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MODULATORY ACTIVITY OF RESVERATROL AND ENVIRONMENTAL ENRICHMENT ON BEHAVIOURAL AND NEUROINFLAMMATORY RESPONSES OF MICE TREATED WITH ALUMINIUM CHLORIDE

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ABSTRACT

The study investigated the modulatory activity of resveratrol and environmental enrichment (EE) on behavioural and neuroinflammatory responses of male mice treated with aluminium chloride ($AlCl_3$). Sixty-three Swiss albino mice were divided into nine groups of seven animals each as follows; 0.2 mL normal saline/kg (control), 0.2 mL Carboxymethyl cellulose (CMC)/kg, Resveratrol (200 mg/kg), CMC 0.2 mL/kg + EE, $AlCl_3$ (50 mg/kg), Resveratrol (200 mg/kg) + EE, $AlCl_3$ (50 mg/kg) + Resveratrol (200 mg/kg), $AlCl_3$ (50 mg/kg) + EE, and $AlCl_3$ (50 mg/kg) + Resveratrol (200 mg/kg) + EE, respectively. All treatments were given oral and lasted for 8 weeks. Assessments of behaviour were carried out at 0, 4 and 8 weeks after treatments, followed by biochemical analyses. $AlCl_3$ significantly ($p < 0.05$) induced motor endurance deficits at the fourth week which was improved by Resveratrol and EE. The concentration of NF-Kβ significantly decreased in $AlCl_3$ when compared to $AlCl_3$ + EE + resveratrol treated group. Furthermore, the concentration of TNF-α was significantly decreased in Resveratrol, and $AlCl_3$ + EE treated groups, when compared to $AlCl_3$ + EE + Resveratrol treatment group. $AlCl_3$ induced motor function deficits, which was improved by Resveratrol and EE. Resveratrol treatment alone and EE + $AlCl_3$ decreased biomarkers of neuro-inflammation.

Keywords: Alzheimer's disease, Environmental enrichment, Neuroinflammation, Resveratrol, Aluminium Chloride

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease and the major cause of dementia caused by interactions among genetic and epigenetic factors (Stelzmann et al., 1995). It affects millions of people globally, and has been projected to affect about 115 million people by 2050 (Mohandas et al., 2009; Prince et al., 2013). The common characteristics associated with the disease include severe cognitive impairment and hallucinations, microscopically visible cortical atrophy without macroscopic focal degeneration; disintegrated neurones, presence of neuritic/senile plaques, and intracellular neurofibrillary tangles (Stelzmann et al., 1995). People aged 60 and above are mostly

affected by AD, with an estimated prevalence of 25-50% in people over the age of 85 (Hong-qi et al., 2012) . The prevalence of dementia associated with AD is expected to increase in the near future, sequel to the steady growth of the ageing population in the world (Aprahamian et al., 2013) . The principal histological hall-marks of AD are the presence of aggregated amyloid-beta (Aβ)-laden plaques and hyperphosphorylated tau-laden neurofibrillary tangles (NFTs) (Selkoe, 2001) . Other factors that induce neurodegeneration that consequently led to AD include neuroinflammation, oxidative stress, vascular disease, accumulation of metals, such as aluminium (Al) (Armstrong, 2011; Mohandas et al., 2009).

Aluminium (Al) ion possesses strong affinity for bio-membranes and may induce neurotoxicity via increased generation of free radicals and increasing cellular oxidative damage and neuroinflammation by potentiating the pro-oxidant properties of transition metals (Bondy, 2021). Exposure to Al has been associated with the impairment of mitochondrial functions and the antioxidant defence systems; leading to oxidative stress and lipid peroxidation, neuroinflammation which eventually promote Aβ peptide formation, deposition, and AD-like amyloidosis (Kumar et al., 2008) . Neuroinflammation has been considered as a key neuropathological feature of AD aside from the accumulation of Aβ and NFTs (Kinney et al., 2018) . Neuroinflammatory mediators like tumour necrotic factor-α (TNF-α) and nuclear factor kappa-β (NF-kβ) have been demonstrated to play key roles in the pathogenesis of AD through alteration of various processes in the brain such as neurogenesis, survival, proliferation, and maturation (Sung et al., 2020) . This is achieved due to the ability of NF-kβ as an immune transcriptional factor to trigger the activation of astrocytes and microglia leading to increased generation of several proinflammatory cytokines such as IL-1β, IL-6 and TNF-α that consequently led to progressive increased in Aβ and tau-laden NFTs (Sung et al., 2020).

Resveratrol, (*trans*-3,5,4'-trihydroxystilbene), is a polyphenolic flavonoid found in the seeds and skins of grapes, red wine, mulberries, peanuts and rhubarb (Roy et al., 2011) . It is found both in *trans*- and *cis*- forms, with the trans-resveratrol having greater biological beneficial activity (Baur & Sinclair, 2006) . Over the last few decades, scientific research evidence both *in vivo* and *in vitro* revealed the therapeutic and prophylactic potentials of resveratrol both in health and numerous pathological conditions, such as ageing and cardiovascular diseases (Tamaki et al., 2014; Zordoky et al., 2015). These could be through modulation of Nrf2/antioxidant defence pathway, peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α), Sirt1 and 2/NAD^+-dependent histone deacetylase mechanism, cyclooxygenases (COX), phosphodiesterases, NF-κB, phosphoinositide 3-kinase (PI3K/Akt) signalling, mTOR signalling, oestrogen receptors, and MAPK signalling (Park et al., 2012; Tamaki et al., 2014; Zordoky et al., 2015).

Environmental enrichment (EE) is defined as a continuous increase in cognitive and sensorimotor stimuli, coupled with accumulated voluntary physical activity and dynamic social interactions (Anastasía et al., 2009). It produces wide variety of changes in the brain at behavioural, cellular and molecular networks. These changes driven by processes that alter brain networks, neurotransmitters, neuronal morphology, neurogenesis, neurotrophic factors and behavioural correlates of learning and memory (Kotloski & Sutula, 2015) . Most pronounced investigations on the beneficial role of EE on health and disease conditions

revealed the therapeutic potential of EE on numerous disorders such as stroke, Huntington's disease, Parkinson's disease, spinal cord injury, drug abuse and AD (Hakon et al., 2018; Kotloski & Sutula, 2015) . It increases the quantity of dendritic spines and cell survival molecules by increasing the levels of brain derived neurotrophic factor (BDNF), phospho-Akt and phospho- Mitogen activated protein kinase 1/2 (MAPK1/2), in middle-aged BALB/c mice, hence improving cognitive functions (Ramírez-Rodríguez et al., 2014) . It also significantly increases the expression of IGF-1 gene in the frontal cortex of piglets (Brown et al., 2017).

The involvement of neuro-inflammation in the pathogenesis of AD may contribute to cognitive impairment and play a significant role in AD progression. However, suppression of specific pro-inflammatory mediators by resveratrol and EE may be neuroprotective by mitigating the neuropathological process involved in the pathogenesis of AD. Both resveratrol and EE were found to independently modulate neuroinflammation and improve Aβ-peptide clearance by activating SIRT1 and AMPK (Keymoradzadeh et al., 2020; Yang et al., 2021). The research question that came to our mind was that, will the combined treatment of resveratrol and EE improve behavioural and neuroinflammatory responses of mice treated with $AlCl_3$. In view of this, therefore, the objective of the study was to investigate the modulatory role of resveratrol and EE on behavioural and neuroinflammatory responses of Swiss albino mice treated with $AlCl_3$.

MATERIALS AND METHODS

Animals and Management

A total of 63 male Swiss albino mice, 8-12 weeks of age, and weighing 22-27 g, were used for the study. They were purchased from the Animal House Facility of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. They were given free access to feed and water. The mice were allowed to acclimatise to the environment for two weeks before starting the experiment. The Ahmadu Bello University Research policy, ethics, and regulations governing the care and use of experimental animals (NIH Publication no. 85-23, revised 1996) was followed during experimental procedures. Ethical approval was received from Ahmadu Bello University Committee on Animal Use and Care; ethical clearance approval number: ABUCAUC/2018/056. The experiments were conducted in a quiet laboratory from 9: 00 h to 16: 00 h.

Preparation and Administration of Drugs

Resveratrol (200 mg/kg/body weight/day), was suspended in 10 g of CMC/L of normal saline because it is poorly soluble in water, and administered 0.2 ml/kg orally (Emlia Juan et al., 2002; Roy et al., 2011) . Aluminium chloride (50 mg/kg/body weight/day) was dissolved in normal saline and administered 0.2 ml/kg orally (Bihaqi et al., 2009).

Animal Groupings

The mice were divided into nine groups of seven animals (Table 1). All drugs were administered via the oral route.

All treatments lasted for 8 weeks as previously described (Prakash & Sudhandiran, 2015) . Neurobehavioural studies to assess endurance strength, memory and learning were carried out in three phases: 7 days before treatment (at base-line), and after weeks 4 and 8 of the treatment. The animals were sacrificed 24 hours following the last neurobehavioural assessment. The hippocampus was collected for biochemical analysis.

Environmental Enrichment Housing

The enriched housing (66 cm long × 46 cm wide × 38 cm high) used in keeping the mice was constructed as described by Harburger et al (Harburger et al., 2007). The cage contained cues and toys of different varieties. The number of toys were increased in every two days and replaced continuously to avoid contamination by faeces and urine, and to ensure maximal exploration of the EE housing by the animals (Anastasía et al., 2009).

Neurobehavioral Assessments

Spatial Working Memory Using T-Maze Test

Test for cognitive abilities using T-maze to assess spatial and working memory as described by Deacon and Rawlins was used (Deacon & Rawlins, 2006) . The maze composed of start alley 30 x 10 cm, goal arm (2) 30 x 10 cm, food well (diameter 1 cm, height 1 cm), wall height (enclosed maze) 20 cm, guillotine doors (cut to fit maze), central partition (extend 7 cm into start arm). Each mouse was introduced from the wooden base platform of the T-maze and allowed to choose one of the goal arms, abutting the other end of the stem. The trial was carried out twice in quick succession. At the second trial, the ability of the mice to choose the arm not visited before reflected a memory of the first choice. This is called 'spontaneous alternation'. The spontaneous alternation is very sensitive to dysfunction of the hippocampus, and other brain structures. Each trial was completed in less than 2 min (Deacon & Rawlins 2006).

Assessment of Endurance Strength Using Hang Test

The endurance strength of mice in the study was evaluated using a protocol described by Mohanasundari et al. (Mohanasundari et al., 2006). The apparatus consists of a horizontal grid (grid 12 cm^2 opening 0.5 cm^2). The grid was mounted 20 cm above a hard surface, to discourage jumping off or injury in case of falling. The apparatus was equipped with a 3-inch wall to prevent animals from traversing to the upper side of the grid. The mice were placed on the horizontal grid and supported until they held the grid. The grid was then inverted so that the mice were allowed to hang upside down. The mice were allowed to stay on the grid for 30 s and ten trials were given with 1 min interval and the best maximum hanging time was recorded. The percentage of success was recorded as maximum time hanging/30 s × 100 (Tillerson et al. 2002).

Biochemical Assessments

All the mice were anaesthetized with ketamine (75 mg/kg) + diazepam (25 mg/kg) and humanely sacrificed. The hippocampus was removed from each brain sample using surgical procedure, and kept in an ice-cold isotonic saline. The hippocampus was macerated in a glass mortar and homogenised with ice-cold 100 mM phosphate buffer (pH 7.4; 1 g/9 mL). The homogenates (10 % w/v) were then centrifuged at 1000 g for 20 min and the supernatants were used for the analyses of nuclear factor-kappa B concentration and tumour necrotic factor-α concentrations.

Quantification of Mouse Nuclear Factor-Kappa B and Mouse Tumour Necrotic Factor-α Concentrations

The Mouse NF-κB ELISA assay kit (GA-E1271MS) and TNF-α Elisa assay kit (GA-E0130MS) were purchased from GenAsia Biotech Co., Ltd and used to assess the concentration of NF-κB and TNF-α in mouse hippocampal samples on the bases of biotin double antibody sandwich technology as described in (Table 2). (Maeda et al., 2005; Lee et al., 2006)

Table 1: Animal Groupings

Groupings	Treatment
1	0.2 ml normal saline/kg body weight (negative control)
2	0.2 ml CMC/kg body weight
3	Resveratrol (200 mg/kg)
4	CMC + Enriched housing
5	$AlCl_3$ (50 mg/kg)
6	Resveratrol (200 mg/kg) + Enriched environment
7	$AlCl_3$ (50 mg/kg) + Resveratrol (200 mg/kg)
8	$AlCl_3$ (50 mg/kg) + Enriched environment
9	$AlCl_3$ (50 mg/kg) + Resveratrol (200 mg/kg) + Enriched environment

Table 2: Quantification of mouse nuclear factor-kappa B and mouse tumour necrotic factor-α concentrations

Item	Blank wells	Standard wells (pre-coated with NF-κB and TNF-α monoclonal antibodies)	Samples wells (pre-coated with NF-κB and TNF-α monoclonal antibodies)
Samples	-	-	40 µL
Standard	-	50 µL	-
Mouse NF-κB and TNF-α antibodies labelled with biotin	10 µL	-	10 µL
Streptavidin-HRP		50 µL	50 µL
Covered, incubated at 37°C for 60 minutes and then washed 5 times			
Chromogen A	50 µL	50 µL	50 µL
Chromogen B	50 µL	50 µL	50 µL
Incubated at 37°C for 10 minutes			
Stop solution	50 µL	50 µL	50 µL
Colour changes was observed from blue to yellow and the optical density (OD) sample concentrations were measured and calculated using My Assays software			

Statistical Analyses

The analysis of data was done using Statistical Product and Service Solutions (SPSS) version 22 (NY: IBM Corp, 2013) and values obtained were expressed as mean ± standard error of mean (SEM). All analyses were done using one-way analysis of variance (ANOVA) for biochemical parameters and

mixed analysis of variance for neurobehavioural evaluation, followed by Tukey's and Bonferroni *post-hoc* tests in order to evaluate the significance of the differences between the means, respectively. "p-value" < 0.05 were considered significant.

RESULTS

Assessment of Memory and Learning Deficits Induced by $AlCl_3$ in Mice

There was a significant ("p-value" < 0.05) difference in percentage alternation across the three stages of the study: [F (2, 70) =19.44; "p-value" = 0.01] (Figure 1). A significant decrease in percentage (%) alternation was observed in CMC; 4 weeks (40.00 ± 15.58) and 8 weeks (30.00 ± 14.54), EE; 8 weeks (40.00 ± 14.54), $AlCl_3$; 8 weeks (40.00 ± 14.54) and $AlCl_3$ + EE + resveratrol; 4 weeks (30.00 ± 15.58) treatment groups, when compared to their respective base-line mean scores. There was no significant difference observed in spontaneous alternation between groups; [F (8, 35) = 0.94; "p-value" = 0.49], and the interaction between groups and time was insignificant; [F (16, 70) = 0.64; "p-value" = 0.84] in all the three stages of the study.

Assessment of Endurance Strength Deficits Induced by $AlCl_3$ in Mice

A significant difference in endurance strength was observed across the three time points (0, 4, and 8 weeks) of the study; [F (2, 72) = 3.16; "p-value" = 0.05], (Table 3). Bonferroni *post-hoc* test showed a significant decrease in percentage hanging in $AlCl_3$ at base-line (77.46 ± 11.87) and 4 weeks (25.98 ± 10.08), and EE + resveratrol at 4 (68.88 ± 9.20) and 3 weeks (39.48 ± 7.40), respectively. There was no significant change between groups [F (8, 36) = 1.29; "p-value" = 0.29] and the interaction between group and time [F (16, 72) = 1.59; "p-value" = 0.09] in all the stages of the study was insignificant.

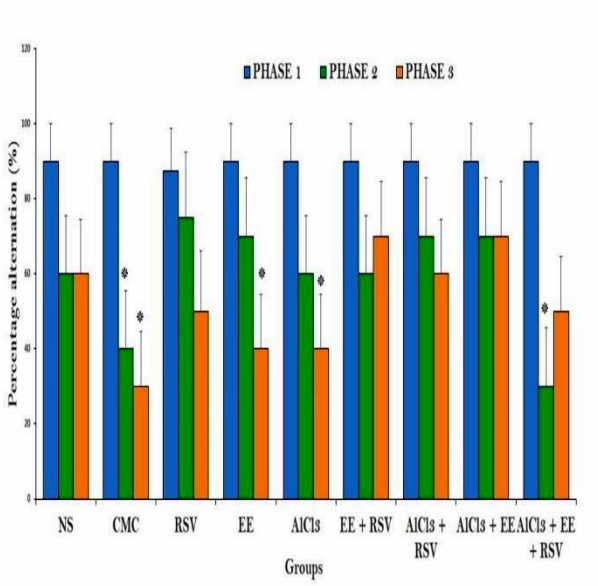

Figure 1: Effect of resveratrol and environmental enrichment on aluminium chloride-induced learning and memory induced deficits using T-maze Spontaneous alternation test for memory in mice.

*p < 0.05 = Indicate significant difference when compared to phase 1 (base-line). CMC = Carboxymethyl cellulose, RSV = Resveratrol, EE = Environmental enrichment, $AlCl_3$ = Aluminium chloride, Phase 1 = base-line, Phase 2= after four weeks of the study, Phase three= after eight weeks of the study, n = 7

Assessment of Nuclear factor kappa β in $AlCl_3$-induced neurotoxicity

Results of NF-kβ concentration (ng/ml) obtained during the study are represented in Figure 2. There was no significant change observed in the concentrations (ng/ml) of NF-kβ in all the treatment groups, when compared to the controls (normal saline). However, the concentration of NF-kβ (ng/ml) was higher in the $AlCl_3$ + EE + Resveratrol (3.97 ± 0.26) [F (8, 24) = 3; "p-value" = 0.02], when compared to the $AlCl_3$ (2.71 ± 0.13) group.

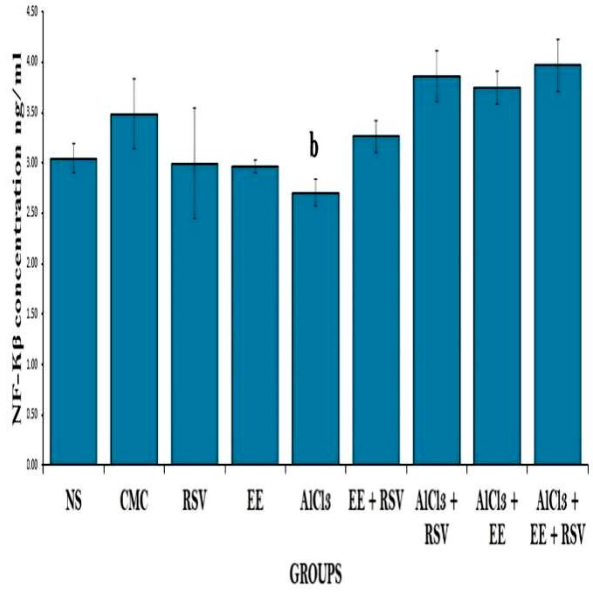

Figure 2: Effect of resveratrol and environmental enrichment on nuclear factor kappa-beta concentration in aluminium chloride induced neurotoxicity in mice.

[b] Indicate significant ($p < 0.05$) difference, when compared to $AlCl_3$ + EE + Resveratrol. CMC = Carboxymethyl cellulose, RSV = Resveratrol, EE = Environmental enrichment, $AlCl_3$ = Aluminium chloride, NF-Kβ= Nuclear factor-kappa β, n = 4

Table 3: Effect of resveratrol and environmental enrichment on endurance strength deficits induced by aluminium chloride model of Alzheimer's disease using hang test

Groups	Percentage Hanging Phase 1 (base-line)	Percentage Hanging Phase 2 (Four weeks)	Percentage Hanging Phase 3 (Eight weeks)
Normal Saline	69.00 ± 11.87	51.32 ± 10.08	39.98 ± 8.10
CMC	38.68 ± 11.87	43.34 ± 10.08	27.32 ± 8.10
Resveratrol	42.58 ± 13.28	43.33 ± 11.27	45.80 ± 9.06
EE	56.68 ± 13.28	53.40 ± 11.27	49.18 ± 9.06
$AlCl_3$	77.46 ± 11.87	25.98 ± 10.08*	51.32 ± 8.10
EE + Resveratrol	62.83 ± 10.84	68.88 ± 9.20	39.48 ± 7.40[#]
$AlCl_3$ + Resveratrol	59.50 ± 10.84	54.47 ± 9.20	34.45 ± 7.40
$AlCl_3$ + EE	48.58 ± 13.28	34.15 ± 11.27	45.83 ± 9.06
$AlCl_3$ + EE + Resveratrol	41.65 ± 10.84	66.12 ± 9.20	53.92 ± 7.40

* Indicate significant ($p < 0.05$) difference when compared to phase 1, [#] significant ($p < 0.05$) difference when compared to phase 2, CMC = Carboxymethyl cellulose, EE = Environmental enrichment, $AlCl_3$ = Aluminium chloride, n = 7

Assessment of Tumour Necrotic Factor-α in $AlCl_3$-Induced Neurotoxicity

Results of TNF-α concentration (ng/L) obtained during the study are represented in Figure 3. There was no significant change observed in the concentration (ng/L) of TNF-α in the treatment groups, when compared to the normal saline (control). However, a significant [F (8, 19) = 2.54; "p-value" = 0.045] difference was observed in concentrations (ng/L) of TNF-α in Resveratrol (218.95 ± 34.50) and $AlCl_3$ + EE (162.80 ± 1.83) groups, when compared to that of $AlCl_3$ + EE + Resveratrol (1027.63 ± 102.40).

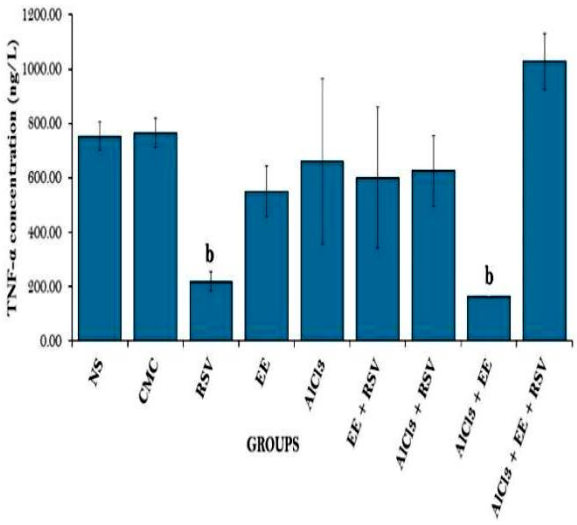

Figure 3: Effect of resveratrol and environmental enrichment on tumour necrotic factor-alpha concentration in aluminium chloride-induced neurotoxicity in mice.

[b] Indicate significant ($p < 0.05$) difference, when compared to $AlCl_3$ + EE + Resveratrol. CMC = Carboxymethyl cellulose, EE = Environmental enrichment, $AlCl_3$ = Aluminium chloride, TNF-α = Tumour necrotic factor-alpha, n = 4.

DISCUSSION

The study demonstrated that resveratrol and environmental enrichment modulate behavioural and neuroinflammatory responses of mice treated with aluminium chloride.

The decrease in spontaneous alternation observed over time in the various treatment groups which was more pronounced in CMC group, indicating a decline in memory. This finding demonstrated that CMC generally regarded to be inert exert some activity especially on memory and learning in this study. This agrees with the study of Isa et al. (2019) . who revealed that CMC possesses some biological activities by enhancing motor endurance in Wistar rats. To the best of our knowledge, there was no evidence for the biological activities of CMC on memory based on available literature, thus warranting further investigation into its physiological activity. Cognitive abilities recede with normal aging due to failure of information processing, and degeneration of neuronal networks which may hinder the execution of cognitive functions such as spatial memory (Korman et al., 2019) , as observed from the present study.

There were no significant differences observed in spontaneous alternation between all the groups and the interaction between groups and time was not significant in all the stages of the study. This result is in disagreement with the finding of Al-Amin et al. (2016) , who reported memory impairment after oral administration of $AlCl_3$ to albino mice for forty-two (42) days, compared to the controls. However, the difference between the present studies and that of others is that we administered 50 mg/kg/oral $AlCl_3$ to Swiss albino mice for eight weeks, while other investigators administered higher doses of (200 mg/kg) for 10 weeks to Wistar rats intraperitoneally (Mahdi et al., 2021) . The model of assessing cognitive function should also be taken into consideration. While T-maze spontaneous alternation was used to assess spatial working memory in the current study, radial arm test and Morris water maze were used to assess cognitive deficits by Al-Amin et al. (2016) , and Cao et al. (2016) respectively. These differences could be responsible for the variations observed in the findings between the studies. Extending the studies, a little bit further using higher doses could probably result to significant differences in spatial working memory between the control and the various treatment groups.

The significant decrease in percentage hanging observed in $AlCl_3$ group between the

base-line and fourth week demonstrated deficits in endurance strength, induced by $AlCl_3$ treatment over time. The result agrees with the study of Lakshmi et al. (2014) who reported significant decrease in muscular and motor activities of rats, exposed to $AlCl_3$ treatment. However, the decline observed was improved by resveratrol and EE treatments with trend towards significance at the fourth week of the study. This demonstrated the possible beneficial role of resveratrol and EE in modulating motor functions as observed from the present study.

The results of NF-kβ and TNF-α concentrations as indices of neuroinflammation obtained in the study are in disagreement with the findings of Shunan et al. (2021) who reported increased mRNA levels of COX-2, iNOS, TNF-α IL-6, Il-1β of rats exposed to 100 mg/kg $AlCl_3$, indicating their role in inducing neuroinflammation. However, the dose used by Shunan et al. (2021) was higher than that of the present study (50 mg/kg). Furthermore, other investigators administered $AlCl_3$ (200 mg/kg) orally once daily for 10 weeks in combination with D-galactose 60 mg/kg intraperitoneally to achieve AD phenotype (Mahdi et al., 2021). The difference in dosage and duration between our studies and of Shunan et al. (2021) and Mahdi et al. (2021) could account for the reason why $AlCl_3$ failed to increase NF-kβ and TNF-α concentrations beyond that of the control.

The significant decreased in the concentration of TNF-α obtained in the resveratrol alone and $AlCl_3$ + EE groups could be as a result of the individual ability of both resveratrol and EE to decrease TNF-α concentration. This agrees with previous investigations on the beneficial role of resveratrol and EE by decreasing IL-1α IL-1β and TNF-α concentrations (Keymoradzadeh et al., 2020; Yang et al., 2021). Furthermore, the observed increase in TNF-α and NF-Kβ concentrations in $AlCl_3$ + EE + Resveratrol group beyond the individual treatments, and to some extent $AlCl_3$ group and the controls, could be as a result of cumulative stress, resulting from the combination of two mild stressors; resveratrol and EE with the neurotoxin $AlCl_3$, thereby cumulatively leading to oxidative stress that consequently induced neuroinflammation. Investigations have revealed that proinflammatory mediators such as IL-6, COX-2, TNF-α and iNOS are known to be induced via the redox sensitive transcription factors such as NF-kB, which is triggered by ROS and turn on the expression of genes, associated with the cellular and immunological defence systems (Baetz et al., 2005; Nishanth et al., 2011).

CONCLUSION

Based on the results of this study, Aluminium chloride induced motor function deficits over time which was improved by Resveratrol and EE at the fourth week of the study. Independent treatment with resveratrol, and or EE + $AlCl_3$ decreased markers of neuro-inflammation. However, the combined treatment of Resveratrol + EE + $AlCl_3$ failed to ameliorate neuro-inflammatory biomarkers demonstrating the beneficial role of individual treatments over the combined treatment with resveratrol and EE in ameliorating $AlCl_3$ induced neurotoxicity in the present study.

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