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Research Article

A Novel Curcumin-Based Formulation Offers Protection Against Neurodegeneration in Rat Models of $AlCl_3$-Induced Alzheimer's Disease

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Abstract

The exact mechanisms underlying Alzheimer's disease (AD) pathogenesis are not fully understood, and effective disease-modifying treatments remain lacking despite decades of AD research. Therefore, alternative therapeutic approaches that may target multiple mechanisms of action underlying AD and have a better safety profile than synthetic drugs, such as phytotherapy, are being explored. We aimed to assess the neuroprotective properties of a novel curcumin formulation fortified with andrographolides and piperine (MAG XXI) in rat models of aluminium chloride–induced AD. Overall, 30 male Wistar rats were included and divided into five groups (a healthy control group, a non-treated AD group, and three AD groups treated with donepezil or 200/400 mg/kg body weight of MAG XXI). The Morris water maze, passive avoidance, and elevated plus maze tests were performed on the rats. Tissue samples from the cortex and hippocampus of the rats were then subjected to biochemical evaluation of neuronal, oxidative stress, and inflammatory markers. Compared to non-treated rats, donepezil-treated rats and high-dose (400 mg/kg body weight) MAG XXI–treated rats showed a mild but significant improvement in the Morris water maze and elevated plus maze test findings and a marked and significant improvement in the passive avoidance task results. Furthermore, oxidative stress markers, inflammatory markers, and neuronal markers improved overall in the donepezil-treated group and in both MAG XXI–treated groups. Notably, the improvement in the oxidative stress markers was more marked with MAG XXI (both doses) than with donepezil. Histopathological examination revealed lower incidence rates of neurofibrillary tangles, gliosis, and neuronal degeneration in the high-dose MAG XXI and donepezil groups. Notably, cresyl staining revealed minimal-to-mild cell dispersion in the donepezil group, whereas normal neuronal cells with well-lineated cell bodies and Nissl substance were observed in the high-dose MAG XXI group. No adverse events were noted in the MAG XXI groups. MAG XXI could be a promising alternative for AD treatment because it appears to exhibit neuroprotective properties, as demonstrated by its ability to alleviate oxidative stress and neuroinflammation. However, further clinical trials involving humans are necessary to corroborate the present study's results.

Keywords: *Andrographolides, Alzheimer's Disease, Curcuminoids, Memory, Neurodegeneration, Phytotherapy*

Background

Alzheimer's disease (AD) is a common chronic neurodegenerative disorder characterised by a progressive deterioration of cognitive function, especially memory, in the aging population globally [1]. The characteristic symptoms of AD include cognitive impairment, memory loss, and mild behavioural changes [2]. The prevalence of various dementia forms has increased to more than 47 million worldwide and is estimated to reach approximately 152 million by the year 2050, and this is a concerning statistical fact for AD clinicians [3]. Several hypotheses regarding AD pathogenesis have been proposed, and these hypotheses by and large focus on amyloid-beta (Aβ) plaque deposition and tau hyperphosphorylation, which cause various cognitive dysfunctions in patients with AD. One such hypothesis is that oxidative stress is induced by increased accumulation of reactive oxygen species (ROS) and neuroinflammation, and oxidative stress in turn leads to the apoptosis of nerve cells in the brain. Another hypothesis is that the deposition of Aβ plaques and neurofibrillary tangles (NFTs) is initiated by the degeneration of cholinergic neurons in the cerebral cortex, hippocampus, basal ganglia, and amygdala, which subsequently triggers stress on the endoplasmic reticulum and mitochondria and causes AD [4-6]. The relationship between mitochondrial dysfunction and AD is bidirectional. Mitochondrial dysfunction leads to compromised adenosine triphosphate (ATP) production, increased ROS production, and impaired calcium haemostasis. These changes lead to further neuronal cell damage and result in AD progression [7, 8]. The production of ATP in the mitochondria occurs via oxidative phosphorylation; this process is impaired by the deposition of Aβ plaques and tau tangles in several regions of the brain in patients with AD [9, 10]. The process commences with a decreased production of ATP, leading to the overproduction of ROS, which signifies the failure of the mitochondria to maintain cellular energy. In addition, the mitochondria contribute to intracellular calcium ion signalling as a sensor, buffer, and modulator [11]. In patients with AD, there is an increase in the cytosolic levels of calcium ions, which are quickly absorbed by the mitochondria to avoid an overload in the cytosol, thereby increasing the production of ROS and inhibiting ATP synthesis. Owing to this collective impairment, mitochondrial dynamics are significantly compromised in patients with AD. Additionally, previous studies have suggested that exposure to aluminium causes pathological changes that contribute to the potential onset and progression of AD [12].

As the pathogenesis of AD is complex, AD presents significant challenges to patients and their healthcare providers, thus highlighting the need for early interventions such as enhanced diagnostic and therapeutic options. Furthermore, these challenges have become more relevant because the prevalence of AD has increased rapidly among young adults in recent times. Commercially available medications, such as donepezil, galantamine, and rivastigmine, provide temporary symptomatic relief but are frequently associated with considerable side effects such as insomnia, muscle spasms, and fatigue [13]. Given the complex underlying pathological mechanisms and the absence of highly effective therapeutic options, there is renewed interest in exploring alternative therapeutic approaches, including phytotherapy, which may have multi-targeted mechanisms of action and may be accompanied by fewer and less severe side effects than synthetic drugs. Phytotherapy, treatment with a plant-based derivative, could be a good alternative option that may alleviate symptoms and address the underlying pathogenesis of AD [14]. While several preliminary studies have suggested neuroprotective properties of certain herbs [15, 16], to the best of our knowledge, comprehensive investigations into the efficacy and mechanisms of action of these herbs in animal models of AD are lacking. After proper research on and screening for potential herbs and bioactive compounds, we identified a mixture of curcuminoids (extracted from *Curcuma longa*) and andrographolides (extracted from *Andrographis paniculata*), which showed potential for AD management. Curcuminoids and andrographolides have been reported in the literature to exhibit anti-inflammatory, anti-apoptotic, and anti-mitotic properties, which help minimize neuronal damage in the brain [17, 18]. *C. longa*, from the *Zingiberaceae* family, has been reported to be capable of exhibiting medicinal properties [19]. Curcuminoids, derived from an ancient Indian spice commonly known as turmeric, are a mixture of 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin. Turmeric has a distinctive yellow colour and is used as both food colouring and flavouring agents [20]. Additionally, turmeric has historically been used to treat respiratory conditions, abdominal pain, and sprains, and research has been conducted to understand the positive effects of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) on cognitive improvement in humans [21]. *A. paniculata* (green chiretta) is from the *Acanthaceae* family and is widely distributed in China and South and East Asia [22]. It has been shown to treat various respiratory disorders, inflammation-related disorders, cancer, and rheumatoid arthritis [23], and recent studies have attempted to understand the anti-inflammatory and neuroprotective properties of these herbs, which may help in the treatment of AD [24].

Oxidative stress has been implicated in mitochondrial dysfunction, and it has also been established that mitochondrial degeneration leads to neuronal degeneration. It is known that excess ROS disrupts the electron transport chain, collapses the mitochondrial membrane potential (MMP), and promotes cytochrome-c release, thereby triggering the cascade of apoptosis [25]. Dysfunctional lysosomes and dysregulated autophagy/mitophagy are known to cause accumulation of Aβ and NFTs, which leads to progressive neurodegeneration [26]. Therefore, we hypothesized that improving the mitochondrial antioxidant system and mitochondrial health and simultaneously improving lysosomal function would lead to improved autophagy and prevent substrate accumulation and neurodegeneration. In this context, curcumin is known to improve the antioxidant system and improve mitochondrial health [27]. It is also known to promote lysosomal function (increased lysosomal acidification and enzyme activity) and to improve lysosomal biogenesis and promote autophagy [28]. Similarly, andrographolides have been shown to affect lysosomes through the regulation of autophagy and lysosomal function, particularly through their interaction with the PI3K/Akt/mTOR pathway and their effect on autophagosome–lysosome fusion [29]. Therefore, for the synergistic effect on the mitochondria–lysosome and autophagy/mitophagy, we chose these two ingredients, curcumin and andrographolide, to prevent neurodegeneration. Additionally, we chose piperine because it has been suggested by a previous study to enhance curcumin's bioavailability [30]. Altogether, based on the literature, this composition targets the mitochondria–lysosome–autophagy axis implicated in AD pathogenesis.

Although previous studies have demonstrated the potential benefits of *C. longa* and *A. paniculata* individually, to the best of our knowledge, there are no animal studies on the neuroprotective properties of a mixture of these herbs. Therefore, the present study aimed to assess the neuroprotective properties of a novel phytoactive formulation (MAG XXI) comprising curcuminoids, andrographolides, and piperine in rat models of aluminium chloride ($AlCl_3$)–induced AD. In particular, this study assessed the ability of the herbal formulation to ameliorate free radical accumulation and consequently halt the progression of AD. These rat models were chosen for this study because aluminium is a potential cholinotoxin that is known to promote degeneration in neuronal cells and lead to the disruption of the cellular signalling pathway through the formation and aggregation of Aβ plaques and tau tangles, which are the hallmark features of AD [31].

Methods

Drug formulation

A formulation of *Curcuma longa* rhizome extract with 95% curcuminoids (physicochemical properties and high-performance liquid chromatography [HPLC] results in Supplementary Figure 1 [32]), *Andrographis paniculate* extract with 95% andrographolides (with the sum of the volumes of curcuminoids and andrographolides constituting 98%; physicochemical properties and HPLC results in Supplementary Figure 2 [33]), and *Piper nigrum* extract 2%; (physicochemical properties and HPLC results in Supplementary Figure 3 [34]) was poured into a double-cone blender and mixed for 1 hour [35]. *Curcuma longa* extract and *Piper nigrum* extract were purchased from All Season Herbs Private Limited, Karnataka, India, and andrographolides were purchased from Nutra Extract Private Limited, Uttar Pradesh, India. These phytocompounds were extracted using the hydroalcoholic method from the plants by the manufacturers, not the authors [36]. This process was carried out at a controlled temperature and humidity (temperature not exceeding 24°C and a relative humidity of 25%–30%) [38].

Experimental protocol for the *in vivo* study

In total, 30 male Wistar rats aged 8–12 weeks and weighing approximately 250–350 g were included in the study. The rats were housed in groups in polypropylene cages and provided with standard rodent pelleted feed and purified water ad libitum. All animal experiments complied with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and were approved by the animal ethics committee of Sri Ramachandra Institute of Higher Education and Research (Approval number: Institutional animal ethics committee (IAEC) IAEC/66/SRIHER/776/2022; approved on 15.03.2022). Additionally, the study complied with the ARRIVE guidelines. The experiment was conducted for 28 days. The rats were allowed to acclimatise for 5 days; at the end of the acclimatisation period, individual animal body weight was recorded, and the rats were randomised into one of five groups, with six rats in each group. $AlCl_3$ (Sisco Research Laboratories Pvt. Ltd., Chennai, India) was injected intraperitoneally once daily for 4 weeks, and the rats' responsive clinical signs were recorded. Additionally, 1 hour before $AlCl_3$ administration, the test drug (MAG XXI; 200 and 400 mg/kg; the doses were based on findings of the *in vitro* screening analysis) formulated in a vehicle (0.03% carboxymethyl cellulose) was administered orally, which is the intended route of administration in humans, every day for 28 days, and their responses were monitored throughout the study period.

The rats were randomised to one of the following groups: group 1—exposed to saline, not $AlCl_3$ (control group); group 2—500 µL of $AlCl_3$ (100 mg/kg body weight); group 3—500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4—$AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5—$AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

All rats were observed for mortality and morbidity twice daily from acclimatisation to the end of the study. They were examined daily for clinical signs and local reactions after drug administration. Individual body weight was also recorded during acclimatisation, on the first day of treatment, and at every week of treatment until necropsy.

Behavioural analyses

All animals underwent behavioural analyses once a week throughout the study period.

Morris water maze test

The Morris water maze test was performed to assess the rats' spatial memory and learning abilities. A pool containing water and housing a hidden escape platform beneath the water surface was set up. The rats were introduced into the water to swim and search for the hidden platform to exit the water. They were trained three to five times daily for at least 3 days on the escape routes before the start of the actual test session. Subsequently, the time taken by the rats to reach the platform (escape latency) was noted, and a total of four trials were performed to assess escape latency. Learning and retrieval processes were evaluated via training and probe or transfer trials. Once a week, a probe trial was performed, during which the number of times the rats crossed the centre of the pool within 1 minute was recorded (Supplementary Figure 4). Spatial memory was assessed separately in a probe trial, without a platform. Learning and re-learning experiments were performed by switching the position of the platform, and the total number of times that the animal reached the target escape latency (3 minutes) was recorded once weekly.

Passive avoidance task

The passive avoidance task was performed to assess the preservation of the avoidance memory. A dual-chambered, trough-like corridor was installed, with the two sections separated by a movable barrier. The bright chamber was free of aversive stimuli, while the dark chamber was equipped with the capacity to deliver electric shocks. All animals underwent 6 to 7 days of training before the test trial. During the training session, the rats were placed in the bright chamber facing the door while having the door open to the dark chamber. When the rat stepped into the dark chamber with all four paws, the door was closed. Subsequently, a foot shock lasting 1 to 2 seconds was delivered (electric shock ranging between 0.2 and 0.5 mA, which is the minimum intensity required to provoke reactions such as running, jumping, flinching, and/or vocalisation). They remained in the dark chamber for an additional 10 to 30 seconds after aversive stimulus termination. The same procedure was followed in the test trials, and the latency (time taken) to re-enter the dark chamber was noted (Supplementary Figure 5). However, no aversive stimuli were applied to the rat upon re-entering the dark chamber during the trial session.

Elevated plus maze test

The elevated plus maze test was conducted to assess the rats' anxiety-related behaviours. The setup comprised open and closed arms, which were placed perpendicularly to each other and crossed in the middle, as well as a central area. The animals were allowed to move freely between the two arms, and the number of entries into the open and closed arms was recorded (Supplementary Figure 6).

Biochemical analyses

After the behavioural analyses, the rats were sacrificed and their brain samples were collected. The brain samples were weighed, and half of the collected brain tissue was stored at −80°C for further analysis. Subsequently, 5% potassium chloride was added, and the mixture was homogenised using a tissue homogeniser. All biochemical analyses were performed using tissue samples from both the cortex and hippocampus. Most of the reagents used in the biochemical analyses were purchased from Sisco Research Laboratories Pvt. Ltd. (Chennai, India). For reagents/kits purchased from other manufacturers, the manufacturer details are provided next to the corresponding reagent/kit names in the text.

Oxidative Stress Markers

Superoxide dismutase (SOD) levels were determined by adding 0.05 mL of 5% brain homogenate to 0.3 mL of sodium pyrophosphate buffer (0.025 M; pH, 8.3), 0.075 mL of nitroblue tetrazolium (300 µM; pH, 8.3), and 0.025 ml of phenazine methosulfate (186 µM). The reaction was started by adding 0.075 mL of nicotinamide adenine dinucleotide (780 µM; pH, 8.3). The absorbance was measured at 560 nm by using a microplate reader [39].

Lipid peroxidation (LPO) status was determined by adding 0.2 mL of 10% brain homogenate to 0.8 mL of saline, 0.5 mL of butylated hydroxytoluene, and 3.5 mL of 2-thiobarbituric acid for 90 minutes in a water bath. The absorbance was measured at 532 nm using a microplate reader [40].

Protein estimation was performed by using the biuret method; 0.2 mL of saline was added to 10% homogenate, and subsequently 1.25 mL of the biuret reagent was added. The absorbance was measured at 540 nm [41].

Glutathione peroxidase (GPx) activity was assessed by adding the mixture of 200 µL of Tris-HCl buffer, 200 µL of potassium ethylenediaminetetraacetic acid, 100 µL of sodium azide, and 200 µL of the 10% brian enzyme preparation to 200 µL of the reduced glutathione solution and 0.1 mL of H_2O_2. The reaction was terminated by adding 0.5 mL of 10% trichloroacetic acid. To evaluate the non-enzymatic reaction rate, the enzyme sample was substituted with buffer. The solution was then centrifuged at 4000 rpm for 10 minutes in order to remove the precipitate. Subsequently, the concentration of the reduced glutathione in the supernatant was quantified by adding 1 mL of 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent). The absorbance was measured at 412 nm.

Glutathione (GSH) content was estimated by using Ellman's reagent (0.6 mM; pH, 8.0). The absorbance was measured at 405 nm [42].

Inflammatory biomarkers

Inflammatory biomarkers— interleukin-6 (IL-6), tumour necrosis factor–alpha (TNF-α), and nuclear factor kappa light chain enhancer of activated B cells (NF-κB)—were analysed in the cortical and hippocampal regions.

TNF-α levels were evaluated using the TNF ALPHA ELISA Kit from Krishgen Biosystems (Mumbai, India) per the manufacturer's instructions. IL-6 levels were determined using the IL6 ELISA Kit from Krishgen Biosystems as per the manufacturer's instructions. NF-κB levels were evaluated using the NF K BETA ELISA Kit from KinesisDx (Brea, CA, USA) according to the manufacturer's instructions.

Neuronal biomarkers

Amyloid precursor protein (APP) levels were evaluated using the APP ELISA Kit from KinesisDx per the manufacturer's instructions. Aβ levels were determined using the Amyloid Beta ELISA Kit from Elabscience (Houston, TX, USA) per the manufacturer's instructions. Beta-secretase (β-secretase) levels were determined using the Beta Secretase ELISA Kit from ELK Biotechnology CO., LTD (Denver, CO, USA) according to the manufacturer's instructions. Gamma-secretase (γ-secretase) levels were evaluated using the Gamma Secretase ELISA Kit from ELK Biotechnology CO., LTD per the manufacturer's instructions. Acetylcholinesterase (AChE) activity was evaluated using the Ellman method, with 5,5'-dithiobis-(2-nitrobenzoic acid) as the chromogenic reagent. The absorbance was measured spectrophotometrically at 412 nm [43].

Gross pathological and histopathological examinations

The animals were weighed and euthanised using CO_2 after the behavioural analyses. A CO_2 flow meter was used to ensure a gradual displacement rate of approximately 30%–40% of the chamber volume per minute (13–15 L/min) as per the guidelines set forth by the American Veterinary Medical Association. The animals underwent detailed gross pathological analyses, which included gross examination of the external orifices, including body cavities, and inspection of the thoracic, abdominal, cervical, and cranial cavities for gross pathological changes. After gross examination, a part of the brain was collected and fixed with neutral buffered formalin [44], and the fixed tissues were embedded in paraffin wax and sectioned at approximately 3–4 µm. The fixed tissues were cleared in xylene and dehydrated in absolute alcohol.

The sliced brain tissues (coronal tissue sections) were then stained with haematoxylin–eosin for evaluating the tissue morphology.

Results

Using 5–10 µg/mL as a minimal anticipated effective concentration and conservative oral bioavailability assumptions for a non-nano solid blend (F = 5%–10%) with a V_d of 1 L/kg, the corresponding oral rat dose was found to be approximately 100–200 mg/kg. Thus, we selected 200 mg/kg body weight as the low dose and 400 mg/kg body weight as the high dose for the *in vivo* study. The acute oral toxicity data (OECD-423) indicated an LD_{50} greater than 2000 mg/kg; thus, the doses of 200 and 400 mg/kg remain well within the no-mortality dose, providing the authors with a comfortable safety cushion for efficacy testing in rat models.

Physical examination

No mortality or morbidity was observed in the animals during acclimatisation or following drug administration until the completion of the experiment (Supplementary Table 1). All animals appeared to be normal and did not show any clinical signs from days 1 to 28 following AD induction and drug administration. However, abdominal discomfort, mild tremor, and piloerection were noted in groups 2, 3, and 4 after 30 minutes of $AlCl_3$ administration every day and resolved within 1 hour of administration (Supplementary Table 1). The body weights of all animals were found to be normal throughout the experimental period; however, in group 3, body weight decreased on day 28 ($P < 0.05$). No significant body weight changes were noted in group 2 ($AlCl_3$-exposed animals) compared to group 1 (control group; Supplementary Table 2).

Behavioural analyses

Morris water maze test

There was a significant decrease in the number of times the animals crossed the centre of the pool, as well as escape latency, in group 2 compared to group 1. Additionally, compared to group 2, groups 3 and 5 showed a mild but significant increase in the number of times the animals crossed the centre of the pool as well as escape latency (Figure 1).

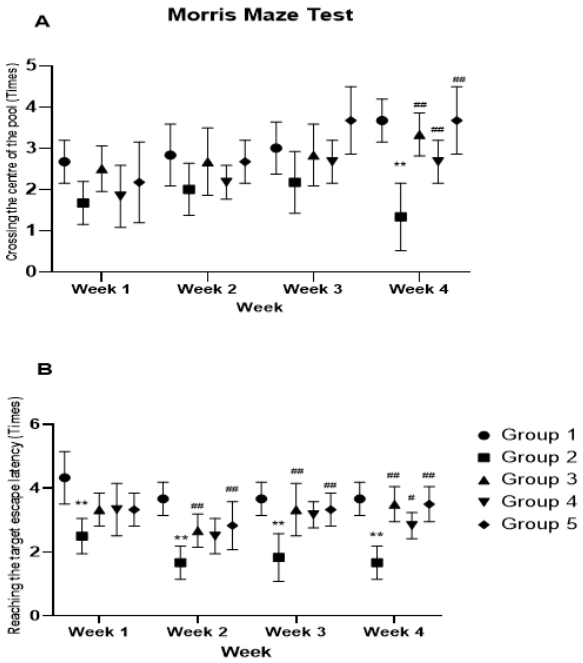

Figure 1. Dose-dependent effect of MAG XXI on spatial memory, as assessed using the Morris maze test

A) The number of times the rats crossed the centre of the pool and B) the number of times the rats reached the target escape latency of 3 min on the Morris maze test at weeks 1 to 4 are presented. Data from six rats in each group are presented as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively). # and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

$AlCl_3$: aluminium chloride

Passive avoidance test

Significant changes in the latency to enter the dark chamber were observed in group 2 compared to group 1. Groups 3 ($P < 0.01$) and 5 ($P < 0.01$) showed a significant improvement in latency compared to group 2 ($P < 0.01$ for both; Figure 2).

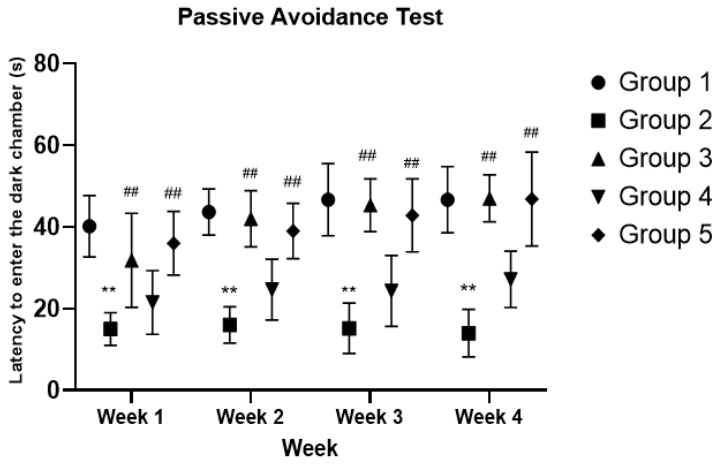

Figure 2. Dose-dependent effect of MAG XXI on non-declarative memory, a form of long-term memory

The time taken to enter the dark chamber on the passive avoidance test at weeks 1 to 4 is presented. Data from six rats in each group are presented as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively). # and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$, respectively).

$AlCl_3$: aluminium chloride

Elevated plus maze test

The number of entries into the open and closed arms in the elevated plus maze are presented in Figure 3 and Supplementary Table 3. Compared to group 1, group 2 showed a decrease in the number of entries into the open and closed arms at all weeks, which was significant at weeks 1 and 4. Compared to group 2, groups 3 and 5 showed a mild but significant increase in the number of entries at week 4.

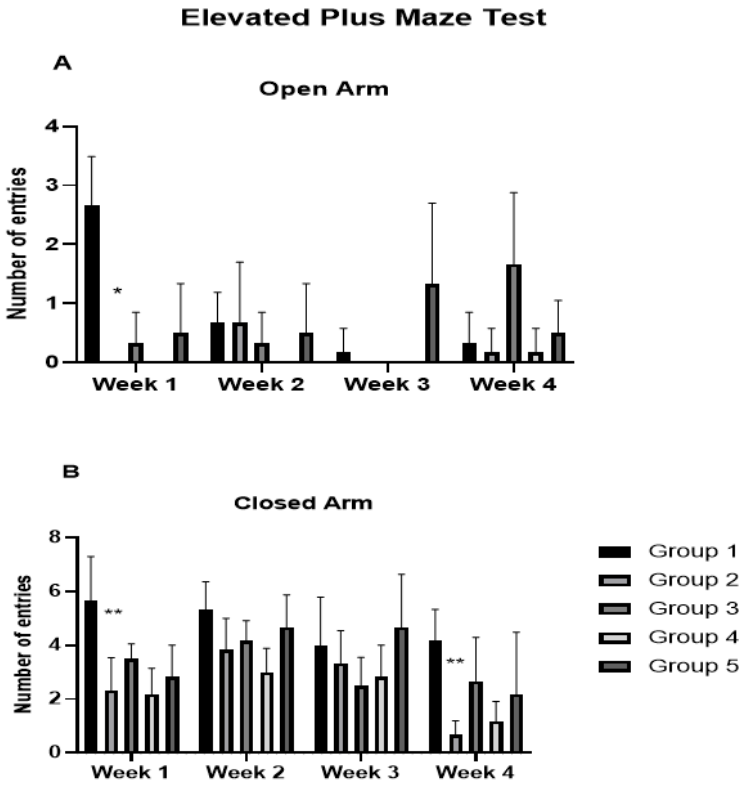

Figure 3. Dose-dependent effect of the drug on anxiety behaviour in rats with AD.

The number of entries into the A) open and B) closed arms on the elevated plus maze test at weeks 1 to 4 are presented. Data from six rats in each group are presented as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively). # and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$, respectively).

$AlCl_3$: aluminium chloride

Biochemical analyses

Oxidative stress markers: Antioxidant levels

In the hippocampus, group 2 showed a significant increase in LPO degree and a significant decrease in GPx, SOD, and GSH levels compared to group 1 ($P < 0.05$ and $P < 0.01$; Figure 4). Additionally, the levels of these oxidative stress markers improved significantly in groups 3, 4, and 5 ($P < 0.05$ and $P < 0.01$) compared to group 2. In the cortex, group 2 showed a significant increase in LPO degree and a decrease in GPx, SOD, and GSH levels compared to group 1 ($P < 0.05$). The levels of these oxidative stress markers showed a mild but significant decrease in groups 3, 4, and 5 ($P < 0.05$) compared to group 2. Furthermore, the levels of the antioxidant markers were significantly higher in groups 4 and 5 ($P < 0.05$ and $P < 0.01$) than in group 2 (Figure 4).

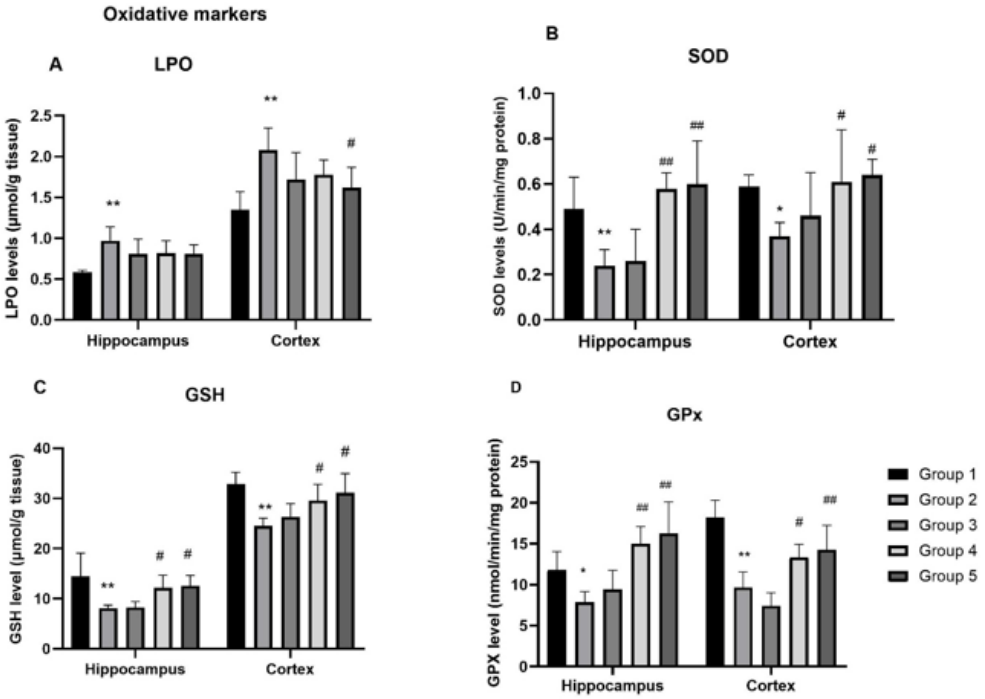

Figure 4. Effects of MAG XXI on the levels of oxidative stress markers in rats with AD

A) LPO, B) SOD, C) GSH, and D) GPx levels noted in the cortex and hippocampus of the five groups are presented. Data from six rats in each group are expressed as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively). # and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$, respectively).

AD: Alzheimer's disease, LPO: lipid peroxidation, SOD: superoxide dismutase, GSH: glutathione, GPx: glutathione peroxidase, $AlCl_3$: aluminium chloride

Inflammatory markers

The levels of the inflammatory markers noted in the five groups are presented in Figure 5. Group 2 had significantly higher levels of TNF-α, IL-6, and NF-κB in the hippocampus and cortex than group 1 ($P < 0.01$). The levels of the inflammatory markers showed a significant decrease in groups 3 and 4 compared to group 2 ($P < 0.05$ and $P < 0.01$). Additionally, a marked and significant reduction in the levels of the inflammatory markers was observed in group 5 compared to group 2 ($P < 0.05$ and $P < 0.01$).

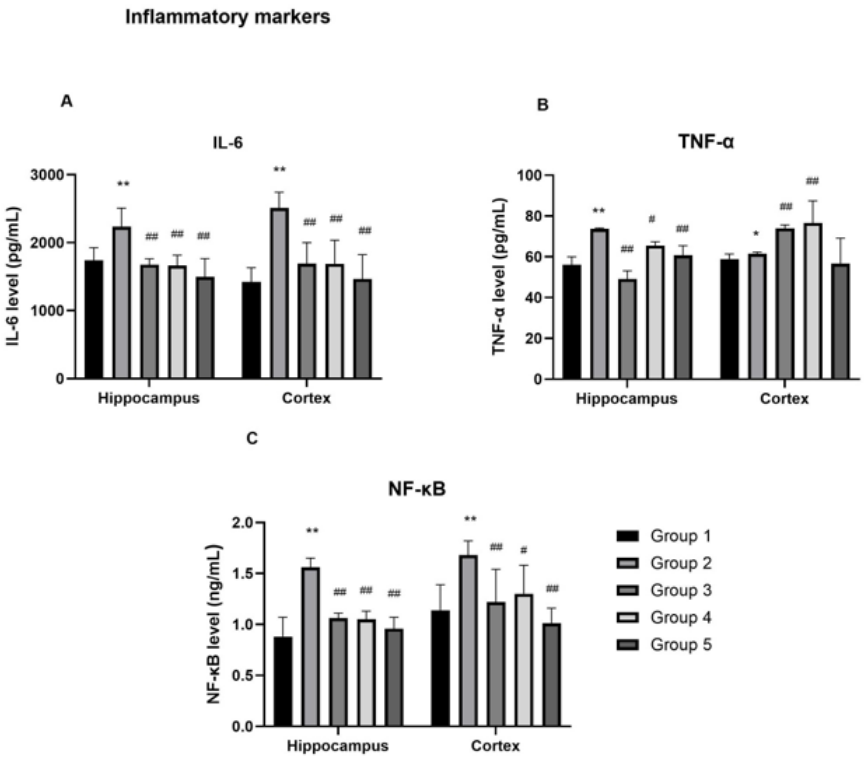

Figure 5. Dose-dependent effects of MAG XXI on the levels of inflammatory markers in rats with AD.

A) TNF-α, B) IL-6, and C) NF-κB levels observed in the cortex and hippocampus of all groups are presented. Data from six rats in each group are expressed as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively). # and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$, respectively).

AD: Alzheimer's disease, TNF-α: tumour necrosis factor-alpha, IL-6: interleukin-6, NF-κB: nuclear factor kappa light chain enhancer of activated B cells, $AlCl_3$: aluminium chloride

Neuronal markers

Group 2 showed a notable increase in AChE content as well as Aβ, γ-secretase, β-secretase, and APP levels, mainly in the hippocampal region. There was a mild decrease in the levels of the neuronal markers in groups 3 and 4 compared to group 2 ($P < 0.05$ and $P < 0.01$). Additionally, an improvement in the neuronal markers to near-to-normal levels was observed in group 5 compared to group 2 ($P < 0.01$; Figure 6).

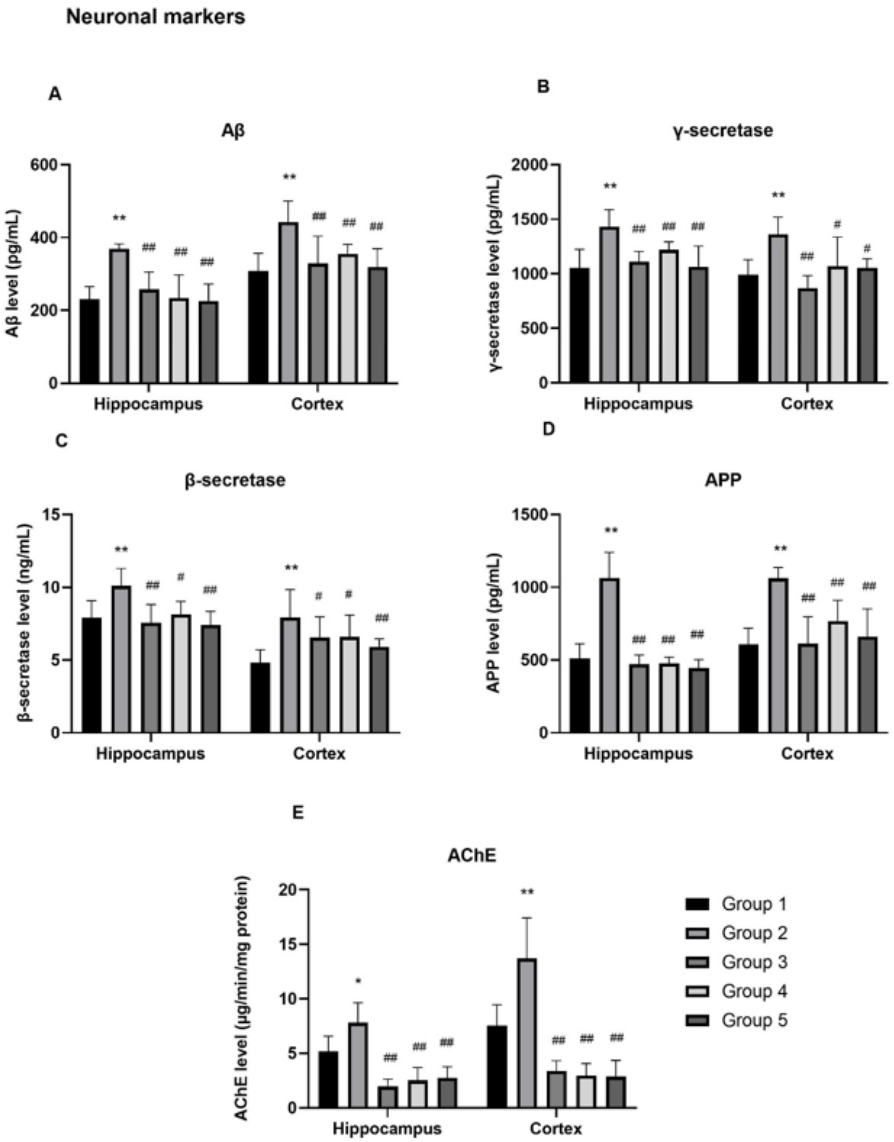

Figure 6. Dose-dependent effects of MAG XXI on the levels of neuronal markers in rats with AD.

A) Aβ, B) γ-secretase, C) β-secretase, D) APP, and E) AChE levels noted in the hippocampus and cortex of the five groups are presented. Data from six rats in each group are expressed as mean ± standard deviation (one-way analysis of variance followed by Tukey's post hoc test).

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

* and * *: Values are statistically significant between group 1 and group 2 ($P < 0.05$ and $P < 0.01$, respectively).

and ##: Values are statistically significant between group 2 and group 3, 4, or 5 ($P < 0.05$ and $P < 0.01$).

AD: Alzheimer's disease, AChE: acetylcholinesterase, Aβ: amyloid-beta, γ-secretase: gamma-secretase, β-secretase: beta-secretase, and APP: amyloid precursor protein, $AlCl_3$: aluminium chloride

Gross pathology and histopathology

No gross lesions were observed in any of the animals in the five groups during necropsy (Supplementary Table 6). No remarkable histopathological findings were observed in any of the animals in group 1. Minimal-to-mild NFTs were noted in the hippocampus of six, five, five, and four animals in groups 2, 3, 4, and 5, respectively, and in the cerebral cortex of all animals in all groups. Gliosis was observed in three, two, two, and zero animals in groups 2, 3, 4, and 5, respectively.

Glial nodules were noted in one animal in group 2; however, these findings were absent in the other groups. Additionally, mononuclear cell infiltration was observed in one animal in group 2 and one animal in group 4, but it was absent in the other two groups. Furthermore, neuronal degeneration was noted in two animals in group 2; notably, it was absent in the drug-treated groups. Of note, cresyl staining revealed mild-to-moderate cell dispersion in group 2, minimal-to-mild cell dispersion in group 3, minimal-to-normal cell dispersion in group 4, and normal neuronal cells with well-lineated cell bodies and Nissl substance in group 5. The incidence of remarkable histopathological findings was lower in group 5 than in groups 2 and 4. Representative images of haematoxylin–eosin staining of the cortex and hippocampus are presented in Figure 7 and Supplementary Figure 11, respectively. Similarly, representative images of cresyl staining of the cortex and hippocampus are presented in Figure 8 and Supplementary Figure 12, respectively.

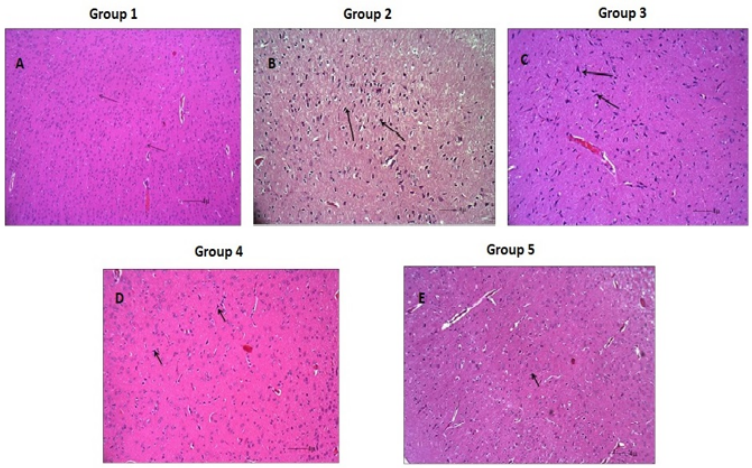

Figure 7. Representative images of H&E staining performed on cortical samples from rats with AD.

A) A photomicrograph of an H&E-stained section (×100) showing normal neuronal cells of the cerebral cortex in group 1. B) A photomicrograph of an H&E-stained section (×200) showing neurofibrillary tangles in the cerebral cortex of an animal from group 2. C) A photomicrograph of an H&E-stained section (×200) showing neurofibrillary tangles in the cerebral cortex of a rat from group 3. D) A photomicrograph of an H&E-stained section (×200) showing neurofibrillary tangles in the cerebral cortex of a rat from group 4. E) A photomicrograph of an H&E-stained section (×200) showing mild neurofibrillary tangles in the cerebral cortex of a rat from group 5.

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

H&E: haematoxylin and eosin, $AlCl_3$: aluminium chloride

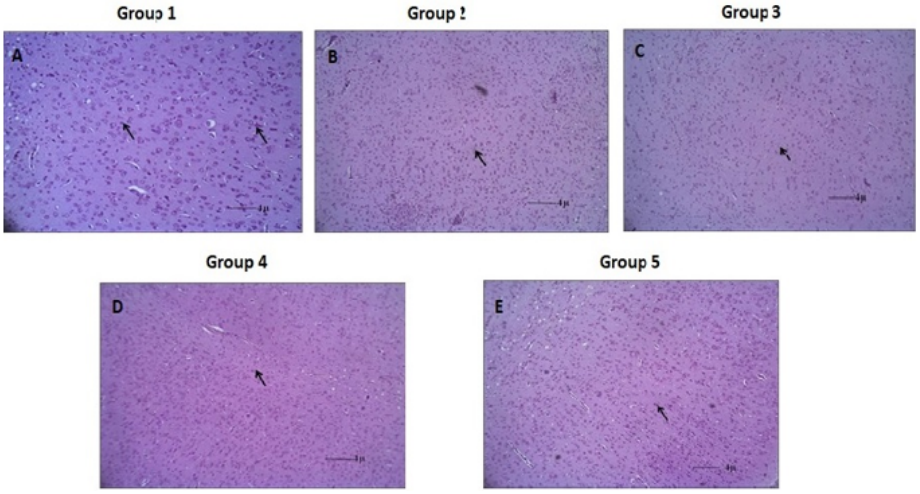

Figure 8. Representative images of cresyl staining performed on cortical samples from all groups.

A) A Photomicrograph of a cresyl violet–stained section (×100) showing normal appearance of neuronal cells in the cortical region of a rat from group 1. B) A photomicrograph of a cresyl violet–stained section (×200) showing moderate neuronal cell dispersion in the cortical region of a rat from group 2. C) A photomicrograph of a cresyl violet–stained section (×200) showing mild neuronal cell dispersion in the cortical region of a rat from group 3. D) A photomicrograph of a cresyl violet–stained section (×200) showing mild neuronal cell dispersion in the cortical region of a rat from group 4. E) A photomicrograph of a cresyl violet–stained section (×200) showing a normal appearance of neurons in the cortical region of a rat from group 5.

Group 1: exposed to saline, not $AlCl_3$ (control group); group 2: 500 µL of $AlCl_3$ (100 mg/kg body weight); group 3: 500 µL of $AlCl_3$ plus donepezil (10 mg/kg body weight; standard treatment group); group 4: $AlCl_3$ plus MAG XXI (200 mg/kg body weight; treatment group 1); and group 5: $AlCl_3$ plus MAG XXI (400 mg/kg body weight; treatment group 2).

$AlCl_3$: aluminium chloride

Discussion

The present study aimed to assess the neuroprotective effects of MAG XXI, a novel herbal formulation comprising curcuminoids, andrographolides, and piperine, against Alzheimer's disease, a condition currently treated with therapeutic agents with limited efficacy such as donepezil. The results showed that MAG XXI could improve memory and anxiety-related behaviours, as evidenced by the findings pertaining to the behavioural analyses, as well as improve AD-related neuronal, inflammatory, and oxidative stress markers in the hippocampal and cortical regions of rat models of $AlCl_3$-induced AD.

$AlCl_3$ has been widely used to establish rat models of AD. $AlCl_3$ administration (optimal dosage: 100 mg/kg body weight orally for 42 to 60 days) has been shown to cause AD-associated pathological events, including an elevation of Aβ and AChE concentrations in the hippocampus and cortex as well as an increase in the extents of neuroinflammation and oxidative stress in the hippocampus and cortex [45, 46]. Two previous studies have reported that $AlCl_3$-exposed animal models exhibit reduced locomotor activity, memory deficits, and learning impairments (evaluated by using behavioural tests such as the Morris water maze, Y-maze, and open field tests) [46, 47]. Additionally, $AlCl_3$ has been reported to be present in the brain tissues of patients with age-related dementia and AD [48]. The exact mechanism of action of aluminium in causing AD-like pathology is unclear. However, evidence suggests that it may enter the brain through a high-affinity receptor–ligand system that is similar to that used for the delivery of iron to neurons and glial cells [49], contribute to the formation of AD-associated structures and processes, and then potentially activate the classical complement cytolytic pathway, leading to neuroinflammation and cell death [50]. Based on these previous observations, in the present study, a pilot experiment was performed on six healthy young adult rats that were injected with $AlCl_3$ (100 mg/kg body weight) intraperitoneally once daily for 4 weeks. The rats showed memory deficits and learning impairments after exposure to $AlCl_3$—as evidenced by the Morris maze test—and exhibited AD-like brain changes, as confirmed by histopathological analysis.

The Morris water maze, passive avoidance, and elevated plus maze tests were performed on the rats in order to evaluate their short-term and long-term memory, learning ability, and anxiety-related behaviours, respectively. It is notable that compared to non-treated rats, donepezil-treated rats and high-dose MAG XXI–treated rats showed a mild but significant improvement in the Morris water maze test result and elevated plus maze test finding as well as a pronounced and significant improvement in the passive avoidance task result. These findings suggest that MAG XXI can reverse or improve the behavioural defects associated with AD. Notably, the effect of MAG XXI, especially at the high dose, was comparable to that of donepezil.

Biochemical analyses of oxidative stress, inflammatory, and neuronal markers were performed on tissue samples from both the hippocampus and cortex, which are the main regions of interest for AD. AD is associated with elevated oxidative stress and alterations in antioxidant defence mechanisms. In a previous study, a decrease in the activity of erythrocyte SOD and GPx as well as reduced concentrations of plasma non-enzymatic antioxidants such as GSH were noted in the blood of patients with AD [48]. Likewise, the activities of the antioxidant enzymes (SOD, GPx, and catalase) as well as GSH concentrations were statistically significantly lower in the saliva of patients with AD than in the saliva of the healthy control groups [51]. In addition, the extent of lipid peroxidation (as measured based on malondialdehyde levels) was significantly increased in both saliva and plasma of patients with AD; this observation indicates a shift in redox balance towards oxidative reactions both locally in the oral cavity and systemically [51]. In the present study, MAG XXI significantly increased the levels of GSH, GPx, and SOD and decreased the degree of lipid peroxidation in both the hippocampus and cortex. Interestingly, the improvements in these markers, especially SOD and GPx, were more pronounced with MAG XXI than with donepezil.

Neuroinflammation is an important player in the initiation and pathological progression of neuronal ailments and leads to loss of learning difficulties and memory. Particularly, in the context of tauopathies, tau-activated NF-κB signalling promotes the spreading of tau and neurotoxicity medicated by microglia; the activation of NF-κB in microglia has been shown to increase the seeding and spreading of tau, whereas the inhibition of NF-κB in microglia reduces these effects and reverses tau-mediated learning and memory deficits in mouse models [52]. In addition, TNF-α and IL-6, which are both regulated by NF-κB, are inflammatory cytokines whose levels are elevated in patients with AD; elevation of the levels of these cytokines leads to chronic inflammation in the brain, which is a hallmark of AD pathology [53]. In our study, MAG XXI significantly decreased the levels of IL-6, TNF-α, and NF-κB in the hippocampus and cortex. Notably, although both donepezil and MAG XXI significantly decreased the levels of these markers, the decrease observed with MAG XXI was marginally higher. Interestingly, cortical TNF-α levels were higher in the donepezil and low-dose MAG XXI groups than in the other groups; however, the reason for this observation is unclear.

AD pathogenesis involves complex interactions between several important proteins and enzymes. APP is subjected to sequential cleavage by β-secretase and γ-secretase in order to generate Aβ peptides, which are the main components of senile plaques [54, 55]. APP's amyloidogenic processing is initiated by β-secretase (specifically BACE1), and the process is completed when γ-secretase generates Aβ peptides of varying lengths [55]. Additionally, AChE accelerates the degradation of acetylcholine, which leads to neurotransmitter deficiency and cognitive impairment. MAG XXI significantly decreased the levels of these AD markers in the hippocampus and cortex, and the effects of MAG XXI and donepezil were comparable.

An in vitro screening study conducted by us before the commencement of the in vivo study showed that MAG XXI reversed mitochondrial dysfunction in an SH-SH5Y neuroblastoma cell model of rotenone-induced Parkinson's disease. The drug significantly increased cell viability (potential ability to regenerate neurons) and, importantly, reversed the disruption of mitochondrial membrane potential—as assessed based on rhodamine-123 uptake—and improved pyruvate and ATP generation. As mitochondrial dysfunction is a key driver of AD progression, because it hinders the body's ability to fight against the disease, it is promising to note that the drug is able to reverse the mitochondrial dysfunction related to AD. These findings hint at the potential mechanism of action of the drug—reversal of mitochondrial dysfunction—and at the drug's ability to improve not only AD but also possibly other neurodegenerative diseases.

Histopathological examination also showed a lower rate of remarkable findings (neurofibrillary tangles, gliosis, and neuronal degeneration) among rats treated with the high dose of MAG XXI and donepezil than among untreated rats exposed to $AlCl_3$. Notably, gliosis, neuronal degeneration, glial nodules, and mononuclear cell infiltration were not observed in any of the animals treated with the high dose of MAG XXI. Another notable finding was that while minimal to mild cell dispersion was noted in the donepezil-treated group, normal neuronal cells with well-lineated cell bodies and Nissl substance were observed in the group treated with the high dose of MAG XXI.

The additive effect of the herbal formulation compared to the efficacy of its individual constituents was assessed. In the present study, a separate experiment assessing the efficacy of the individual constituents showed that andrographolides and curcumin plus piperine improved the oxidative and neuronal markers by 10%–42.6% and 8%–45.8%, respectively, whereas MAG XXI improved these parameters by 38.7%–150% (Supplementary Table 9).

The strength of this study lies in the fact that a comparative analysis of the novel drug and a standard-of-care drug, donepezil, was performed. To the best of our knowledge, no recent relevant previous studies on AD have performed such a comparative analysis, which provides insights into whether a novel herbal drug is comparable to or superior to current commercially available drugs. Interestingly, in the present study, MAG XXI was marginally more effective than donepezil in improving certain AD markers. The limitations of the present study include the inherent limitations of small-animal studies; although rats are a common model for AD research, the findings might not completely translate to humans owing to physiological and genetic differences. Additionally, this in vivo study did not delve deeply into the mechanism of action of MAG XXI; however, we hypothesize that the drug reverses the mitochondrial dysfunction related to AD. Additionally, curcumin, one of the components of the formulation, has been demonstrated to initiate autophagy and activate the function of lysosomes by inhibiting the Akt-mTOR signalling pathway and by directly targeting and activating transcription factor EB [57]. Furthermore, it has been shown that the reduction of γ-secretase activity, which was achieved by MAG XXI in the present study, ameliorates autophagy defects in monogenic AD neurons [58, 59]. The exact mechanisms of action of the drug is be verified in a separate study.

Neuroinflammation is an important player in the initiation and pathological progression of neuronal ailments and leads to loss of learning difficulties and memory. Particularly, in the context of tauopathies, tau-activate

Conclusions

This in vivo study suggests that MAG XXI exhibits neuroprotective properties and is safe and effective in rat models of AD. Thus, this herbal formulation may serve as a promising alternative for AD management by mitigating oxidative stress and neuroinflammation via multiple mechanisms of action. The study emphasizes the need for clinical trials to verify the efficacy and safety of MAG XXI in human patients with AD, highlighting its potential role in the development of new, natural therapeutic strategies for AD management.

List of Abbreviations

Aβ: Amyloid beta
ACHE: Acetylcholinesterase
AD: Alzheimer's disease
$AlCl_3$: Aluminium chloride
APP: Amyloid precursor protein
ATP: Adenosine triphosphate
DMEM: Dulbecco's modified Eagle medium
FBS: Foetal bovine serum
GPx: Glutathione peroxidase
GSH: Glutathione
IL-6: Interleukin-6
LC: Liquid chromatography
LPO: Lipid peroxidation
MMP: Mitochondrial membrane potential
MS: Mass spectrometry
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB: Nuclear factor kappa light chain enhancer of activated B cells
NFT: Neurofibrillary tangles
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TNF-α: Tumour necrosis factor alpha

Ethics Approval and Consent to Participate

All animal experiments complied with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and were approved by the animal ethics committee of the institution (Approval number: IAEC/66/SRIHER/776/2022; approved on 15.03.2022).

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

Mr. Mohamed Arif is Chief Scientific Officer at Gidaa Life Sciences Private Limited, which funded this study.

Funding

This study was funded by Gidaa Life Sciences Private Limited. The funder had a role in the herbal formulation (MAG XXI), conceptualization and design of the study.

Authors' Contributions

MA: Study conceptualization and design, herbal drug formulation, and writing; **VG:** In vivo study: Methodology, statistical analysis, result compilation, data curation, writing—review, data visualization, formal analysis, and validation; **PK**: In vivo study methodology and investigation; **SR:** Animal monitoring and in vivo study methodology and investigation; **ST:** In vivo histopathological analysis. All authors read and approved the final manuscript.

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