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Neuro-Protective Effect of Ethanolic Extract of *Trombidium Grandissimum* Koch Against Aluminium Chloride (Alcl3) Induced Neurotoxicity in Albino Rats

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Abstract:

The current goal of this investigation is to assess the ethanolic extract of T. grandissimum (EETG) against $AlCl_3$-initiated neurotoxicity, where AlCl3 (100 mg/kg/p.o) is administered for 42 days by provoking oxidative stress followed by neurotoxicity. The evaluation of behavioural parameters such as locomotor activity, elevated plus maze, and motor incoordination, as well as biochemical parameters such as reduced glutathione, lipid peroxidation, Superoxide dismutase (SOD), Catalase (CAT), total Protein, and Acetylcholinesterase in the brain, was performed. According to the study report, behavioural changes such as dropped locomotor movements, enhanced transfer latency, and muscle weakness were often noted. Significant rising of lipid peroxides and AChE activity in rat brain tissue was observed, and activity of antioxidant enzymes like SOD, Catalase, and Reduced Glutathione decreased substantially in alcl3 treated rats compared to control, whereas EETG at both dose and standard treated rats showed a significant reduction in LPO, AChE activity and significant elevation in SOD, CAT, and GSH in brain tissues compared to AlCl3 treated rats. The findings are further supported by histopathology of the brain. This is due to the extract's enormous amount of bioactive components. Through in-silico analysis, isolation, and characterisation, future research will be required to pinpoint the chemical component responsible for the extract's observed biological effect.

Keywords: *Trombidium grandissimum, $Alcl_3$, Anti-oxidant, Entomotherapy, AChE*

1. Introduction

Neuroprotection is a field of research that focuses on preventing future harm to the damaged nerve cells that are still there. Additionally, the inflammation around the spinal cord is minimized, as well as cell death. It is a well-researched therapy option for a variety of central nervous system (CNS) problems, including neurodegenerative diseases, stroke, traumatic brain injury, spinal cord injury, paralysis, and acute management of neurotoxic ingestion. Any chemical or biological molecule that exhibits neuroprotective properties in the nervous system against neurodegenerative disorders, brain trauma, or paralysis is known as a neuroprotective molecule. This result could manifest as the protection of neurons from apoptosis or degeneration (Djaldetti, *et al*,2003; Ha, K.Y.*et al*, 2011). In several parts of the central nervous system (CNS), neurodegenerative illnesses are characterized by a slow and progressive loss of neurons. Common pathways include elevated oxidative stress, mitochondrial failure, excitotoxicity, inflammatory alterations, iron buildup, and protein aggregation. (Seidl *et al*, 2011; Dunnett *et al*,*1999;* Andersen *et al*, 2004). Neuroprotective therapies frequently target excitotoxicity and oxidative stress, two of these mechanisms that are closely linked to CNS diseases. Limiting excitotoxicity and oxidative stress is thus a crucial component of neuroprotection. Antioxidants and glutamate antagonists, which work to reduce excitotoxicity and

oxidative stress respectively, are common neuroprotective therapies. (Zadori *et al*, 2012; Zhang *et al*, 2012). The earth's crust has the highest concentration of aluminium. In addition to being a natural part of drinking water and many unprocessed foods like fruits, vegetables, grains, toothpaste, and pharmaceuticals, they are also naturally present in ambient air. (Rute *et al.*, 2022). Aluminium can pass across the blood-brain barrier and build up in brain tissues, with the hippocampus having the highest quantities (Hussein *et al.*, 2020). Aluminium is a well-known neurotoxin that contributes to the onset of neurodegenerative conditions like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD).

Due to its high cholinotoxin level, aluminium supplementation results in cognitive impairment, neurodegeneration, and neuronal death due to apoptosis. (Madhu *et al.*, 2022) An important factor in the development of AlCl3-induced neurotoxicity is oxidative stress. It has been established that the underlying mechanisms of $alcl_3$-induced neurotoxicity include oxidative and endoplasmic reticulum stress, mitochondrial dysfunction, inflammation, cell death, interaction with Aβ and α-synuclein, cytoskeletal abnormalities, alteration of synaptic plasticity, and signal transduction through interference with neurotransmitter systems. Treatment of Aluminium neurotoxicity and related illnesses may benefit from focusing on these processes at various stages (Anatoly *et al.*, 2021). Zootherapy is a type of treatment or cure that is described as "the treatment of human diseases by use of therapeutic substances obtained or derived from animals." Animals do have a remarkably diverse range of therapy alternatives that significantly contribute to the global healing of humans (Natarajan *et al.*, 2021).

The tribes of Chhattisgarh rub this oil on the male genitalia to prolong sexual activity. One hour before sexual activity, it is administered. The oil lengthens the retention period, delaying ejaculation to improve pleasure, etc. For the treatment of paralysis both internally and topically, Chhattisgarh's traditional healers developed an oil from the mite *T.Grandissimum.* To get a full recovery, the oil is heated and massaged into the affected area regularly for a few weeks. More than ten serious illnesses, including malaria, urogenital problems, and paralysis, can be effectively treated with the oil produced by the red velvet mite (*T.Grandissimum*). Due to their capacity to stimulate sexual desire, these mites are known as "Indian Viagra."(Annandale, 1906; Hill, 1905; Costa-Neto, 2005). The red velvet mite *T.Grandissimum* is traditionally used to cure malaria, urogenital disorders, and paralysis but lacks of scientific validation. So the present study aimed to provide scientific validation for the Neuro-protective activity of red velvet mite *T.Grandissimum.*

2. Materials and Methods

Insect collection

From November to January, fresh *T. grandissimum* mites were collected in the sandy soil of Parvathamalai highlands in Tamilnadu's Tiruvannamalai district. Dr M. Gabriel Paulraj of Loyola College, Entomology Research Institute (registered with DSIR), Chennai-60034, identified and authenticated the mites. A Voucher Specimen No: ESI/L/08-02-2020/01.

Extraction

The mites were manually removed from the soil and placed in a collection jar and suspended in a sealed container in 95% ethanol. The mites in the container were removed, aseptically crushed using a mortar and pestle, and then kept in reflux for 2 h with 95% ethanol (Lighty George *et al*., 2010; Natarajan *et al*., 2019).

Experimental animals

The Wistar albino rats that weighed 150-180gm were housed in a controlled environment for 12 hours while receiving water and hygienic food. The research was carried out in a soundproof research lab. The animal ethics committee's clearance was obtained by their approval number, KPCP/IAEC/2022/01.

Pharmacological screening

Experimental Design Aluminium chloride (AlCl3) induced neurotoxicity in rats Total 30 rats were randomly divided into five groups of 6 rats each and treated for 42 days as follows:

Table 1: Neuroprotective activity treatment protocol

Group	Treatment

Normal control	Normal saline (5ml/kg, p.o)
Negative control	AlCl3 (100mg/kg, *p*.o.) + Normal saline(5ml/kg, p.o)
Standard	AlCl3 (100mg/kg, *p*.o.) + Donepezil hydrochloride 1mg/kg/day (p.o)
EETG 200	AlCl3 (100mg/kg, *p*.o.) + EETG (200mg/kg, p.o)
EETG 400	AlCl3 (100mg/kg, *p*.o.) + EETG (400mg/kg, p.o)

The ethanolic extract of *T.Grandissimum* (EETG) was administered before 1 hour of administration of AlCl3 100 mg/kg (p.o). During the drug treatment, rats were observed for behavioural changes for 50 min daily. On 43th day, rats were evaluated for General behavioural studies such as locomotor activity, rota-rod performance and elevated plus maze test. On the 44th day, rats were sacrificed under mild anaesthesia and their brain was isolated for estimation of different biochemical parameters such as reduced glutathione (GSH), lipid peroxidation (LPO) and superoxide dismutase (SOD). Total protein was estimated using rat serum and histopathological study (Debashish *et al.*, 2022; Roja *et al.*, 2022; Auti, S.T. and Kulkarni, 2019).

Monitored Parameters

Rota Rod

A rota-rod apparatus was used to measure the impact on motor coordination. The rota rod is made of a non-slip iron rod with a 3 cm diameter and 30 cm length. Three disks separated this rod into four equal pieces, allowing four rats to walk on it simultaneously at a pace of 22 rpm while being observed. The duration of the performance was measured as the time between the animal being mounted on the rod and falling off of it (Madhu *et al*, 2022; Rabbani *et al*., 2008).

Actophotometer

Locomotor activity using an actophotometer with infrared-sensitive photocells, the spontaneous locomotor activity was recorded. The apparatus was set up in a dark, light, and well-ventilated testing room. Animals were first placed individually into the activity meter for 2 minutes for habituation. Thereafter, their locomotor activity was recorded during a 5-minute test session. Locomotor activity was expressed as the number of total photo beams / 5 min (Auti, S.T and Kulkarni, Y.A.2019).

Elevated Plus-Maze Test

Memory was evaluated by the elevated plus maze (EPM) apparatus. The maze consists of two closed arms (50×10 cm) with two opposite open arms (50×10 cm) which are plus-shaped and remain elevated above the floor level. Before the experiment, each rat was trained by placing them at the end of an open arm and by using a stopwatch. Transfer latency time (s), can be defined as the time taken by a rat to enter (with all four paws) into either of the closed arms. The transfer latency was observed and noted. The maze was cleaned with 70% ethanol between runs. The time taken by each animal to enter into the closed arm with all its four limbs when positioned at the edge of one open arm facing away from the central platform was recorded as the initial transfer latency. The 60-second cut-off was set. The rat was then allowed to move freely inside the maze regardless of open and closed arms for another 10 sec. After 24 hours the retention transfer latency test was performed in the same way as in the acquisition trial. If the rat did not enter the enclosed arm within 60 sec of 2nd trial, the transfer latency (day 0) was assigned 60 sec. The rats were again put into the elevated plus maze on day 43 to evaluate the transfer latency. (Auti, S.T and Kulkarni, Y.A. 2019).

Biochemical estimations:

On the 44th day, rats from each group were sacrificed under diethyl ether anaesthesia following overnight fasting. The brain was quickly isolated and stored at −20°C for subsequent analysis.

Tissue Processing Methods

Following sacrifice, the brain was excised immediately, One part was kept in 10% neutral-buffered formalin for histopathological study. The other part was rinsed with saline (0.9% NaCl), dried, and homogenized using handheld homogeniser with phosphate buffer (10 mM, pH 7.4) that contained 1.15% potassium chloride and 1.15% ethylene-diamine tetra-acetic acid (EDTA), and centrifuged at 3000 rpm for 15 min to obtain tissue lysates. The tissue lysates were collected and kept at −20°C to be used for the estimation of several endpoint parameters like Reduced glutathione, Lipid peroxidation, Superoxide dismutase (SOD), Catalase (CAT), Protein concentration and Acetylcholinesterase in the brain. (Usama, *et al*,2017).

Determination of Acetylcholinesterase

250 µL of brain homogenate with 25 µL 5, 5'-dithio-2-nitrobenzoic acid (0.82 mg/mL) followed by the addition of 25 µL of the enzyme-substrate, acetylthiocholine (6.7 mg/mL). The change in absorbance/min during a 5-minute interval was measured at 405 nm. Enzyme activity was calculated using the estimated extinction coefficient ε=14,150 $M^{-1}cm^{-1}$. Enzyme activity was expressed in µmoles of acetylthiocholine hydrolyzed/min/mg tissue (Usama *et al.*,2017).

Histopathological studies:

The brain tissue was dissected out and fixed in 10% formalin. The paraffin sections were prepared and stained with dyes haematoxylin and eosin and examined using light microscopy.

Statistical analysis

Statistical analysis Values were expressed as Mean ± SEM. The Mean difference in behavioural and biochemical parameters was analysed using One-way ANOVA followed by Dunnett's test. Analysis was performed using GraphPad Prism statistical software (Version 5.03).

3. Results and Discussion

Behavioural Assessment

Loco-motor activity

Locomotor activity is considerably lower in alcl3-treated groups compared to control. The locomotor score improves after treatment with EETG at doses of 200 and 400 mg/kg, as well as standard treatment, as shown in Figure 1.

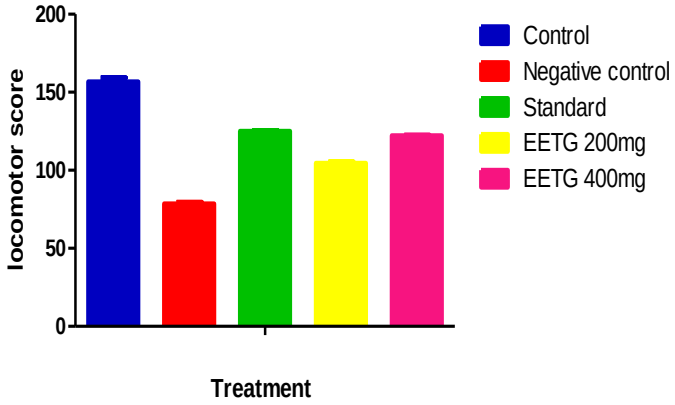

Figure 1: Effect of ethanolic extract of *T.grandissimum* on the locomotor score in $AlCl_3$ induced Neurotoxicity in Albino rats.

Muscle Relaxant Activity

When compared to the control group, the $AlCl_3$-treated group has a much shorter period of fall from the rotating rod. Figure 2 depicts the effects of EETG treatment at both doses and Standard.

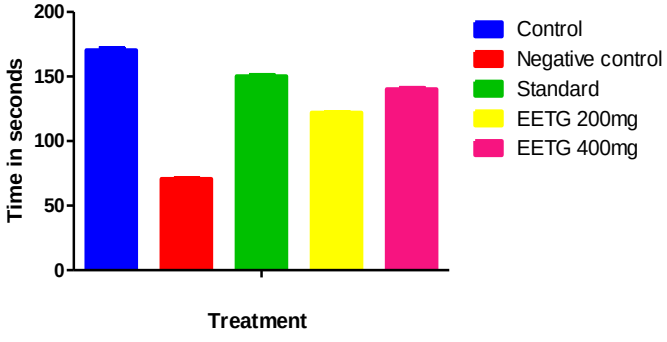

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Figure 2: Effect of ethanolic extract of *T.grandissimum* on Muscle Relaxant activity in $AlCl_3$-induced Neurotoxicity in Albino rats.

Elevated Plus Maze test

In the EPM test, the negative control group had a significant increase in transfer latency when compared to the normal control group. EETG at both doses significantly reduced transfer latency when compared to the disease control group. Transfer latency was dramatically reduced in animals treated with EETG when compared to rats treated with standard. Figure 3 depicts the circumstances.

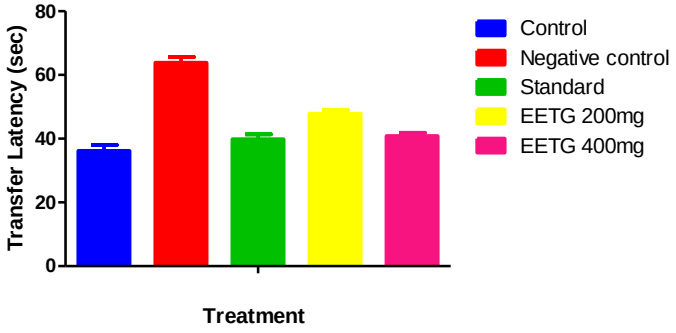

Figure 3: Effect of ethanolic extract of *T.grandissimum* on transfer latency in $AlCl_3$-induced Neurotoxicity in Albino rats.

Anti-oxidant Parameters

LPO

When compared to the control group, the LPO level of the negative control group was possibly higher. When compared to the negative control group, the elevated level of LPO in the Standard treatment group was reduced. When compared to the negative control group, the EETG-200mg/kg p.o and EETG-400mg/kg p.o treatment groups reduced LPO levels. The LPO level in the standard and EETG-400mg/kg p.o groups was reduced and returned to normal. Figure 4 depicts the outcomes.

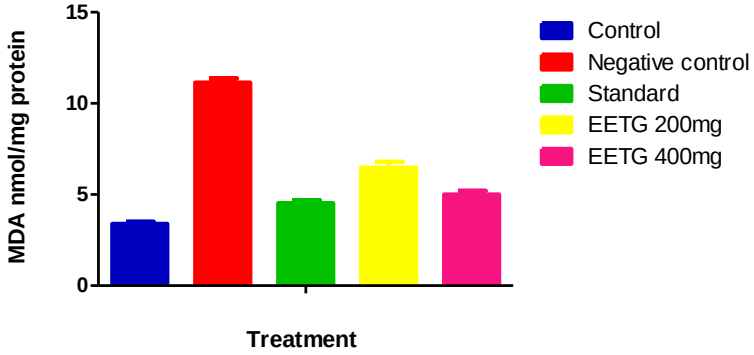

Figure 4: Effect of ethanolic extract of *T.grandissimum* on LPO Levels in $AlCl_3$-induced Neurotoxicity in Albino rats.

GSH

When compared to the control, the GSH level in the negative control group was possibly lower. When compared to the negative control group, the standard treatment group showed higher levels of GSH. When compared to the negative control group, the EETG-200mg/kg p.o and EETG-400mg/kg p.o treated groups had higher GSH levels. Figure 5 depicts the results.

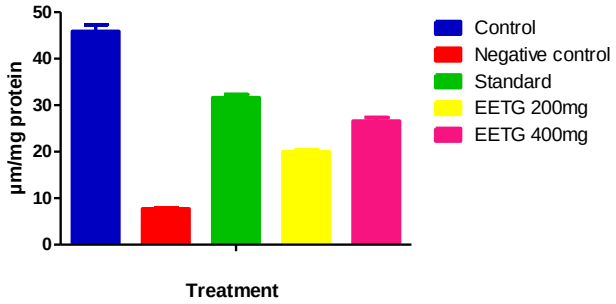

Figure 5: Effect of ethanolic extract of *T.grandissimum* on GSH Levels in $AlCl_3$-induced Neurotoxicity in Albino rats.

Catalase

When compared to the control group, the CAT level of the negative control group was possibly lower. When compared to the negative control group, the standard treatment group exhibited a higher level of CAT. When compared to the negative control group, the CAT level was enhanced in the EETG-200 and EETG-400 treatment groups. Figure 6 depicts the results.

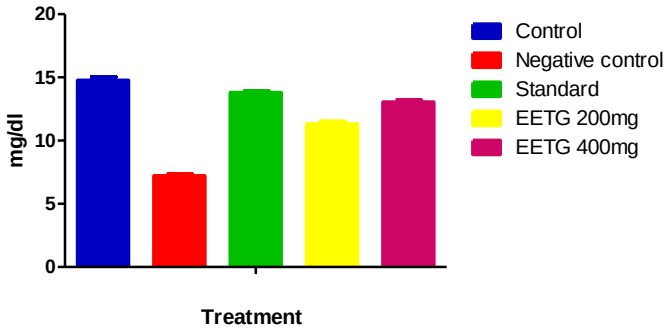

Figure 6: Effect of ethanolic extract of *T.grandissimum* on cat Levels in $AlCl_3$-induced Neurotoxocity in Albino rats.

SOD

When compared to the control group, the SOD level of the negative control group was possibly lower. When compared to the negative control group, the standard treatment group exhibited a higher level of SOD. When compared to the negative control group, the SOD level was enhanced in the EETG-200 and EETG-400 treatment groups. Figure 7 depicts the results.

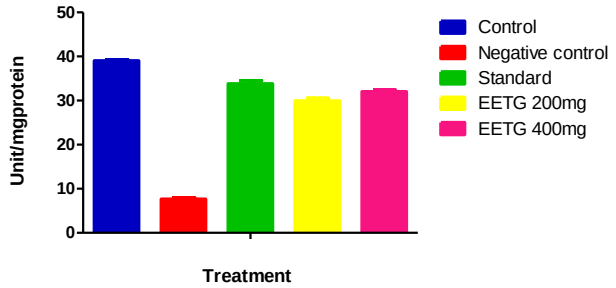

Figure 7: Effect of ethanolic extract of *T.grandissimum* on SOD Levels in $AlCl_3$-induced Neurotoxicity in Albino rats.

Total protein

When compared to the control group, the negative control group's total protein level was possibly lower. When compared to the negative control group, the lowered amount of total protein in the Standard treatment group was increased. The total protein level was enhanced in the EETG-200 and EETG-400 treated groups as compared to the negative control group. The total protein level in the standard and EETG-400 groups was enhanced. Figure 5 depicts the outcomes.

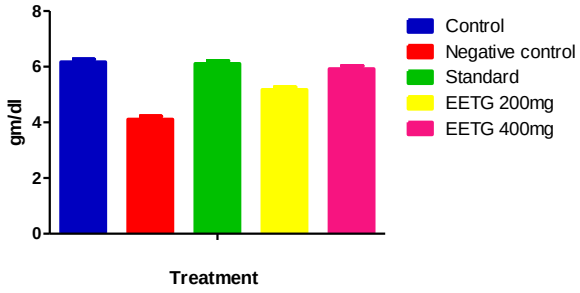

Figure 8: Effect of ethanolic extract of *T.grandissimum* on total protein Levels in $AlCl_3$-induced Neurotoxicity in Albino rats.

Effect of EETG on AChE activity in treated groups

The Alcl3 group had significantly higher AChE levels than the control group. However, when compared to the AlCl3 group, both the standard and EETG groups had a considerable drop in AChE activity. Figure 9 depicts the outcomes.

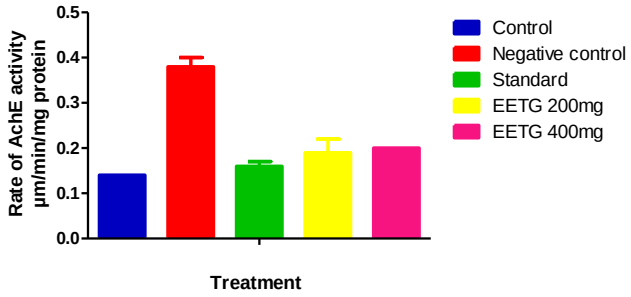

Figure 9: Effect of ethanolic extract of *T.grandissimum* on Acetylcholinesterase Levels in $AlCl_3$-induced Neurotoxicity in Albino rats.

Effects on histopathology of the Brain tissue

The negative control group shows that there is mild meningeal vessel congestion with mild neuronal degeneration, a cyst present in the meninges is observed. Standard and EETG 400 mg/kg treated groups show moderate neuronal degeneration in the brain. Whereas the EETG 200mg/kg treated group showed mild meningeal vessel congestion and cyst present in the meninges and the control group showed no abnormality in the brain tissue. The results are shown in Fig 10,11,12,13,14.

Control

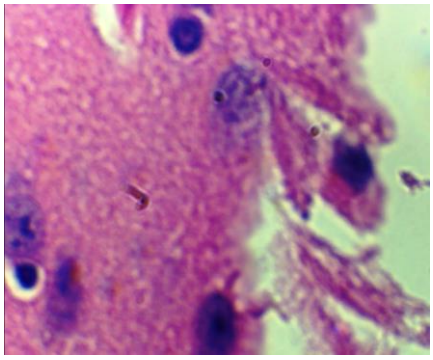

Figure 10: Histopathology of the control group showing no abnormality in the brain tissue.

Negative control

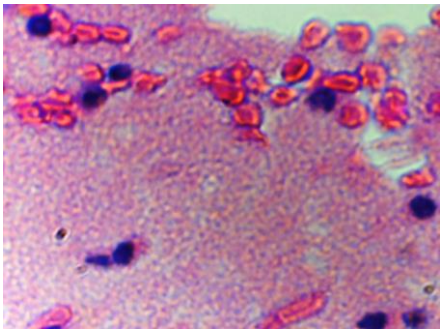

Figure 11: Histopathology of the negative control group showing mild neuronal degeneration with cyst present in the meninges of brain tissue.

Standard

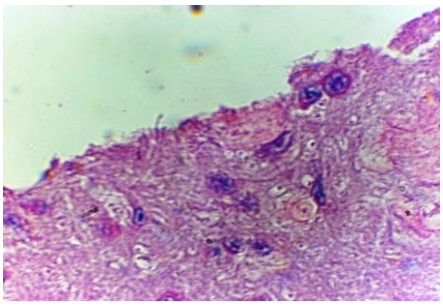

Figure 12: Histopathology of the standard group showing moderate neuronal degeneration in the brain tissue.

EETG 200MG

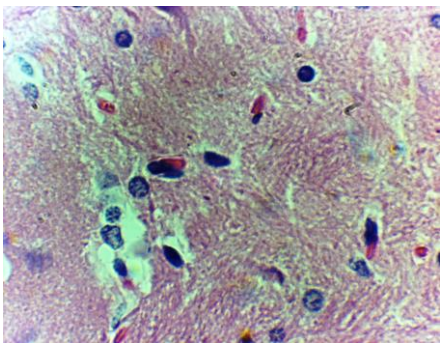

Figure 13: Histopathology of EETG 200 group showing mild meningeal vessel congestion and cyst present in the meninges in the brain tissue.

EETG 400MG

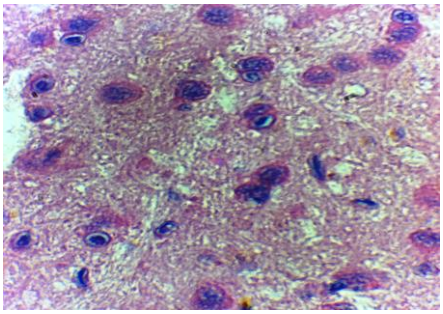

Figure 14: Histopathology of EETG 400mg group showing moderate neuronal degeneration in the brain tissue.

Significant accumulation of lipid peroxides in brain tissue of rats was observed and activity of antioxidant enzymes like SOD, Catalase and Reduced Glutathione was significantly decreased in the alcl3 treated group as compared with control, whereas EETG and standard treated rats showed a significant reduction in LPO activity and significant elevation in SOD, CAT and GSH in brain tissues as compared with $AlCl_3$ treated rats.

Aluminium binds with the cholinergic system as a cholinotoxin (neurotoxin), causing modifications in cholinergic, dopaminergic, and noradrenergic neurotransmission; as a consequence, it can impair the transmission of cholinergic signals by influencing neurotransmitter synthesis as well as release (Hassanin et al., 2017). According to published research, the long-term usage of aluminium causes oxidative damage to cellular lipids, proteins, and DNA. One example is lipid peroxidation, which can harm tissues over time. As a result, aluminium might be regarded as a risk factor for neurological disorders. Following AlCl3 treatment, it accumulates in all brain regions, with a greater concentration in the cortex, causing damage. These areas are critical for learning and memory (Kangtao et al., 2017). The involvement of free radicals in neurodegeneration and decline in cognition has previously been researched, and outcomes highlight the ROS role in the brain and found to increase neuronal function. According to the previous investigations, the concept of memory problems occurring after aluminum chlorides administration was caused by mitochondrial malfunction, which is thought to be the major factor for ROS formation and ultimately triggering oxidative damage to the brain's neurons. As a result, oxidative stress was identified as one of the main factors of cognitive dysfunction.

Aside from long-term stress, it also boosts oxidative stress and disrupts the brain's antioxidant defence mechanism (Ramachandran et al., 2017). In the current investigation, Alcl3-induced physiological and behavioural alterations such as reduced locomotor movements, greater transfer latency, and muscle weakness

were frequently observed. The reduced locomotor activity relative to the control is attributed to free radical destruction of the dopaminergic neurons. Standard and EETG could mitigate the negative effects of Alcl3 by reducing free radical damage. When compared to the control group, the Alcl3-treated group had a considerably shorter period of fall from the revolving rod. When compared to the Alcl3-treated group, EETG therapy at both doses and standard medication considerably extended the time of fall off the rotating rod. The EPM test was employed to assess transfer latency, which aided in the understanding of spatial memory as well as learning. Chronic AlCl3 proximity diminished open-arm exploration and enhanced transfer delay, demonstrating anxiogenic effects (Murugaiyan and Bhargavan, 2021). The results of the research suggest that treatment with EETG extract dropped transfer delay. Thus, the restoration of Alcl3-induced diminished locomotor exertion, loss of muscle grip exertion, and transfer delay with EETG therapy may be owing to its antioxidant and neuroactive effectiveness which protects against neurotoxicity.

In the current investigation, a rise in brain LPO levels, together with a decline in GSH, SOD, and CAT levels, is regarded as an indication of oxidative damage and neuronal injury. Raised LPO levels cause neuronal membranes to erode function and integrity, increasing indiscriminate permeability to ions and disrupting the structure of the membrane. The treatment with standard and EETG diminished LPO levels in the Alcl3-treated group, implying that EETG protects against Alcl3-induced oxidative stress. When compared to control animals, animals treated with Alcl3 had considerably lower levels of GSH. GSH is a key cytoplasmic scavenger of radicals that are free and an important modulator of free radical-mediated lipid peroxidation. Excitotoxicity-induced glutathione depletion in rat neural tissue is caused by a collapse of the glutamate-cystine antiporter system, resulting in reductions in the amount of GSH. GSH levels were significantly greater in standard and EETG-treated rats at both 200 and 400 mg/kg doses in comparison to the Alcl3-treated group, demonstrating that restoring GSH levels protected neurons against Alcl3-induced neurotoxicity. SOD and CAT levels were considerably lower in the Alcl3-treated group compared to the untreated group. SOD protects cells from free radical damage by converting O2 radicals to H2O2 and preventing the production of OH radicals via the O2-driven Fenton reaction. The CAT removes the H2O2 produced by SOD.

As a result, if the activity of CAT is insufficient to break down H2O2, more H2O2 is transformed into harmful hydroxyl radicals (Viswanatha Swamy et al., 2013). The current investigation found that Alcl3-induced oxidative stress dramatically lowered SOD and CAT activity. SOD and CAT levels increased significantly in both the EETG and standard treatment groups as compared to the Alcl3-treated group. Thus, EETG's protective mechanism could be related to its powerful neuroactive, anti-inflammatory, and antioxidant properties. Acetylcholine is a critical neurotransmitter in the CNS that plays an important role in behaviour, learning and memory, and neurodegenerative disorders. Our findings indicate that Alcl3 treatment increases AChE levels in the brain, which may lead to a drop in cholinergic neurotransmission performance due to a fall in acetylcholine range within the synaptic cleft, causing gradual cognitive decline (Weil et al., 2008; Jiang et al., 2008). Furthermore, Alcl3 produces oxidative stress by raising lipid peroxides and the formation of reactive oxygen species (ROS), which could boost the level of AChE.

The anti-inflammatory, analgesic, and neuroactive properties of bioactive components reported for pharmaceutical applications, such as 1-hexyl-2-nitrocyclohexane, have been mentioned (Thirumalaisamy et al., 2018; Bundy et al., 2019). Cis-9,10-epoxyoctadecan-1-ol acts as a microbicide, antifungal, antioxidant, and anti-inflammatory agent (Chandralega, 2020). Antihyperlipidemic, nematicide, antioxidant, androgen antagonist, haemolytic, and pesticide properties have been demonstrated for hexadecanoic acid ethyl ester (Mohamed et al., 2014; Elaiyaraja, 2016). Docosanoic acid ethyl ester has cosmetic, antimicrobial, and antioxidant properties (Sharad, 2017; Jayanthi et al., 2020).In the current investigation, EETG at doses of 200 and 400 mg/kg showed profound neuroprotection. This activity could be attributed to the extract's neuroactive, antioxidant, and anti-inflammatory properties.

4. Conclusion

As an outcome, we believe that the results obtained during this study convincingly suggest that the ethanolic extract of *T.Grandissimum* contains a neuro-protective effect at both doses comparable to the conventional medicine donepezil. Because AlCl3 is exceedingly harmful, its use should be forbidden. This effect may be due to the presence of active components such as 1-Hexyl-2-nitrocyclohexane, Cis-9,10-epoxyoctadecan-1-

ol, Hexadecanoic acid, ethyl ester, Docosanoic acid, ethyl ester with antioxidants, neuroactive and antiinflammatory properties present within the extract, which support the mite's traditional use for paralysis. In the future, additional research such as *in silico* analysis, isolating and characterizing the chemical component responsible for the reported biological activity of the extract will be necessary.

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Conflict Of Interests
No conflicts of interest have been disclosed by the research's creators.

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