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An Evaluation Of The Neuroprotective Activity Of Nerium Oleander Leaves Extract In Dementia Associated With Alzheimer Disease

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ABSTRACT

Nerium oleander Linn., a member of the Apocynaceae family, is frequently found in Indian gardens and is widely valued in Ayurvedic treatment. In addition to its many medicinal applications—which include treating dyspepsia, malaria, a variety of mental illnesses, and STDs—this plant is prized for its aesthetic attractiveness. It functions as an insecticide, molluscicide, diuretic, heart stimulant, and abortifacient. Moreover, it's used to treat skin disorders like ringworm, scabies, ulcers, leprosy, and itching. It's also used to treat neurological diseases like epilepsy.Its ability to improve cognitive functioning has also been noted in earlier studies, especially when it comes to extracts made from its leaves. The current study investigates the effectiveness of a methanol extract made from the leaves of Nerium oleander in treating Alzheimer's disease (AD)-related dementia. Using an 8-arm radial maze test, the study's method of inducing dementia in lab animals by high consumption of copper and aluminium was confirmed. The evaluation of several biochemical indicators, such as reduced glutathione (GSH), myeloperoxidase (MPO) activity, acetylcholinesterase (AChE) activity, and

superoxide dismutase (SOD), was done in addition to this.To support the findings, lipid profiles were investigated in conjunction with histological examinations. The results showed that the methanol extract from Nerium oleander leaves had neuroprotective properties and seemed to lessen dementia symptoms. This implies that it may play a therapeutic role in the management of Alzheimer's disease by utilising its natural abilities to improve cognitive abilities.

Keywords: Nerium oleander, Alzheimer's disease, Radial 8 arm maze, Dementia, acetylcholinesterase, neuroprotective, superoxide dismutase.

INTRODUCTION

A variety of symptoms linked to cognitive loss that are severe enough to interfere with day-to-day functioning are together referred to as dementia. Impaired memory, reasoning, problem-solving, and other cognitive abilities are its defining characteristics. With Alzheimer's disease responsible for 60–80% of cases, it is the most frequent cause of dementia. Alzheimer's disease is a degenerative neurological condition that causes the brain to shrink (atrophy) and causes brain cells to die. Usually, the illness begins slowly and gets worse over time, greatly impairing a person's capacity to function on their own [1]. Early Alzheimer's symptoms include forgetfulness, difficulty executing routine tasks, and disorientation about time and place. As the illness worsens, patients may have profound memory loss, confusion about events, time, and place, mood swings, and behavioural abnormalities. Alzheimer's disease can cause significant impairments in body functions in its latter stages, which can eventually result in mortality from complications including infections[2–5]. Alzheimer's disease does not presently have a cure, however there are therapies that can help control its symptoms. To improve treatment options, postpone the disease's development, and stop it from developing, research is still being done. Extensive scientific research is still being conducted in an effort to better understand the mechanisms underlying Alzheimer's and develop viable therapies [6–10].

The herb Nerium oleander Linn. (Apocynaceae Family) is used traditionally in Ayurveda. It is also referred to as Surkh Kaner in Unani and Kaner in Hindi. Ayurvedic names for this plant include Viraka, Karavira, Hayamaaraka, Ashvamaaraka, Divyapushpa, Gauripushpa, Shatakumbha, Raktapushpa, Siddhapushpa (white-flowered variation), Ravipriya (red-flowered variety), Raktaprasava, etc[11, 12]. It's referred to as Rose Bay or Red Oleander in English. Common oleanders are evergreen ornamental shrubs that flourish in subtropical and temperate climates[13, 14]. In Indian gardens, the plant is also frequently planted as an ornamental. In humid coastal regions like Assam, Arunachal Pradesh, Uttar Pradesh, Himachal Pradesh, Tamil Nadu, Rajasthan and, the plant is also frequently grown [11, 12]. For millennia, oleander preparations have been utilized as traditional and native medicine to treat a wide range of illnesses, including leprosy, dyspepsia, malaria, and mental or sexually transmitted infections. It is reportedly also utilized as an abortifacient[15]. Native Americans used N. oleander as a pesticide, molluscicide, heart tonic, and diuretic to treat skin diseases, snake bites, and epilepsy [16]. Previous research on this plant revealed cardiac glycosides, supporting the hypothesis that oleander extracts can treat cardiac insufficiency [17]. One of N. oleander's most well-known secondary chemicals is oleandrin [13, 18–20].

According to our review of the literature, this plant's extracts have been shown to exhibit biological activity in addition to a variety of phytoconstituents that have been extracted from various plant sections[21].The methanolic extract of Nerium oleander leaves has undergone preliminary screening, which reveals that this plant is a strong cognition enhancer and has strong

anti-inflammatory and free radical scavenging properties. The present study targeted to examine the ability of this plant to reverse Alzheimer disease (AD) dementia in a model of the disease in rats.

MATERIALAND METHODS

Materials: Drugs and chemicals

Our suppliers of tocopherol, gallic acid and other standard drugs were the St. Louis, Missouri-based Sigma Chemicals Company. The following other reagents were obtained in analytical grade for the research: pyridine, sodium nitrate, sulphanilamide, sodium nitrite, naphthylethylenediamine dihydrochloride, hexadecyltrimethylammonium bromide (HTAB), 1,1,3,3-tetramethoxypropane and O-dianisidine. Other reagents used in the study included acetic acid, tris buffer, thiobarbituric acid, n-butanol, chloroform, acetylthiocholine and diethyl ether.These substances were purchased from reputable commercial vendors, guaranteeing the consistency and integrity required for exact scientific research.

Preparation of plant extract

Nerium oleander leaves were collected throughout the months of June and July from Nashik, Maharashtra, India. These samples were verified by renowned Indian botanist Dr. S.K. Sharma using conventional pharmacognostical techniques. A herbarium's crude drug repository housed a voucher specimen from this collection that was stored and catalogued under accession number 96107. The leaves were coarsely powdered after being shade-dried after collecting.To guarantee full extraction of the phytochemicals, about 5 kg of this coarsely ground material were subjected to a rigorous extraction procedure employing methanol in a Soxhlet system. After extraction, a rotary evaporator operating at 45 °C was used to concentrate the resulting solution. Next, to maintain stability and dryness, the concentrated extract was stored in vacuum desiccators that were filled with anhydrous silica gel. Acute toxicity testing was also performed on this produced extract in accordance with OECD requirements to determine its safety for use in additional experiments[22].

Acute toxicity studies

Following Test No. 423, an acute oral toxicity study was carried out in compliance with the OECD's (Organization for Economic Co-operation and Development) criteria for chemical testing. This testis intended to assess the toxicological profile of chemicals when given orally to experimental animals as a single dosage. Using this technique, the lethal dose may be ascertained and the chemical can be categorized according to the degree of acute health risk it poses [22]. Three Wistar rats of each sex were selected at random for the acute toxicity investigation. These animals were fasted for a whole night before the experiment to exclude any nutritional impacts. They were kept in carefully monitored laboratory settings that met all accepted norms for humidity, light levels, and temperature. Each produced extract was given orally for the test in gradually increasing doses, up to a maximum of 2000 mg/kg. By observing any acute hazardous effects at different dosage levels, this approach aids in the establishment of a safety profile for the chemical being studied.

Animals

For the experiment, adult male Sprague Dawley rats weighing between 200 and 250 grammes at 4 months of age were used. The controlled environment in which these animals were housed

followed normal laboratory protocols, with a temperature set at 23 ± 2°C, a 12-hour light/12-hour dark cycle, and a relative humidity of 52 ± 10%. Throughout the trial, the rats had unrestricted access to filtered water and a normal pellet meal. The care and handling of laboratory animals is subject to strong ethical norms enforced by the Institutional Animal Ethical Committee, which operates under the principles of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSSEA) in India. This ensures that study animals receive humane treatment.

Experimental design

Thirty Sprague Dawley rats in all were divided into five groups of six rats each for the experiment. This is how each group was set up and handled:

- Group 1: Normal Control – This group served as the reference point for comparison, getting only regular lab feed and unlimited access to normal drinking water.
- Group 2: Positive Control – To create dementia-like symptoms, rats in this group were given metals (copper and aluminium in micromoles) in their drinking water on a free-choice basis.
- Group 3: Standard Group – Similar to the positive control, this group received metals (Aluminum and Copper in micromoles) in water ad libitum, supplemented with Vitamin E at a dosage of 100 mg/kg to assess its protective effects against metal-induced toxicity.
- Group 4: Test Group (Low Dose) – In addition to the same metal treatment, these rats were given NOLE (Nerium oleander leaf extract) at a dosage of 200 mg/kg.
- Group 5: Test Group (High Dose) – This group also received metals in water like the others, with a higher dosage of NOLE at 400 mg/kg to evaluate dose-dependent efficacy.

Radial 8 arm maze test

Rats' spatial memory was tested in an experiment using a radial arm maze. Eight arms, each with a radial extension from a central circular space of 28.7 cm in diameter, made up this maze. There were nine-centimeter-tall doors dividing the arms from the center platform. To provide visual contrast, black paint was applied to both the arms and the middle region. In the training phase, a number between 1 and 8 was assigned to each arm. There were tiny amounts of food pellets in the food cups at the tips of arms 1, 3, 5, and 7. The rats were put on a limited diet and went through a preliminary molding phase in order to get them ready for the maze assignment. The rats were trained to navigate to the end of the arms in groups of four over the course of four days in order to collect the bait. Bait was initially spread out around the maze but eventually became limited to the meal containers alone. After shaping was finished, private training sessions started.After being put in the middle of the maze, each rat underwent five days of five daily training trials. Every trial had a 5-minute break in between each one until all four baits had been swallowed or until 5 minutes had passed. Before every experiment, the maze was wiped with 70% ethanol and dried to eliminate the possibility of smell marking impacting the outcomes. The same four arms—1, 3, 5, and 7—were continuously baited throughout these trials. There was no bait used on the other four limbs (2, 4, 6, and 8). A rat's admission inside an arm was only recorded when each of its four limbs was within the arm.When a rat entered a non-baited arm, the researchers recorded reference memory errors; when a rat revisited a baited arm it had already visited, they recorded working memory errors. Until all of the baits were eaten, the order and pattern of arm entry were also recorded. The rats received one trial every day following the first intense training session. Rats with excellent memory accuracy—defined as making no more than one error per trial and two or fewer errors across three consecutive trials—were the only ones selected for further behavioural

and pharmacological investigations.The study's rats were carefully trained and chosen to guarantee that they were well-suited to the job, yielding accurate results that could be used to evaluate how different treatments affected memory and learning [23-25].

Biochemical assessments

Estimation of Lipid profile parameters

Blood samples were taken 12 hours after the last dose of the experimental medicine was given, and again 24 hours later to assess the lipid profile of the study's rats. While the rats were sedated with ether, blood was extracted from the retro-orbital plexus as part of the collection procedure. After that, these samples were put into Eppendorf tubes that weren't heparinized.After the blood samples were taken, they were allowed to clot at room temperature for 45 minutes. After clotting, serum was extracted from the clotted blood using centrifugation for 20 minutes at 5000 rpm to guarantee a distinct separation of the blood cells from the serum. This is how the lipid profile analysis was done - Following the manufacturer's instructions, triglycerides were tested using a Necpath triglyceride testing kit, which uses the Glycerol-3-Phosphate Oxidase-Peroxidase (God/Pod) method. A Necpath Total Cholesterol test kit was used to measure total cholesterol levels. This kit uses the Cholesterol Oxidase-Phenol Aminophenazone (Chod-Pap) endpoint method to measure total cholesterol. Using an Enzopak HDL-Cholesterol test kit and the phosphotungstic acid method (PTA), HDL cholesterol was measured.

Additionally, the following formulas were used to determine the levels of VLDL and LDL cholesterol:

VLDL Cholesterol = Triglycerides / 5

LDL Cholesterol = Total Cholesterol - (VLDL + HDL Cholesterol)

Milligrams per deciliter, or mg/dL, was used to express all of the test results. This all-encompassing strategy made it possible to evaluate the lipid profiles in great detail, which is essential for comprehending how the medications being studied affect metabolism.

Brain homogenate preparation

The rats were beheaded, their brains removed, and they were then submerged in ice-cold normal saline before being exposed to a pH 7.3, 0.2 M solution. Weighing the Tris-HCl buffer. Using the previously mentioned procedure, 10% w/v of homogenate was produced in 0.15 M Tris-HCl buffer and subjected to lipid peroxidation measurement[26]. After precipitating proteins with trichloroacetic acid (TCA), a portion of the homogenate was used to estimate glutathione using the previously mentioned procedure [27]. The remaining homogenate was centrifuged for 15 minutes at 4°C at 15,000 rpm. Superoxide dismutase (SOD) was estimated using the supernatant so obtained and the previously mentioned technique[28].

Estimation of superoxide dismutase (SOD)

The previously described method was applied to assess the brain tissue's SOD activity [28]. A specific methodology was used to assess the activity of superoxide dismutase (SOD) in brain homogenates. This methodology entailed mixing several precisely measured components into an assay mixture. 0.1 mL of the sample, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 mL of phenazine methosulphate (186 µM), 0.3 mL of nitro blue tetrazolium (300 µM), and 0.2 mL of NADH (750 µM) were the components comprised in the mixture. NADH was added to start the reaction, which was then allowed to run for 90 seconds at 30°C. 0.1 mL of glacial acetic acid was added to halt the reaction.After the reaction was stopped, the mixture was agitated briskly

with 4.0 milliliters of n-butanol so that the reaction's-coloured formazan product could be removed into the butanol layer. The mixture was centrifuged after standing for ten minutes to make sure all the separation was complete. After that, the butanol layer was separated for examination. SOD activity was determined by measuring the color's intensity using a spectrophotometer set to 560 nm.The efficiency of the enzyme in converting superoxide radicals into less reactive molecular species is indicated by this measurement. The antioxidative defense systems found in brain tissues were quantitatively evaluated by expressing the quantity of SOD in units per milligram of protein. Understanding how therapies affect oxidative stress and neuroprotection in experimental models requires the use of this technique.

Assessment of Protein and GSH levels

Bovine serum albumin was used as a reference when measuring the protein content using the previously mentioned method [29]. The tissue homogenate was extracted and placed in 0.1 M phosphate buffer (pH 7.4) in order to quantify the reduced glutathione (GSH) level. At first, the process was carried out as previously said [27].An elaborate process of preparation and measurement was used to examine the glutathione (GSH) levels in the brain tissue homogenates. First, the homogenate was mixed with an equal amount of 20% trichloroacetic acid (TCA) containing 1 mM EDTA in order to precipitate the proteins. Before centrifuging this mixture to extract the precipitate and supernatant, it was allowed to stand for five minutes to allow for full precipitation. 200 µL of the clear supernatant was cautiously placed into a fresh set of test tubes following centrifugation. Ellman's reagent (5, 5'-dithio bis-2-nitrobenzoic acid, DTNB) was added to this in a volume of 1.8 mL.A yellow chemical is created when GSH and Ellman's reagent react. The reagent is made at a concentration of 0.1 mM in 0.3M phosphate buffer with 1% sodium citrate. For all samples, the total volume in each test tube was set at 2 mL to guarantee consistency. After the reaction, the solutions' absorbance was evaluated using spectrophotometry at 412 nm, comparing the results to a produced blank that took background absorption into account. The absorbance measurements from the test samples were evaluated against a standard curve in order to determine the GSH concentration.The GSH levels in the samples could be accurately determined thanks to this curve, which was previously created using known quantities of GSH and expressed in micromoles of GSH per milligramme of protein. Since GSH is essential for counteracting oxidative stress in cells, this process is critical for evaluating the antioxidative potential of brain tissue.

Assessment of acetylcholinesterase (AChE) activitylevels

Enzymes were generated using brain homogenates, which were dissolved in 1.0 mL of 0.1 M phosphate buffer pH 8.0 (50 mg/mL) and left for five minutes in an ice bath. Acetylcholinesterase activity was measured using the previously described method [30]. Acetylcholinesterase (AChE) activity was measured using a particular procedure under carefully monitored circumstances. The measurement was conducted at 25°C in a cuvette with a 10 mm route length. Several carefully measured ingredients were included in the incubation mixture for this experiment in order to promote the enzymatic reaction. The concoction contained: Acetylthiocholine iodide, 0.01 millilitres: 0.05 mL of DTNB (5,5-dithio-bis-2-nitrobenzoate) is the substrate that the AChE enzyme uses to catalyze its hydrolysis, with a concentration of 0.5×10^5 M: A chromogenic agent was dissolved in 0.1 M phosphate buffer pH 7.0 at a concentration of 12.5×10^4 M. When DTNB and the thiocholine that AChE's enzymatic action releases combine, A yellow-colored substance is produced along with 0.05 mL of enzyme-containing supernatant: This contains the AChE that was

extracted from the tissue homogenates together with 1.45 milliliters of pH 8.0 0.1 M phosphate buffer.: This buffer aids in preserving the pH required for the best possible enzyme activity. In order to account for any background absorbance, controls for the test were set up by replacing the enzyme-containing supernatant with 0.05 mL of the phosphate buffer.Following AChE's hydrolysis of acetylthiocholine iodide, thiocholine is released into the reaction and combines with DTNB to generate the yellow colour that is detected spectrophotometrically at 412 nm. The color's intensity is directly correlated with the amount of AChE activity in the sample. The quantitative data are given in nanomoles of hydrolyzed substrate per minute per milligram of protein (nmol/min/mg protein). This technique yields a consistent measurement of AChE activity, which is crucial for comprehending the kinetics of the enzyme and how different chemicals affect its activity.

Statistical analysis

The experiment's results were shown as Mean ± Standard Deviation (SD). Graph Pad Prism, a software program well-known for its reliable handling of scientific data—particularly in the domains of biostatistics and biological sciences—was used for the analysis. One-way analysis of variance was used for the statistical evaluation (ANOVA). Tukey's multiple comparison tests were performed after the ANOVA. The study's significance threshold was established at a p-value < 0.05.

RESULTS

Acute toxicity studies

At doses up to 2000 mg/kg, the N. oleander extract did not cause any mortality. Thus, for additional research, 1/8th and 1/4th of the highest dose (250 and 500 mg/kg, orally) were selected.

Radial 8 arm maze test

An evaluation of NOLE's ability to improve memory was conducted utilizing a radial 8-arm maze test. The rats showed a decrease in spatial memory after being given aluminium and copper to induce dementia. This was demonstrated by the rats taking longer to find the baited arms than the control group, which did not exhibit any changes. Interestingly, the amnesia caused by aluminium and copper was significantly reversed in the rats given NOLE (at doses of 250 and 500 mg/kg). As seen in results, this improvement in memory function indicates that NOLE has a memory-enhancing impact in this experimental model [31-34].

Lipid profile

The present investigation revealed notable modifications in the lipid profile, specifically impacting the amounts of LDL and HDL particles (Figure 3). When compared to the control group, the administration of copper and aluminium resulted in a statistically significant ($P < 0.001$) increase in LDL levels and a decrease in HDL levels, respectively. In contrast, compared to the positive control group (PC), vitamin E administration dramatically boosted HDL levels and significantly decreased triglycerides (TG), total cholesterol (TC), and VLDL. These improvements were very significant ($P < 0.001$).Moreover, a noteworthy decrease in LDL levels ($P < 0.05$) was seen in contrast to the PC group. When compared to the PC group, the 500 mg/kg dose of NOLE therapy led to a highly significant rise in HDL ($P < 0.001$) and substantialreductions in VLDL, LDL, and TC levels ($P < 0.01$). With the exception of HDL, where a highly significant increase ($P < 0.001$) was seen in comparison

to the PC group, no significant changes were observed in the other lipid parameters at the 250 mg/kg dose.

Table 1. Findings of the different lipid profile parameters. The units of measurement are mg/dl.

	Control	Positive Control	Vitamin-E	NOLE 250	NOLE 500
HDL	44 ± 0.98	33 ± 0.99	50 ± 1.01***	39 ± 1.02***	43 ± 1.00***
Triglyceride	56 ± 0.88	60 ± 1.05	55 ± 1.04***	59 ± 1.08	60 ± 1.01
Total Cholesterol	78 ± 0.79	84 ± 1.01	80 ± 1.06***	85 ± 1.04	77 ± 1.02***
VLDL	10 ± 0.27	10 ± 0.75	9 ± 0.84***	10 ± 0.99	10 ± 1.01**
LDL	78 ± 0.99	84 ± 1.07	80 ± 1.02*	83 ± 1.01	82 ± 1.06

NOLE: Methanolic extract from the leaves of Nerium oleander L. For n = 6, the data are given as mean ± SD. One-way Tukey post hoc ANOVA: $p < 0.05$, $p < 0.01$ and $p < 0.001$ for all.

Assessment of GSH activity in the brain

Specific details regarding the impact of methanol extract on the glutathione (GSH) content of the brain are given in Table 2. Over the course of 42 days, from the first to the 35th, the administration of Copper and Aluminium led to dementia and a significant reduction in GSH levels in the brain homogenate of the positive control group, to 6.8 ± 0.91, from 9.6 ± 0.98 in the normal group. GSH levels rose dramatically ($P \leq 0.001$) following treatment with the methanol extract at doses of 250 and 500 mg/kg from the 36th to the 42nd day in comparison to the positive control group. The increases were 10.9 ± 0.21 and 13 ± 0.28, respectively. GSH levels of 11.8 ± 0.97 were observed in Vitamin E, which was utilized as a reference. This suggests that both Vitamin E and the methanol extract efficiently.Likewise, Table 2 presents the impact of the methanol extract on brain superoxide dismutase (SOD) activity, utilising the identical treatment regimen as the GSH activity investigation. SOD levels in the positive control group were 52 ± 1.02, substantially lower than in the normal group (92 ± 1.08). Following a 7-day course of therapy with the methanol extract, SOD levels rose significantly ($P \leq 0.001$), to 65 ± 1.09 for the 250 mg/kg dose and 77 ± 1.08 for the 500 mg/kg dose, respectively. When compared to the methanol extract treatments, the rise in SOD levels with Vitamin E treatment (83 ± 1.03) did not exhibit a significant difference, indicating that both treatments were effective in increasing SOD activity, albeit to varying degrees.

Assessment of the AChE activity in the brain

In comparison to the normal group, the study found that the positive control group's brain homogenates had significantly higher levels of acetylcholinesterase (AChE) ($P<0.001$). Increased AChE activity in this elevation suggests a negative impact of aluminium and copper used to cause dementia. AChE levels, however, were significantly reduced upon the administration of a 500 mg/kg dosage of N. oleander methanol extract. In particular, Table 2 shows that this decrease was statistically significant at $P \leq 0.05$ and $P \leq 0.001$, respectively, when compared to the positive control group. This indicates that the methanol extract at this dosage successfully offsets the increase in enzyme activity in the brain caused by exposure to metals.

Table 2. Impact of Vitamin-E and NOLE on AD biomarkers

	Control	Positive Control	Vitamin-E	NOLE 250	NOLE 400
AChE (nmol/min/mg of protein)	18 ± 0.88	21 ± 0.21[a]	19 ± 0.84***	21 ± 0.76	21 ± 0.19*
GSH (µmol/mg of protein)	9.6 ± 0.98	6.8 ± 0.91	11.8 ± 0.97***	10.9 ± 0.21***	13 ± 0.28***

SOD (Units/mg of protein)	92 ± 1.08	52 ± 1.02	83 ± 1.03***	65 ± 1.09***	77 ± 1.08***
MDA/TBARS (nmol MDA/gm tissue)	112 ± 1.17	295 ± 1.31	205 ± 1.01***	285 ± 1.75***	246 ± 1.22***

NOLE: Nerium oleander L. leaf methanolic extract. The information is given as mean ± SD (n = 5).

One-way Dunnett's post hoc ANOVA: $p < 0.05$, $p < 0.01$ and $p < 0.001$ for all.

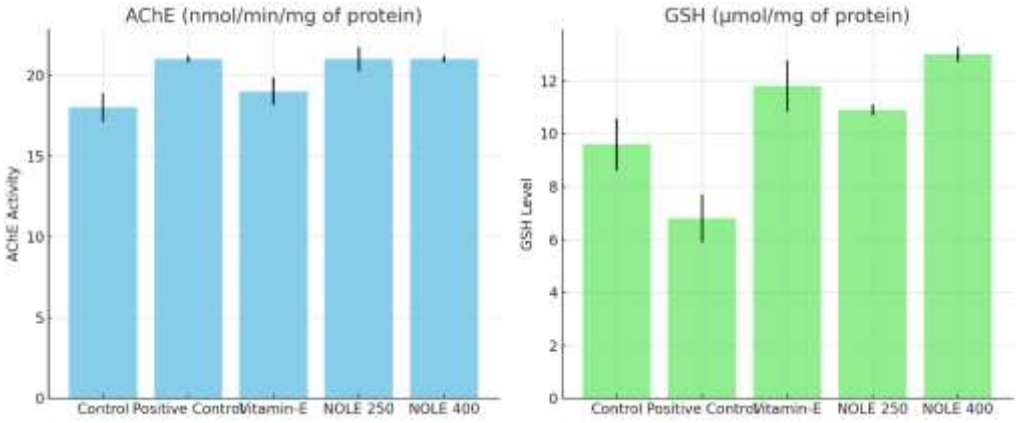

Figure 1. Impact of Vitamin-E and NOLE on AChE and GSH activity in the brain.

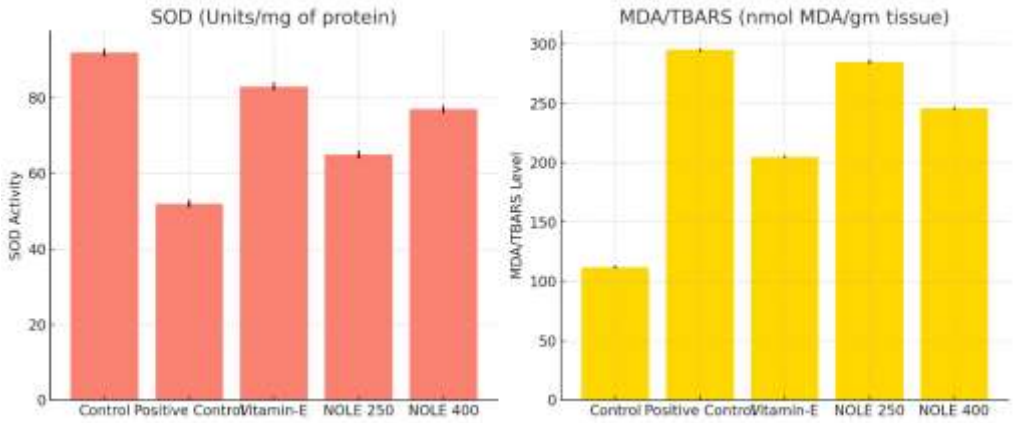

Figure 2. Impact of Vitamin-E and NOLE on SOD and MDA/TBARS activity.

CONCLUSIONS

The current study effectively showcased the neuroprotective properties of the methanol extract derived from Nerium oleander L. leaves. It also highlighted how effective this extract might be in treating dementias, particularly those brought on by Alzheimer's disease. The results point to the extract's potential to lessen neurodegenerative alterations, opening up a promising new line of inquiry into the illnesses it can treat.

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