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Immunomodulatory and Antioxidant Effects of Eugenol on Aluminium Chloride-Induced Neurotoxicity in Wistar Rats

Samuel B. Mesole[1], Uthman A. Yusuf[2], Alfred O. Okpanachi[1], Elvis T. Godam[3], Nsoni F. Bumbangi[4], Abdulrazak Animoku[5]

[1]Department of Clinical Medicine, School of Medicine, Eden University, Lusaka, Zambia; [2]Department of Human Anatomy, Mulungushi University, Livingstone, Zambia; [3]Department of Human Anatomy, Rivers State University, Port-Harcourt, Nigeria; [4]Department of Disease Control and Prevention School of Medicine, Eden University, Lusaka, Zambia; [5]Department of Human Anatomy, Kogi State University, Ayingba, Nigeria

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ABSTRACT

Aluminium and its compounds when taken orally can have negative impacts on a variety of enzymes leading to neurotoxicity. Apurinic endonuclease 1 (APE-1), a protein that performs multiple functions, including deoxyribonucleic acid (DNA) repair, is essential for cell survival. Thus, the determination of APE-1 with aluminium chloride ($AlCl_3$) induced neuro-inflammation in Wistar rats was performed in this study. Wistar rats were divided into four groups, each with six animals. Group 1 received 100 mg/kg $AlCl_3$ orally, Group 2 received 150 mg/kg eugenol, Group 3 received both 100 mg/kg $AlCl_3$ and 150 mg/kg eugenol, and Group 4 received 0.8 ml/kg distilled water. Rats were euthanized after eight weeks, and brain tissues were homogenized and analysed. Oral administration of $AlCl_3$ resulted in significant ($p<0.01$) decreased APE-1, superoxide dismutase (SOD) and amyloid beta-40 (Aβ-40), and significant ($p<0.01$) increased tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), inducible nitric oxide synthase (iNOS), myeloperoxidase, and nitric oxide (NO). Co-administration of $AlCl_3$ and eugenol significantly increased ($p<0.01$) APE-1, Aβ-40 and SOD, while significantly decreasing ($p<0.01$) TNF-α, IL-1, iNOS and MPO levels. Our findings suggest that eugenol, by targeting these most potent hallmarks of neurodegeneration could be an effective alternative in its treatment.

Key words: *Apurinic endonuclease 1; Eugenol; Neurodegenerative disease; Tumour necrosis factor alpha; Inducible nitric oxide synthase*

INTRODUCTION

Neurotoxins are known to aggravate neuroinflammatory activity within the central nervous system through a variety of mechanisms, with aluminium being one of such. This metal is known to exacerbate a variety of processes that promote neuroinflammation, including oxidative stress and the deposition of amyloid beta plaque within the brain, which expresses amyloid precursor and amyloid beta proteins (Praticò et al. 2002). Aluminium and amyloid beta are capable of producing reactive oxygen species, which can cause genotoxicity and deoxy ribonucleic (DNA) damage.

Human apurinic endonuclease 1 is a multifunctional ubiquitous protein that plays a critical role in base excision repair for damaged DNA bases and single-strand DNA breaks caused by oxidative and various alkylating agents (Izumi et al. 2003). It also binds to, and protects the DNA from damage that can be triggered by inflammatory cytokines resulting in the reduction of amyloid beta-40 (Aβ-40) levels, and ultimately tissue damage (Okazaki et al. 1994; Tell et al. 2005). Different studies have reported that

Correspondence: Samuel B. Mesole, PhD; Department of Clinical Medicine, School of Medicine and Health Sciences, Eden University; P.O. Box 37727, Lusaka, Zambia. E-mail: ms361450@gmail.com; ORCID: 0000-0002-0062-1646

oxidative stress caused by traumatic brain injury reduces the activity of apurinic endonuclease 1 in the hippocampus, a condition linked to neuronal cell apoptosis (Edwards et al. 1998; Daniele et al. 2021). Mantha et al. (2012) reported that apurinic endonuclease 1 plays a neuroprotective role as a result of its various alliances with intracellular proteins in amyloid beta-treated experimental rats, and that these could regulate cell functions during amyloid beta-mediated neurotoxicity.

When dangerous microbes, damaged cells, or toxic substances invade a biological system, the inflammatory response is usually a protective mechanism used by the biological system to get rid of all dangerous or harmful stimuli from the system, which improves healing (Ferrero-Miliani et al. 2012; De Cássia da Silveira e Sá, et al. 2013). However, these responses must be tightly regulated and controlled, to last a short period of time; otherwise, it may result in pathological disorganization of the immune system (Tung et al. 2008). Inflammation can be divided into two types: acute and chronic. As an initial response, the acute inflammatory response includes activation of resident cells as well as the production of pro-inflammatory chemokines and cytokines, culminating in the recruitment of neutrophils from the innate immune system to the site of injury. The acute inflammatory response also promotes inflammation-related symptoms such as pain and oedema (Stone 2017). Chronic inflammation, on the other hand, causes slow changes within the various types of cells present at the site of inflammation, resulting in permanent irreversible tissue damage over time. A pro-inflammatory mediator is produced as a by-product of both chronic and acute inflammation (De Cássia da Silveira et al. 2013; Nizamutdinova et al. 2016).

The nuclear factor kappa B (NF-kB), which is very important in the transcription of cytokines such as myeloperoxidase, nitric oxide, tumour necrosis factor, and interleukins, is a very important component of the signalling pathway of the immune response. Various substances, including the naturally occurring such as eugenol, have the ability to inhibit this pathway, and such substances are of particular interest (Wang et al. 2014; Polesso et al. 2017; Islam et al. 2018). Non-steroidal anti-inflammatory drugs are commonly used to treat inflammatory conditions, and have been linked to potentially fatal side effects such as gastrointestinal lesions and bleeding. Non-steroidal anti-inflammatory drugs also have low therapeutic efficacy, which can cause patients to discontinue use; as a result, numerous new bioactive molecules have been sought after (Bermas 2014). Plants have been identified as an essential source of these active biomolecules such as phenylpropanoids which is a life-saving pharmacological component. Phenylpropanoids are normally present in its oxidized form with a hydroxyl at the aromatic ring (Rajput et al. 2018).

Eugenol (4-allyl-2-methoxyphenol) is a significant component of cloves and is classified as a phenolic compound in the phenylpropanoids class. It contains 45–90% of the essential oil of the plant (Zhang et al. 2013). Eugenol is largely employed as a preservative in the food sector, due to its antioxidant characteristics. Soya bean, coffee, and basil all contain eugenol (Marrotti et al. 2005; Mesole et al. 2020b).

The current study explores the therapeutic potential of eugenol in providing better therapeutic value and little to no side effects. This study aimed to investigate the role of eugenol in improving apurinic endonuclease 1 expression and its therapeutic or protective activity against aluminium chloride-induced neurotoxicity in rats. In addition, the expression of pro-inflammatory cytokines like tumour necrosis factor-, interleukin-1 (IL-1), inducible nitric oxide synthase (iNOS) and nitric oxide, as well as myeloperoxidase, Aβ-40, and superoxide dismutase levels in response to aluminium chloride ($AlCl_3$) exposure was also investigated.

MATERIALS AND METHODS

Animals and Induction of Neuroinflammation

A total of 24 adult male Wistar rats (150-180 g) were reared and maintained at the animal house of Eden University School of Medicine and Health Sciences, Lusaka, Zambia. The guidelines for animal care were strictly followed, and this research was approved by the University's Research Ethics Committee (EUM10A2019). Experimental animals were maintained at room temperature (24-26°C) and allowed free access to standard rat chow and water. Neuroinflammation was induced orally with $AlCl_3$ (#7446-70-0; Guandong Guanghua Sci-Tech Co. Ltd., China) at a dose of 100 mg/kg for a duration of eight weeks (Mesole et al. 2020a). Eugenol (#58-23-4; Yueyang Jiazhiyuan Biological Co Ltd, China) of 99.9% purity level was co-administered orally at a dose of 150 mg/kg for 8 weeks (Mesole et al. 2020a). The 24 animals were divided into four groups with six rats in each group (administrations of eugenol and $AlCl_3$ were carried out once daily at 0600 h and both were dissolved in water) as illustrated in Table 1.

Table 1: Rat Grouping and Dosage

Group	Dose
AC ($AlCl_3$)	100 mg/kg $AlCl_3$
EG	150 mg/kg Eugenol
EG+AC	100 mg/kg $AlCl_3$+150 mg/kg Eugenol
CTRL	0.8 ml/kg distilled water

n=6, AC (Aluminium chloride); Eugenol (EG); EG+AC (Eugenol+ Aluminium chloride); CTRL (Control)

The experimental animals were sacrificed humanely twenty-four hours after the last administrations, with 0.7 ml/kg ketamine (intraperitoneally) as the anaesthetizing agent. A pair of scissors was used to open the skull, the brains were carefully removed and quickly transferred to sample bottles containing 1M phosphate buffered saline at pH 7.4, and the frontal lobe was homogenized. The homogenates were centrifuged at 1200 g for 10 min at 4 ℃. The resulting supernatants were used to estimate various protein evaluations. The total protein content of the homogenates was determined using Lowry et al. (1951) method, which was modified by Al-Olayan et al. (2015) and Mesole et al. (2020a).

Estimation of Apurinic Endonuclease 1
Apurinic endonuclease 1 was quantified using a commercially available rat apurinic site lyase (Apex1) ELISA kit and following the manufacturer's instructions. After the samples were added to the wells and incubated at room temperature for 10 min, 50 µl of diluted anti-APE1 antibody was added to each well, followed by 1 h of co-incubation at room temperature. Samples were washed three times with a 1x wash buffer at a volume of 250 µl for each well. The secondary antibody enzyme, which had been diluted to a volume of 100 µl, was then added to the wells and incubated at room temperature for 1 h, washed three times and a warm substrate with (100 µl) added to each well and incubated at room temperature. To halt the enzymatic reaction, 100 µl of stop solution was added to each well. The obtained results were read in a spectrophotometer at a wavelength of 450 nm (Jia et al. 2017).

Estimation of Amyloid Beta-40
The amyloid beta peptide 1-40 was determined or quantified using a commercially available kit and strict adherence to the manufacturer's instructions. Frontal lobe tissue were washed in saline, then homogenized in an extraction buffer containing 10 mM HEPES, 350 mM sucrose, 5 mM EDTA, pH 7.4, 1% Triton-X100, and a protease inhibitor cocktail. The homogenates were evaluated and the results were calculated as pg/ml/g tissue.

Estimation of Tumour Necrosis Factor
Tumour necrosis factor alpha (TNF-α) quantification in the brain was performed using a rat TNF- ELISA kit supplied by ABCAM and used in strict accordance with manufacturer instructions. TNF- concentrations were expressed in pg/g brain tissue.

Estimation of Nitric Oxide
The brain tissue level of nitric oxide (NO) was analysed spectrophotometrically using an ABCAM-supplied ELISA kit and strict adherence to the manufacturer's instructions. Endogenous nitrite concentration was measured as an indicator of NO production. The absorbance was measured at 540 nm after the addition of a Griess reagent, which converts nitrites produced to a deep purple azo compound.

Estimation of Cyotokine
Interleukin-1 tissue levels were measured using an ABCAM-supplied rat ELISA kit and strict adherence to the manufacturer's instructions, and the results were expressed as pg/ml brain tissue.

Estimation of Inducible Nitric Oxide synthase
Mybiosource provided the appropriate nitric oxide synthase (iNOS) rat ELISA kit for the quantification of iNOS. To 10 mg of tissue homogenate, 100 µl of phosphate buffered saline was added. The resulting homogenate was then centrifuged at 1000xg (3000 rpm) for 20 min. The obtained supernatant was collected and used for the assay in accordance with the manufacturer's instructions.

Estimation of Myeloperoxidase Activity
An ice-cold homogenization buffer containing 50 mM potassium phosphate, pH 7.4, 1.0 mM EDTA, and 1.0 ml/L Triton X-100 was used to homogenize the brain tissue (Hillegass et al. 1990).

Estimation of Anti-oxidative Stress Marker
The antioxidative stress enzyme superoxide dismutase (SOD) activity level was determined using a rat appropriate SOD ELISA kit and the manufacturer's instructions.

Data Analysis
All data were analysed using Just another statistical package (JASP) version 0.14.1. Results were expressed as mean ± standard error of mean (SEM), and the presence of significant differences among the means of groups was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test for significance. Paired samples t-test was employed for comparison of means as appropriate. Values were considered significant when $p \leq 0.01$.

RESULTS

The effect of eugenol on the expression of apurinic endonuclease 1 (APE-1) following the administration of $AlCl_3$ revealed a significant ($p<0.01$) reduction of this enzyme compared to the control group. Co-administration of eugenol and $AlCl_3$ resulted in a significant ($p<0.01$) increase in APE-1 expression compared to the group administered $AlCl_3$. However, the group administered eugenol alone had a significant increased ($p<0.01$) expression of APE-1 compared to the $AlCl_3$ group (Fig. 1).

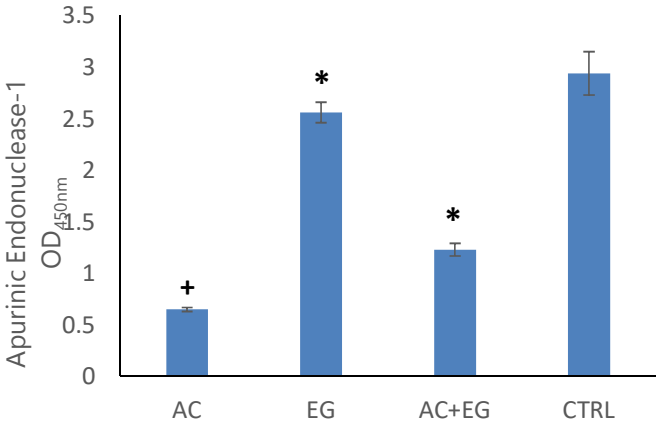

Fig. 1: Effect of eugenol (EG) on the expression of apurinic endonuclease-1. n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

The effect of eugenol on Aβ-40 following administration of $AlCl_3$ showed a significant (p<0.01) reduction of this protein in the $AlCl_3$ group compared to the control. However, co-administration of eugenol and $AlCl_3$ resulted in a significant (p<0.01) increase in Aβ-40 when compared to the $AlCl_3$ administered group. The group treated with eugenol alone showed a significant increase (p<0.01) in Aβ-40 when compared to the $AlCl_3$ treated group (Fig. 2).

The effect of eugenol on tumour necrosis factor-α (TNF-α) level following the administration of $AlCl_3$ showed a significant (p<0.01) increase in the level of TNF-α in the group administered $AlCl_3$ only when compared to the control. However, co-administration of eugenol and $AlCl_3$ resulted in a significant (p<0.01) reduction in the level of TNF-α when compared to the $AlCl_3$ group. In the group administered eugenol only, TNF-α was significantly (p<0.01) reduced compared to the group administered $AlCl_3$ only (Fig. 3).

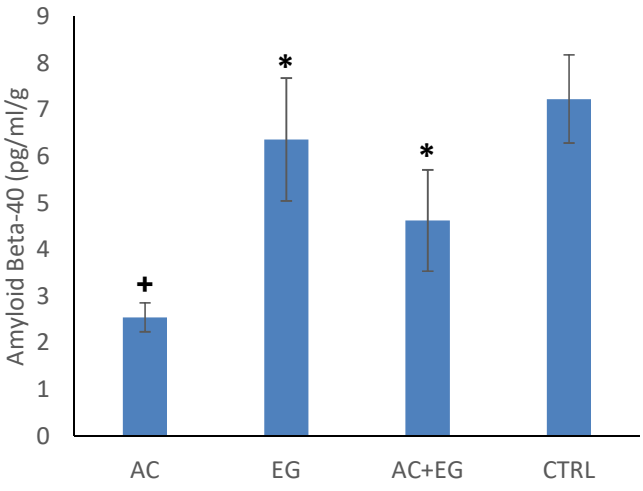

Fig. 2: Effect of eugenol on the level of amyloid beta-40 (Aβ-40). n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

The effect of eugenol on inducible nitric oxide (iNOS) following the administration of $AlCl_3$ showed a significant (p<0.01) increase in the group administered $AlCl_3$ when compared to the control group. Co-administration of eugenol and $AlCl_3$ resulted in a significant reduction (p<0.01) in iNOS level compared to the group administered $AlCl_3$. However, administration of eugenol alone led to a significant (p<0.01) reduction in the level of iNOS compared to the group administered $AlCl_3$ only (Fig. 4).

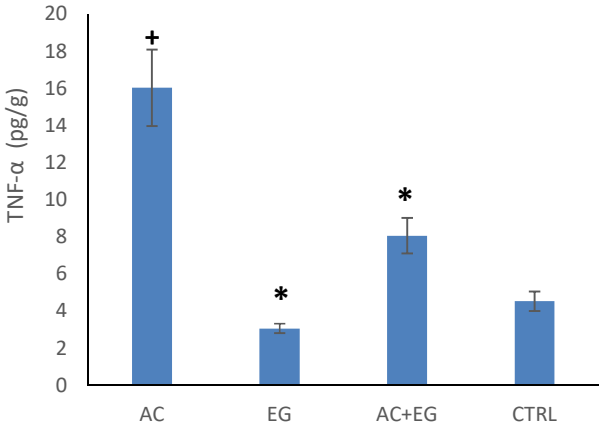

Fig. 3: Effect of eugenol on the activity of tumour necrosis factor-α (TNF-α). n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

The effect of eugenol on the levels of myeloperoxidase (MPO) following the administration of $AlCl_3$ showed a significant (p<0.01) increase in the group administered $AlCl_3$ only when compared to the control group. However, co-administration of eugenol and $AlCl_3$ resulted in a non-significant (p>0.01) reduction in MPO levels when compared to the group administered $AlCl_3$ alone. In the group administered eugenol alone, there was a significant (p<0.01) reduction in MPO levels when compared to the group treated with $AlCl_3$ alone (Fig. 5).

The effect of eugenol on the levels of Interleukin-1 (IL-1) following administration of $AlCl_3$ showed a significant (p<0.01) increase of this cytokine in the group administered $AlCl_3$ when compared to the control. However, co-administration of eugenol and $AlCl_3$ resulted in a significant (p<0.01) reduction in IL-1 levels compared to the group administered $AlCl_3$. In the group administered eugenol only, the level of interleukin-1 was significantly (p<0.01) reduced when

compared to the group administered $AlCl_3$ only (Fig.

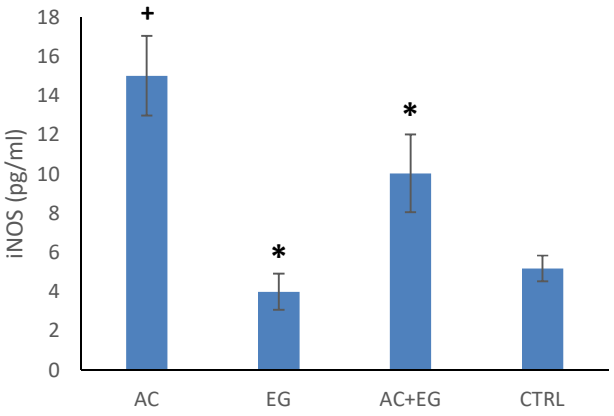

Fig. 4: Effect of eugenol on the activity of inducible nitric oxide (iNOS). n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

6).

The effect of the eugenol on the activity SOD following the administration of $AlCl_3$ showed a significant (p<0.01) reduction in the activity of this enzyme in the group administered $AlCl_3$ compared to the control group. However, co-administration of eugenol and $AlCl_3$ resulted in a significant (p<0.01) increase in the activity of SOD when compared to the group administered $AlCl_3$ alone. Administration of eugenol only resulted in a significant (p<0.01) increase in the activity of SOD when compared to the group administered $AlCl_3$ (Fig. 7).

The effect of eugenol on the levels of nitric oxide (NO) showed a significant (p<0.01) increase of nitric' oxide in the group administered $AlCl_3$ when compared to the control group. However, co-administration of eugenol and aluminium chloride (AC+EG) resulted in a significant (p<0.01) reduction in nitric oxide when compared to the group

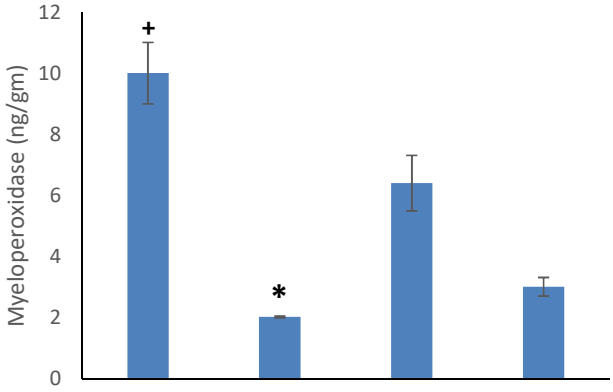

Fig. 5: Effect of eugenol on the activity of myeloperioxidase (MPO). n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

administered $AlCl_3$ only. The group administered eugenol only (EG), the level of nitric oxide was significantly (p<0.01) reduced when compared to the group administered aluminium chloride only (AC) (Fig. 8).

DISCUSSION

Damage to DNA has been linked to oxidative stress and oxidative stress is known to trigger inflammation and hence tissue damage, which is a major factor in the progression of many diseases and is associated with neuronal cell degeneration (Fonken et al. 2011). The role of aluminium compounds in associated with oxidative stress and neuronal loss has aided the understanding of neurodegenerative diseases since neurons are extremely vulnerable to free radicals (Mesole et al. 2020c). Aluminium activates the expression of various genes important for growth

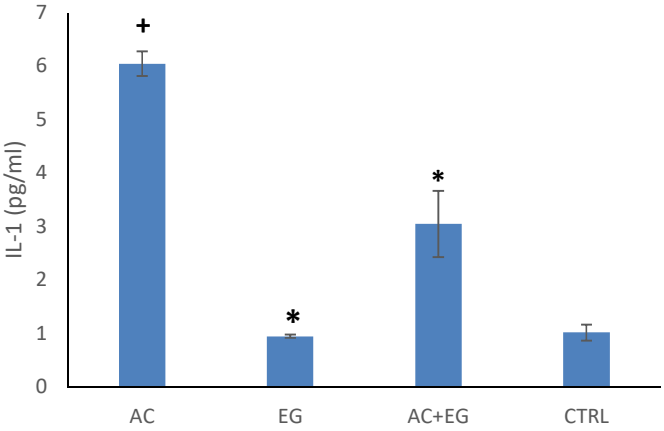

Fig. 6: Effect of eugenol on the levels of Interleukin-1 (IL-1). n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

arrest and DNA damage, as well as inflammatory cytokines known to initiate apoptosis (Smith and Perry 1996). To investigate the efficacy of eugenol in reducing neuroinflammation, the present study showed significant increased neuro-inflammation markers, TNF-α, IL-1, and MPO, following oral administration of AlCl3, which is consistent with the study by Wu et al. (2009). Tumour necrosis alpha and interleukins are two of the most commonly studied proinflammatory cytokines (Capuron and Miller 2011), and are known contribute to the pathology of aging, dementia and epilepsy (factor Vezzani and Granata 2005; Fonken et al. 2011).

Eugenol's anti-inflammatory properties was seen in its effectiveness against the neuro-inflammatory agent, ovalbumin. In this case, eugenol regulated increased levels of IL-4 and IL-5, and subsequently reduced NF-B signalling pathway (Citron 2004).

Among the many immune system cells are macrophages, which contribute to the production of pro-inflammatory cytokines and nitric oxide, both of which are necessary for the progression of the inflammatory process within cells and blood vessels (Arango and Descoteaux 2014). The present results showed that AlCl3 increased the levels of pro-inflammatory cytokines, TNF-α, MPO, IL-1, and iNOS and the ability of Eugenol to reduce the levels of this pro-inflammatory cytokines. The present findings aligns with Yeh et al. (2011), who reported eugenol roles in reducing the production of pro-inflammatory cytokines while inhibiting the production of iNOS and MPO in response to lipopolysaccharide induction. Reactive oxygen species (ROS) initiate oxidative stress, when antioxidants are unable to counteract their activities. Superoxide, hydroxyl radical, and hydrogen peroxide are examples of ROS (Patlevi et al. 2016), whose toxic effects are accompanied by lipid peroxidation and cellular damage (Ayala et al. 2014). Inflammatory events are exacerbated by reactive oxygen species, which also signals the production of pro-inflammatory cytokines, implying that oxidative stress and inflammation are inextricably linked (Biswas and De Faria 2007; Ambade and Mandrekar 2012 and Garcia et al. 2017). The present results showed that eugenol increased the activity of SOD which is consistent with previous studies (Yogalakshmi et al. 2010; Hussain et al. 2011).

Most researches focus on the pathophysiology of elevated amyloid beta with resultant cognitive impairment implicated in Alzheimer's disease. Amyloid precursor proteins (APP) play critical roles in the central nervous system (Dawkins and Small 2014), including the promotion of neurite outgrowth and long-term potentiation via calcium release modulation (Masliah et al. 1992; Kim et al. 2000). Amyloid beta by the way promotes synaptic plasticity with associated long-term potentiation within the hippocampus (Morley et al. 2010; Puzzo et al. 2011), improves the structural integrity of the blood-brain barrier, while protecting the brain from toxic substances (Brothers et al. 2018). In the present study, eugenol increased Aβ-40 levels, which is consistent with a previous study (Akbar et al. 2021).

Amyloid beta-40 is vital in non-pathological condition increasing synaptic activity, neuronal growth, synaptogenesis and cell adhesion. We showed that oral administration of $AlCl_3$ significantly reduced Aβ-40 level, while co-administration with eugenol significantly increased it. Reduction in the levels of Aβ-40 is known to result in production of reactive oxygen species that results in oxidative stress, which is known to result in elevated levels of pro-inflammatory cytokines and hence tissue damage (Puzzo et al. 2011). The present result supports the role of eugenol in regulation of levels of Aβ-40.

APE-1 expression findings revealed that oral administration of $AlCl_3$ significantly reduced APE-1 protein expression, which is consistent with the result of Akbar et al. (2021). Invariably, there was decreased level of the oxidative stress biomarker, SOD. Co-administration of eugenol on the other hand increased APE-1 protein expression and subsequent increased SOD level, which implies a protection of neuronal cells from the deleterious effects of $AlCl_3$.

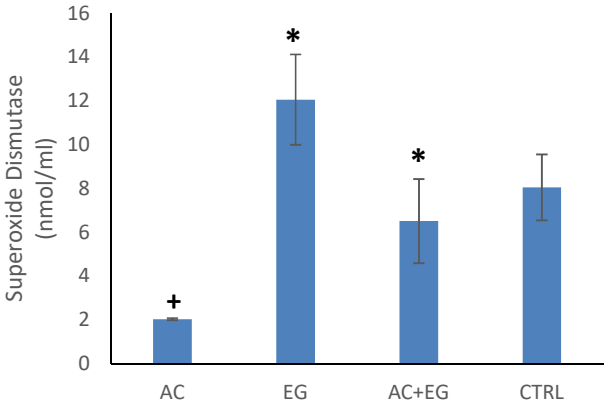

Fig. 7: Effect of eugenol on the activity of superoxide dismutase (SOD) n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

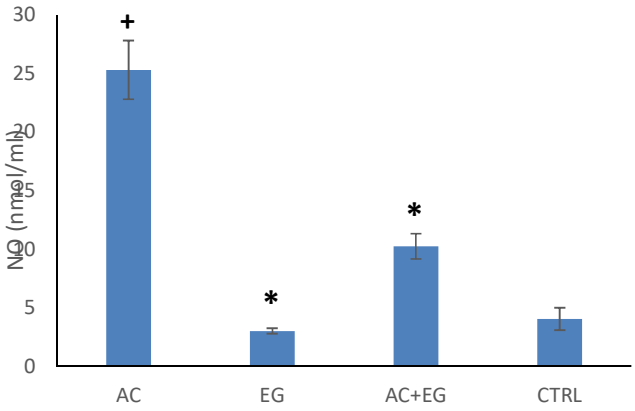

Fig. 8: Effect of eugenol on nitric oxide (NO) level. n=6; Mean±SEM; one-way ANOVA and Tukey's post hoc test: *p<0.01 compared to the $AlCl_3$ (AC) treated group; +p<0.01 compared to the control (CTRL).

Conclusion

Our findings revealed that $AlCl_3$ increased inflammatory biomarkers, IL-1, MPO, iNOS TNF-α, increased in tissue levels of NO and consequently a reduction in antioxidant enzyme SOD, as well as reduction in the tissue level of APE-1 and Aβ-40. Administration of eugenol alone resulted in the reduction of inflammatory biomarkers, increased level of the antioxidant enzyme, and increased APE-1 and Aβ-40 levels. Co-administration of eugenol and AlCl3 reverted deleterious changes associated with oral

administration of $AlCl_3$. Eugenol is therefore indicated as an efficacious immunomodulator and antioxidant in the treatment of neurotoxicity caused by $AlCl_3$.

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Nil.

Conflict of Interest
None declared.

Authors Contribution
BSM - Ideas conceptualization, research design, and write-up; UAY - Data Curation and Methodology; AOO - Data Analysis and Funding Acquisition; TEG - Review and Editing; NFB - Review and Editing; AA - Data Analysis.

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