. guineense*, selenium, and cellgevity had a more pronounced beneficial effect on the reversal of microgliosis. Hence, the effective dose of phenol extract of P. guineense should be considered in order to achieve the desired therapeutic outcome against neuroinflammatory effect of AZT in HIV patients.

Conflicts of Interest

The Authors declared that research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest

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Author Contributions:

FBE: Conception and design, perform the research, wrote the draft; EAE: gave conceptual advice, contributed to the discussion, reviewed, and edited the draft; OAE: analyzed the histology. All authors read and approved the manuscript.

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