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Ameliorative effect of Modified Atkins Diet against aluminium chloride induced cognitive, behavioural and neurochemical impairments in rats

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Dietary and metabolic therapies are emerging Alzheimer's disease (AD) treatment contender due to paucity of effective therapeutic interventions. Modified Atkins Diet (MAD) is a less restrictive version of ketogenic diet and has higher compliance rate. Therefore, in the present study MAD treatment of different durations were investigated for their effect in Aluminium chloride induced AD in rats. Alzheimeric rats received MAD for a period of 4 weeks (treatment 1), 6 weeks (treatment 2) and 4 weeks of MAD + 2 weeks without any treatment (treatment 3). Treatment 2 showed significant decrease in escape latency in MWM while increase in working memory, discrimination ratio and locomotor activity in MWM, NORT and OFT respectively. All the treatments have a mild effect on body weight, body mass index and fasting blood sugar level. Treatment 2 significantly decreased total protein, calcium, lactate dehydrogenase and acetylcholinesterase level in alzheimeric rats. Treatment 2 also improved lipid profile and oxidative stress parameters when compared with toxicant control rats. Treatment 2 also improved brain cell histology in AD rats. Treatment 1 and treatment 3 caused moderate to slight improvement in animal models and biochemical study parameters in alzheimeric rats. Results of the study conclude that MAD treatment for 6 weeks significantly restored cognition, behavioural and neurochemical abnormalities of AD.

Keywords: Alzheimer's disease, Ketogenic diet, Ketosis, Neurodegenerative, Oxidative stress

Alzheimer's disease (AD) is the most prevalent form of dementia that impairs memory and cognition and is a leading cause of dementia in the elderly. It is a progressive neurodegenerative condition that imposes substantial economic and social costs on the society[1]. A complex network of various genetic, neurochemical, biochemical, neurodegenerative pathways and processes contribute to AD[2]. Alzheimer's disease and other forms of dementia claim the lives of one person in every three seconds. The world population of dementia patients is expected to rise to 75.63 million by 2030 and 135.46 million by 2050[3]. Currently no effective treatment is available for AD, and those available can only temporarily improve cognition or relieve symptoms with a variety of side effects. Therefore, non-pharmacological approaches capable of targeting a larger spectrum of targets to not only treat symptoms but also reverse the pathogenesis of AD with fewer or no side effects must be investigated[2]. Non-drug interventions like diet modification aim to delay the loss of mental abilities in AD patients and to increase their well-being and quality of life[4]. Recent studies suggest that nutritional and metabolic therapies may address AD's pathogenic mechanisms, particularly the impairment of brain energy metabolism. Reduced glucose uptake and metabolism are hallmark features of AD, and ketone bodies can serve as an alternative fuel for the brain during energy deficits, potentially protecting neurons

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Abbreviations : ACh, Acetylcholine; AChE, Acetyl Cholinesterase; AD, Alzheimer's Disease; AI, Atherogenic Index; AlCl3, Aluminium Chloride; ANOVA, Analysis of Variance; BMI, Body Mass Index; CMC, Carboxy Methyl Cellulose; CPCSEA, Committee For The Purpose Of Control And Supervision Of Experimental Animals; DA, Dopamine; DR, Discrimination Ratio; DTNB, 5,5′-Dithiobis-2-Nitrobenzoic Acid; EL, Escape Latency; FA, Fatty Acids; Gpx, Glutathione Peroxidase; GR, Glutathione Reductase; GSH, Reduced Glutathione; HDL, High Density Lipoprotein; HMG-CoA, Hydroxymethylglutaryl-coenzyme A; IAEC, Institutional Animal Ethics Committee; KD, Ketogenic Diet; LDH, Lactate Dehydrogenase; LDL, Low Density Lipoprotein; LPO, Lipid Peroxidation; MAD, Modified Atkins Diet; MWM, Morris Water Maze; NEED, Naphthylethylenediamine Dihydrochloride; NORT, Novel Object Recognition Test; OFT, Open Field Test; ROS, Reactive oxygen species; SEM, Standard Error of Mean; SOD, Superoxide Dismutase; UCP, uncoupling protein; VLDL, Very Low Density Lipoprotein

from injury[5]. Currently, numerous keto diets are advised by scientists around the world for treating metabolic disruption in dementia. The ketogenic diet (KD), a high-fat, low-carbohydrate diet, has gained attention for AD prevention and treatment[6] due to its neuroprotective effects, improved mitochondrial function, and reduced inflammation[7]. However, its restrictive nature can lead to complications, making the Modified Atkins Diet (MAD) a more adaptable and palatable alternative[8]. The MAD typically limits daily carbohydrate intake to 10-20 g, with a fat-to-protein-plus-carbohydrate ratio of 1:2:1. Recent research has demonstrated that the degree of ketosis induced by MAD is typically lower than that of KD[9]. MAD has the advantage of maintaining carbohydrates indefinitely, offering variety of food items and no restriction on calories and fluid intake[10]. We hypothesize that dietary carbohydrate reduction and slight ketone body elevation through MAD treatment may protect neurons by improving mitochondrial function, reducing oxidative/nitrosative stress, and decreasing inflammation. Therefore, in the present study MAD treatment of different durations was evaluated for its effect on aluminium chloride induced cognitive, behavioural and neurochemical impairment in rats.

Materials and Methods

Drugs and chemicals

AlCl3, acetylthiocholine iodide, 5, 5'-dithiobis-2-nitrobenzoic acid, and naphthylethylenediamine dihydrochloride (NEED) were procured from CDH, New Delhi, India. Diagnostic kits of ERBA (Mumbai, India) were used for biochemical estimation and diagnostic kit of Beacon (Gujarat, India) was used for LDH estimation. All other chemicals used were of analytical grade and were purchased from Sigma-Aldrich Corp., and Himedia Laboratories, Mumbai, India.

Preparation of MAD

Keto fat powder (Keto Fuel, United States of America), protein powder (Gold Standard Whey, United States of America) and olive oil (Borges, India) were used to make MAD. 13 g of keto fat powder and 6.5 g of protein powder were mixed thoroughly to obtain a uniform mixture pellet (Table 1). The mixture was combined with olive oil (q.s.) to make round pellets of optimal size to feed to the rats of treatment groups. Based on the calculation of calories and nutritional values the self-prepared MAD has 62% fat, 28% protein and 10% carbohydrates which are in accordance of typical MAD nutritional composition as per MAD literature review[9].

Table 1 — Animal diet ingredients

Ingredients (g/kg)	Normal chow diet (g)
Dried skim milk	20.0
Soyabean meal	200.0
Wheat bran	190.0
Corn starch	380.0
Fish meal	100.0
Luceme powder	30.0
Yeast powder	10.0
Zymo protein	30.0
Salt	5
Calcium phosphate	16
Calcium carbonate	6
Choline Cl-70	1
Mineral premix	6
Vitamin premix	6
Ingredients (g/kg)	MAD (g)
Keto fat powder (Keto fuel, USA)	650
Protein powder (Gold standard whey, USA)	325
Olive oil (Borges, India)	q.s.

[MAD, Modified Atkins Diet; q.s., Quantity sufficient; USA, United States of America]

Animals

Adult male Wistar rats weighing 225-250 g and 8-9 weeks of age were procured from SGRR University, Dehradun, Uttarakhand, India and housed in conventional polypropylene cages with food and water *ad libitum*. Rats were kept in a 12-hour light-dark cycle at a regulated ambient temperature (24±2°C) and relative humidity (50-70%). The experiment was conducted in a noise-free environment.. The guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) of Govt. Of India were followed and the experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) with the approval number of CPCSEA/IAEC/SBS/2022/01.

Induction of neurotoxicity

For the induction of alzheimer's disease (neurotoxicity), aluminium chloride (175 mg/kg *p.o.*) was given to the rats for a period of 4 weeks[11]. After 4 weeks, rats showing significant cognitive and behavioural impairments as indicated by animal model studies were selected and included in the study.

Experimental design

Alzheimeric rats were divided arbitrarily into six groups having six animals (calculated using G*Power 3.1 software) in each and received following treatment (Fig. 1). group 1 (normal control)-animals received 1% CMC in distilled water (1 mL/kg) *p.o.*; group 2 (toxicant control)-alzheimeric rats received 1% CMC in distilled water (1 mL/kg) *p.o.* for 4 weeks; group 3 (standard group): alzheimeric rats received donepezil (5 mg/kg) *p.o.* for 4 weeks; group 4 (treatment 1)-alzheimeric rats received MAD for 4 weeks; group 5 (treatment 2)-alzheimeric rats received MAD for 6 weeks; group 6 (treatment 3)-alzheimeric rats received MAD for 4 weeks + 2 weeks without any treatment. Reports from previous studies indicate that duration of MAD intake plays a significant role in exhibiting its pharmacological effects and adverse reactions. Therefore, in the present study treatment 1 and treatment 2 were used to investigate the effect of MAD treatment durations on AlCl3-induced cognitive, behavioural and neurochemical impairment in rats. Treatment group 3 received MAD for 4 weeks followed by a period of 2 weeks without treatment was included in the current study to confirm the relapse phenomena which generally occur with diet intervention.

Body weight, BMI and FBS were checked at regular intervals of 14 days. Behavioural studies using animal models were carried out before and after induction of neurotoxicity and after the MAD treatment period of 4 and 6 weeks. Rats were mildly anaesthetized at the end of the experimental period with diethyl ether and blood samples were collected by retro orbital sinus puncture. Blood was then centrifuged at 10,000 rpm for 10 min using cooling centrifuge (Remi, C24, India) to obtain serum for biochemical estimation of lipid profile, calcium, total protein and LDH. In the end, animals were sacrificed by euthanasia (Phenobarbitone 150 mg/kg, *i.p.*). Brain and hippocampus were excised immediately from all groups of animals, washed with ice-cold saline, weighed, and kept in Tris's buffer (10% w/v). Supernatant of hippocampal homogenate was used for the acetylcholinesterase (AChE) and oxidative stress parameters.

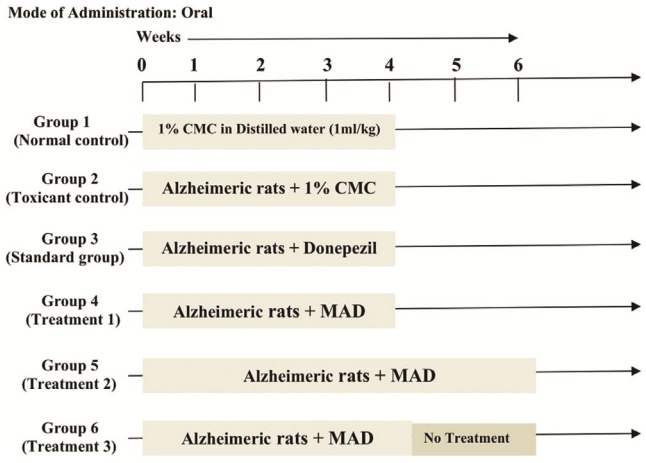

Fig. 1 — Experimental strategy for treatment protocol of alzheimeric rats.This draw highlights the experimental design, treatment protocol along with the mode of administration and time duration of the study.

Animal models for cognition and behaviour

The behavioural responses of the animals before and after induction of neurotoxicity and after MAD treatment were assessed in Open field test (OFT), Morris water maze test (MWM) and Novel object recognition test (NORT). The test sessions were scheduled between 9 a.m. and 2 p.m.

Open field test

An open-field activity test was performed to assess the locomotor and behavioural activity of the rats. Observations were recorded on a wooden apparatus that is divided into 16 (4×4) squares. Rats were kept in the testing room in their home cages for approximately 10 min before the actual experiment for acclimatisation to the novel environment. Animal was placed in one corner of the chamber, and its behaviour was observed for 5 min. The wooden apparatus and its walls were repeatedly cleaned with alcohol (70% v/v) to avoid the olfactory cues. The test measures the number of squares explored in a defined time[12].

Morris water maze test

Morris water maze test was employed to analyse the learning and memorizing ability of the animal. It consists of circular pool (210 cm in diameter, and 51 cm in height) in which opaque water was maintained at room temperature (19–22 °C) for rats and divided into four equal quadrants. A submerged platform of 10 cm diameter was placed inside the target quadrants of this pool. The rats were provided 120 sec to locate the hidden platform. Escape latency time (ELT) was noted as an index of acquisition or learning. On the fifth day, platform was removed, and each rat was allowed to explore the pool for 120 sec. Mean time spent in target quadrant searching for the hidden platform was noted as index of retrieval[13].

Novel object recognition test

Novel object recognition test was performed to evaluate hippocampal function and recognition memory in rats. NORT consist of three sessions: habituation, familiarization, and test session. A black coloured open field box (36×50×36 cm^3) was used and rat was placed in open field and allowed to explore freely for 10 min. After 24 h, test session was conducted by replacing wooden box with a novel object (a plastic box). Each rat was allowed to explore for 3 min in the open field. The time spent (in seconds) exploring the familiar and novel objects is recorded, to calculate the discrimination ratio[14].

$$\text{Discrimination ratio (DR)} = \frac{\text{Total time spend exploring novel object} - \text{Total time spend exploring familiar object}}{\text{Total time spend exploring both novel and familiar object}}$$

Physical parameters

Assessment of body weight and body mass index (BMI)

After the induction of neurotoxicity and the initiation of treatment, body weight and BMI were estimated at a regular interval of 14 days.

$$BMI = \frac{\text{Mass(kg)}}{\text{height(m2)}}$$

$$BMI = \frac{\text{Mass (lbs.)}}{\text{height(inch2)}} \times 703$$

[Where, mass and height are the rat's weight and height respectively]

Biochemical parameters

Estimation of fasting blood sugar

Fasting blood sugar was estimated by GOD-POD method from the serum of alzheimeric rats before and after treatment period[15].

Estimation of lipid profile

Total cholesterol, HDL and triglyceride levels were determined in the serum by CHOD-PAP method[16], phosphotungstic acid method[17] and GPO-Trinder method[18] respectively with the help of Erba diagnostic kits using Erba, Chem 5X Clinical Chemistry Analyser, Mannheim, Germany.

VLDL and LDL were calculated as per Friedewald's equation as follow[19]:

$$\text{VLDL} = \frac{\text{Total serum triglycerides}}{5}$$

$$\text{LDL} = \text{Total serum cholesterol} - \text{VLDL} - \text{HDL}$$

The atherogenic index was calculated by using the formula[20]:

$$\text{Atherogenic index} = \log \frac{[\text{Triglyceride}]}{[\text{HDL Cholestrol}]}$$

Estimation of total protein

The level of total protein in the serum was estimated by Biuret method using Erba Chem 5X Clinical Chemistry Analyser. The colour formed is proportional to the protein concentration and is measured at 546 nm[21].

Estimation of calcium

The level of calcium in the serum was estimated by intensity of the purple-coloured complex formed which was directly proportional to the amount of calcium present in the sample and it was measured photometrically between 540-600 nm[22].

Estimation of lactate dehydrogenase (LDH*)*

The level of LDH in the serum was estimated by the method of IFCC quantitative Kinetic method. The enzymatic function of LDH was examined spectrophotometrically at 340 nm[23].

Estimation of acetylcholinesterase (AChE)

Rats were sacrificed by Phenobarbitone at a dosage of 150 mg/kg via intraperitoneal administration. Hippocampus was quickly isolated and homogenized in ice-cold 0.1 M phosphate buffer, centrifuged and the supernatant was used for the estimation of acetylcholinesterase (AChE). AChE activity was determined quantitatively by Ellman's method. At the end, 20 µL of acetylthiocholine was added to the reaction mixture and change in absorbance was recorded for a period of 5 min at intervals of 1 min[24]. The enzyme activity was calculated using the formula

$$R = \frac{\Delta A/\text{min} \times \text{Vt}}{\epsilon \times \text{b} \times \text{Vs}}$$

[Where, ΔA/min = change in absorbance per min; ε = 1.361 x 104 $M^{-1}cm^{-1}$; b = path length (1 cm); V_t = total volume; V_s = sample volume]

The final reading of enzyme activity was expressed as µ moles/min/mg tissue.

Oxidative/Nitrosative stress

The hippocampal tissue was homogenized with Tris's buffer (10% w/v) at 10,000 rpm for 10-15 minutes. Supernatant was separated (homogenate) and used for estimation of levels of LPO, nitrite, GSH, SOD, GPx and GR.

Estimation of lipid peroxidation (LPO)

LPO was estimated by the method of Slater & Sawyer. The method estimates malondialdehyde,

spectrophotometrically by estimating the amount of lipid peroxides in the hippocampal tissue at 535 nm[25].

Estimation of antioxidant enzymes

Superoxide dismutase (SOD) level was estimated spectrophotometrically by the method of Sun & Zigman[26]. Reduced glutathione (GSH) was assayed spectrophotometrically from the hippocampal supernatant by the method of Moron[27]. Glutathione peroxidase (GPx) function was estimated by the method of Flohe & Gunzler[28]. Glutathione reductase (GR) activity was determined spectrophotometrically at 340 nm *via* Carlberg & mannervik method[29].

Estimation of nitrite/nitrate

Nitrite content in the hippocampus was determined quantitatively by the Griess reagent. Nitrite estimation is an indirect measurement of nitric oxide (NO) content in the biological samples. The reaction is followed by calourimetric detection of nitrates as an azo dye product of Griess reagents at 540-570 nm[30].

Histopathological examinations

Rats were sacrificed and brain was isolated and washed with cold phosphate-buffered saline followed by fixation using 10% formalin. Paraffin-embedded blocks were prepared for brain tissues. After sectioning, the paraffin sections were stained with hematoxylin and eosin and examined using a light microscope for histoarchitectural study. The experiment was performed in triplicates. Blinded experimenters performed the histopathological rating[31]. The histopathological alterations were viewed under light microscope at 100× magnification.

Statistical analysis

The statistical analysis of data was done using Graph-Pad Prism 9.0 software. All the values were presented as Mean ± SEM. Comparisons among groups were done by using two-way ANOVA (Analysis of Variance) followed by Dunnett' Multiple comparison. *P* values less than 0.05 was considered as indicative of significance.

Results

Effect of MAD on cognition and behaviour in alzheimeric rats using MWM, NORT and OFT

Morris water maze test

MWM test was performed to evaluate the effect of MAD on retention of working (reference) and spatial memory in alzheimeric rats. Effects of MAD on escape latency (EL) is summarized in Fig. 2. Administration of AlCl3 (175 mg/kg p.o.) for 4 weeks caused significant increase (P<0.001) in EL as compared to EL before induction in all groups except normal control group. Treatment of alzheimeric rats with MAD for 4 weeks significantly (P<0.01) decreased the EL suggesting that MAD treatment could reverse the spatial memory (day 4) deficits in alzheimeric rats. Result of Treatment 2 group (MAD for 6 weeks) and standard drug showed significant decrease (P<0.001) in EL after treatment. However, animals in treatment group 3 (reversal group) showed less significant decrease (P<0.05) in EL. Results of effect of MAD on mean time spent in target quadrant *i.e*. working memory (day 5) using MWM are summarized in Fig. 3. AlCl3 given to the animals for a period of 4weeks caused most significant decrease (P<0.001) in mean time spent in target quadrant as compared to the results of before induction in all groups except normal control group. Standard drug, treatment 1 and treatment 2 significantly increased (P<0.001) mean time spent in target quadrant after treatment while results of treatment 3 showed moderately increase (P<0.01) in mean time spent in target quadrant after treatment.

Novel object recognition test

NORT was performed to evaluate hippocampal function and recognition memory in alzheimeric rats. The discrimination ratio analysis revealed that after

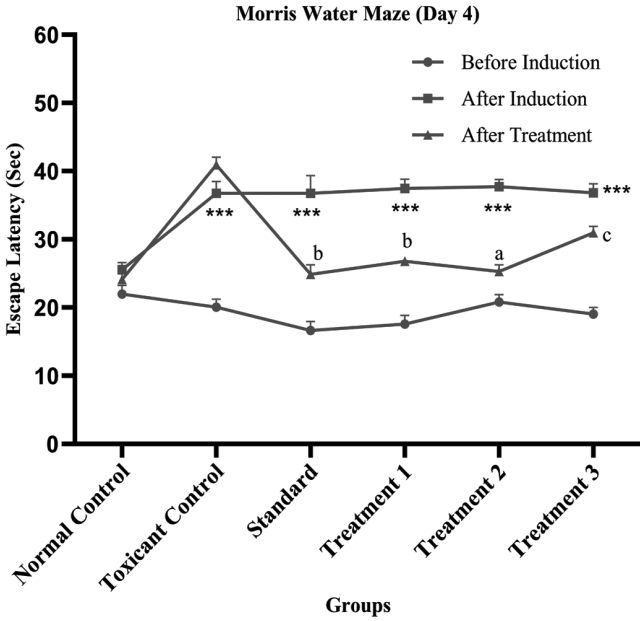

Fig. 2 — Effect of MAD on escape latency in alzhemeric rats. [All values are expressed as Mean ± SEM; n = 6 in each group, two-way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis]

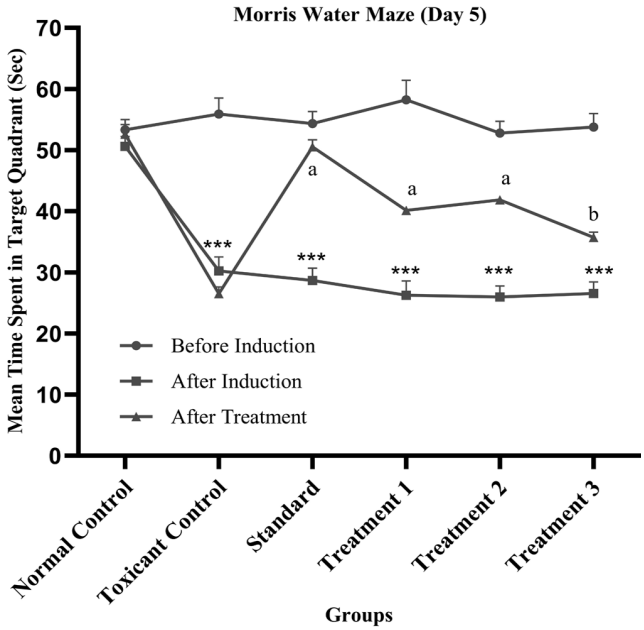

Fig. 3 — Effect of MAD on mean time spent in target quadrant in alzhemeric rats.

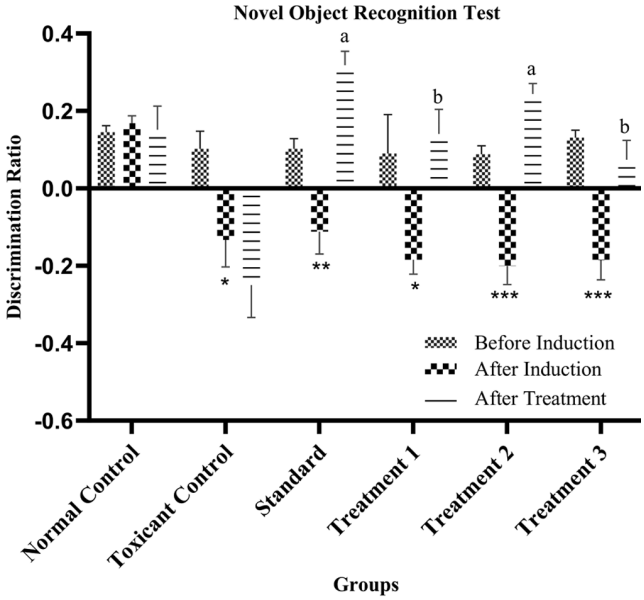

Fig. 4 — Effect of MAD on discrimination ratio in alzhemeric rats.

administration of AlCl3 for a period of 4 weeks, the rats performed very poorly as they were unable to significantly (P<0.001) distinguish between the familiar and novel object as compared to results of before AlCl3 administration indicating induction of Alzheimer's disease (Fig. 4). MAD given for a period of 4 weeks (treatment 1) and 6 weeks (treatment 2) to the alzheimeric rats led to significant increase (P<0.001) in the ability of rats to distinguish between familiar and novel objects in NORT. The animals of

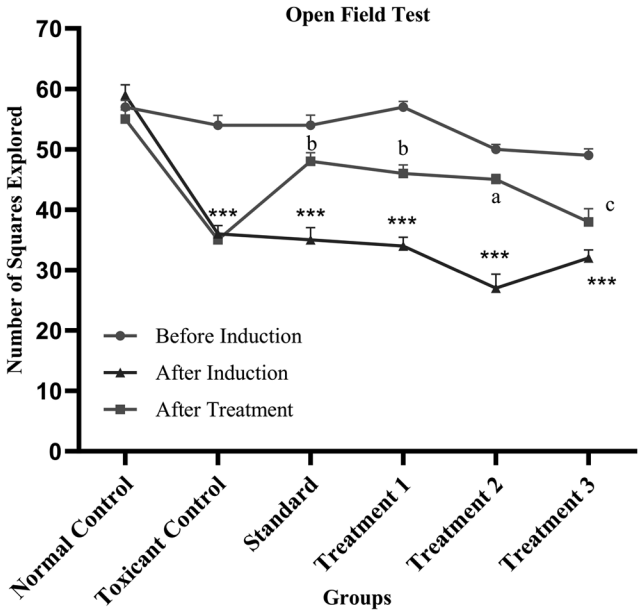

Fig. 5 — Effect of MAD on number of squares explored in alzhemeric rats.

treatment group 3 *i.e*. 4 weeks MAD followed by 2 weeks without any treatment showed moderately significant (P<0.01) enhancement in the ability of animals to discriminate between familiar and novel object compared to before treatment.

Open field test

Locomotor activities of rats were studied through OFT. As shown in Fig. 5, significant (P<0.001) reduction in locomotion was noted in AlCl3-treated rats indicating impairment of locomotor activity. However, treatment of these alzheimeric rats with MAD for a period of 6 weeks caused a most significant (P<0.001) increase in locomotor activity in treatment group 2 followed by moderately significant increase (P<0.01) in locomotor activity after 4 weeks of MAD treatment (treatment 1) and standard drug.

Effect of MAD on body weight and BMI in alzheimeric rats

As the present intervention study is related to diet (MAD), it is vital to monitor body weight and body mass index, as they are the indicators of body fat. The effect of MAD on the body weight and BMI has been depicted in Table 2. Administration of AlCl3 does not have any effect on the body weight and BMI of rats. Results have clearly indicated that treatment of alzheimeric rats MAD for 4 weeks followed by 2 weeks without any treatment (treatment 3) have resulted moderately significant (P<0.01) increase in body weight and BMI in alzheimeric rats as compared with the result of day 0. Treatment ofalzheimeric rats

Table 2 — Effect of MAD on body weight and body mass index (BMI) of alzhemeric rats

Groups	Body weight (grams)			Body Mass Index (Kg/m^2)		
	0 Day	14 Day	28 Day	0 Day	14 Day	28 Day
Normal control	223.33±4.21	231.66±4.77	235±4.28	1.66±0.04	1.70±0.03	1.75±0.06
Toxicant control	208.33±4.77	207±4.28	200.33±4.77^{b}	1.72±0.08	1.74±0.05	1.70±0.05^{c}
Standard group	210.33±3.33	219.33±3.33	221.33±3.07*	1.69±0.05	1.69±0.05	1.72±0.05*
Treatment 1	208.33±4.01	214.66±4.77	220.66±4.77*	1.72±0.04	1.75±0.07	1.80±0.05*
Treatment 2	210±2.58	217.96±1.66	223±2.58*	1.64±0.02	1.69±0.05	1.74±0.04*
Treatment 3	200±3.41	209±4.01	220±4.94**	1.63±0.04	1.67±0.07	1.74±0.08**

[*P* values: **P*<0.05, ***P*<0.01 when results of day 28 were compared with results of day 0 of each group. *P* values: ^{c}P<0.05, ^{b}P<0.01 when the results of toxicant control group were compared with normal control on day 28.]

Table 3 — Effect of MAD on fasting blood sugar (FBS) level of alzhemeric rats

Groups	Fasting Blood Glucose (mg/dL)	
	Before treatment	After treatment
Normal control	95.2±0.41	96.1±0.57
Toxicant control	102±0.73	100±0.79
Standard group	98.4±0.52	104.54±0.35
Treatment 1	99.25±1.33	103.7±0.22
Treatment 2	94.60±1.29	98.08±0.69
Treatment 3	95.35±1.25	120.8±0.80*

[*P* values: **P*<0.05 when results of After treatment were compared with results of Before treatment]

with donepezil, MAD for 4 weeks (treatment 1) and 6 weeks (treatment 2) have resulted in less significant (*P*<0.05) increase in body weight and BMI when results of day 28 were compared with day 0.

Effect of MAD on fasting blood sugar level in alzheimeric rats

The effect of MAD on the fasting blood sugar level has been depicted in Table 3. Result indicates that AlCl3 does not have any significant effect on fasting blood sugar level. Administration of MAD for 4 weeks followed by 2 weeks without any treatment (treatment 3) has resulted in less significant (*P*<0.05) increase in FBS level in alzheimeric rats as compared with before treatment. However, treatment of alzheimeric rats with donepezil, MAD for 4 weeks (treatment 1) and 6 weeks (treatment 2) does not have any effect on FBS level in alzheimeric rats.

Effect of MAD on lipid profile and atherogenic index in alzheimeric rats

Evaluation of lipid profile is an important diagnostic tool for various cardiovascular diseases and several co-morbidities. The effect of MAD on lipid profile of alzheimeric rats has been summarized in Table 4. It is evident from the results that administration of AlCl3 for 28 days caused significant increase in triglyceride, total cholesterol, LDL, VLDL and AI levels and decrease in serum HDL level. MAD treatment given to the alzheimeric rats for a period of 4 weeks (treatment 1) and standard drug caused moderately significant (*P*<0.01) decrease in the level of triglyceride and LDL level as compared to the results of toxicant control. MAD treatment given to the alzheimeric rats for a period of 4 weeks (treatment 1) and standard drug caused mildly significant decrease (*P*<0.05) in total cholesterol and VLDLs level as compared to the results of toxicant control. Treatment 1 and standard drug also increased the level of HDL (43.96±0.98 mg/dL and 37.36±1.47 mg/dL respectively) compared to toxicant control (11.37±0.84 mg/dL). Treatment 1 and standard drug significantly (*P*<0.001) decreased the AI level in alzheimeric rats as compared the results of toxicant control (Fig. 6). Treatment 2 also caused mild to moderately significant (*P*<0.01) improvement in all the parameters of lipid profile. However, treatment 2 most significantly (*P*<0.001) improved the HDL level and significantly (*P*<0.001) decreased AI level in alzheimeric rats as compared to results of toxicant control. Treatment 3 has no significant effect on serum total cholesterol and VLDL level while it causes mildly significant improvement (*P*<0.05) in the level of triglyceride, HDL and LDL and moderately decreased (*P*<0.01) the level of AI in alzheimeric rats.

Effect of MAD on serum total protein and serum calcium levels in alzheimeric rats

The effect of treatment on the level of serum total protein and calcium have been summarized in Table 5. Aluminium chloride caused mildly significant increase (*P*<0.05) in the level of serum total protein and calcium. Alzheimeric rats treated with MAD for 4 (treatment 1) and 6 weeks (treatment 2) showed significant normalization (*P*<0.01) in the values of total protein and calcium as compared to values before treatment. Standard drug donepezil caused most significant decrease in the

Table 4 — Effect of MAD on lipid profile of alzhemeric rat

Groups	Triglyceride (mg/dL)	Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL(mg/dL)
Normal control	80.50±0.61	101.99±1.03	46.12±1.13	49.77±1.78	16.10±0.12
Toxicant control	104.89±1.69^{b}	135.18±2.15^{b}	11.37±0.84^{b}	94.89±4.91^{a}	22.97±0.33^{a}
Standard group	68.91±1.18**	109.81±1.96*	37.36±1.47**	58.66±1.69**	13.78±0.23*
Treatment 1	75.94±1.67**	111.59±2.33*	43.96±0.98*	52.45±2.13**	15.18±0.33*
Treatment 2	72.28±0.79*	109.45±1.64*	47.16±1.13***	47.82±1.38	14.45±0.15**
Treatment 3	79.05±1.45*	117.21±2.90	39.89±1.71*	61.51±4.07*	15.81±0.29

[*P* values: **P*<0.05, ***P*<0.01, ****P*<0.001 when results of each group were compared with toxicant control group. *P* values: ^{b}*P*<0.01, ^{a}*P*<0.001 when the results of toxicant control group were compared with normal control]

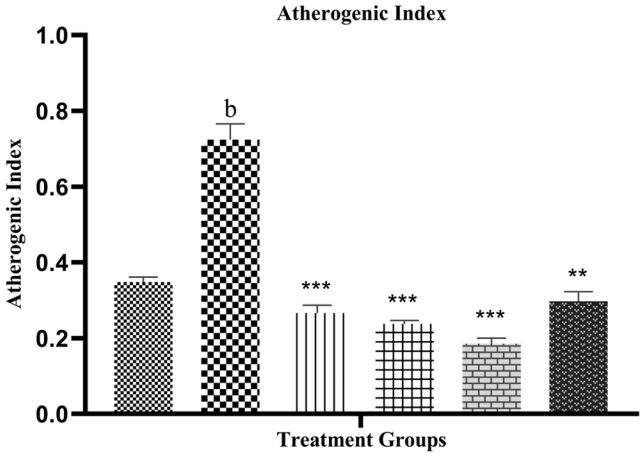

Fig. 6 — Effect of MAD on atherogenic index in alzhemeric rats.

level of total protein (7.27±0.35 g/dL) as compared to results of before treatment (13.44±0.52 g/dL). Animals which received treatment 3 showed moderately significant improvement (*P*<0.01) and mildly significant improvement (*P*<0.05) in total protein and serum calcium levels respectively as compared to before treatment.

Effect of MAD on the level of marker enzymes in alzheimeric rats

Table 6 indicates the effect of MAD on LDH and AChE level in alzheimeric rats. Result shows that AlCl3 given for a period of 4 weeks caused significant increase in the value of LDH (289.47±3.14 U/L)) as compared to normal control animals (235.81±1.58 U/L)). Standard drug given to the alzheimeric rats significantly (*P*<0.001) decreased LDH level as compared to toxicant control. However, treatment 1 (MAD for 4 weeks), treatment 2 (MAD for 6 weeks) and treatment 3 (MAD for 6 weeks followed by 2 weeks without any treatment) moderately decreased (*P*<0.01) the level of LDH as compared to toxicant control. AChE enzyme is responsible for degradation of ACh and plays a very important role in learning and memory. In the present study, AlCl3 caused significant increase (*P*<0.001)

Table 5 — Effect of MAD on serum total protein and serum calcium of alzhemeric rats

Groups	Total Protein (g/dL)		Calcium (mg/dL)	
	Before treatment	After treatment	Before treatment	After treatment
Normal control	8.18±0.61	8.32±0.76	9.87±0.84	9.43±0.48
Toxicant control	10.26±0.73	14.24±0.79*	17.04±0.73	16.18±0.46
Standard group	13.44±0.52	7.27±0.35**	16.47±0.53	11.68±0.53***
Treatment 1	13.72±1.33	8.04±0.22**	16.30±0.95	12.19±1.07**
Treatment 2	14.61±1.29	7.97±0.69**	17.11±1.13	12±0.91**
Treatment 3	15.12±1.25	8.89±0.80**	17.75±0.72	13.40±0.40*

[*P* values: **P*<0.05, ***P*<0.01, ****P*<0.001 when results of After treatment were compared with results of Before treatment]

Table 6 — Effect of MAD on LDH and AChE of alzhemeric rats

Groups	Lactate dehydrogenase (U/L)	Acetylcholinesterase (µmol/min in per gm tissue)
Normal control	235.81±1.58	15.91±0.87
Toxicant control	289.47±3.14^{a}	29.66±0.75^{a}
Standard group	210.83±2.38***	12.93±0.62***
Treatment 1	253.26±1.35**	18.61±0.51**
Treatment 2	242.73±1.91**	17.02±0.42**
Treatment 3	259.06±1.58**	25.31±0.61

[*P* values: ***P*<0.01, ****P*<0.001 when results of each group were compared with results of toxicant control. *P* values: ^{a}*P*<0.001 when results of toxicant control were compared with results of normal control]

the level of AChE as compared to normal animals. Donepezil (Standard group) and MAD given to alzheimeric rats for a period of 4 weeks (treatment 1) caused significant (*P*<0.001) and moderately significant (*P*<0.01) decrease in AChE level in alzheimeric rats respectively as compared to toxicant control. Treatment 2 also restored the value of AChE to normal (17.02±0.42 µmol/min in per g tissue) as compared to toxicant control (29.66±0.75 µmol/min in per g tissue).

Effect of MAD on nitrosative/oxidative stress level in alzheimeric rats

Reactive oxygen species and the resulting oxidative stress play a crucial role in pathology of AD, Parkinson's disease and other neurodegenerative

diseases. Table 7 indicates that administration of AlCl3 significantly increased (P<0.001) the level of TBARS and nitrate and significantly decreased (P<0.001) the level of antioxidant enzymes (GSH, SOD, GPx and GR) as compared to normal control group. Administration of MAD (treatment 1) and standard drug for 4 weeks to the alzheimeric rats significantly decreased (P<0.001) the level of TBARS and nitrate along with the significant increase (P<0.001) in the level of GSH, GPx, GR and SOD as compared to toxicant control. Treatment 2 (MAD for 6 weeks) and treatment 3 (MAD for 6 weeks followed by 2 weeks without treatment) also decreased the level of TBARS and nitrate significantly (P<0.001) along with increase (P<0.001) in the level of GSH, GPx, GR and SOD as compared to toxicant control.

Effect of MAD on the histopathology of brain in alzheimeric rats

Fig. 7 depicts the effect of MAD on the histology of brain cells of rats. Fig. 7A reveal a normal histo-architecture of hippocampus region of rat brains showing occasional neurofibrillary tangles and plaque-like deposition along with occasional microcyst changes in normal control group of animals. Fig. 7B shows the brain tissues of toxicant control with prominent neurofibrillary tangles and significant plaque like deposition possibly neuritic plaques in the hippocampus region. Fig. 7C shows the brain tissues of standard group with very mild neurofibrillary tangles and very few plaques like deposition in the hippocampus region. Fig. 7D shows the brain tissues of treatment 1 group with moderately neurofibrillary tangles and moderate plaque-like deposition in the hippocampus region. Fig. 7E shows the brain tissues of treatment 2 group with mildly prominent neurofibrillary tangles and minimal plaque-like deposition. Reactive glial cells are also seen along with minimal chronic inflammatory infiltrate, a few congestive blood vessels and minimal edema. Fig. 7F shows the brain tissues of treatment group 3 with comparatively more neurofibrillary tangles and plaque-like deposition in the hippocampus region.

Discussion

In the present study, the modulatory role of MAD in AlCl3-induced cognitive, behavioural, and neurochemical changes in rats was evaluated to

Table 7 — Effect of MAD on oxidative stress parameters of alzhemeric rats

Groups	LPO (mol/L)	GSH(µg/mL)	SOD(IU/L)	Nitrite/Nitrate(µmol/L)	GP_x(U/g)	GR(U/g)
Normal control	49.25±1.03	103.05±1.31	97.72±0.81	59.75±0.45	10.57± 0.49	220.12± 0.70
Toxicant control	131.90±0.82[a]	52.29±0.82[a]	45.63±0.83[a]	121.09±0.33[a]	5.03±0.68[a]	102.7±1.52[a]
Standard group	55.27±1.79***	93.50±1.58***	91.91±0.76***	64.26±1.04***	9.17±1.14***	205.11±0.74***
Treatment 1	61.64±1.28***	72.62±0.89***	80.24±0.88***	83.81±0.48***	7.49±0.53**	180.27±0.97***
Treatment 2	69.84±1.00***	74.15±0.84***	81.58±1.10***	77.22±0.31***	8.95±1.01***	196.1±0.44***
Treatment 3	85.18±1.68***	68.33±1.03***	73.84±0.69***	91.37±0.74***	6.63±0.36	171.29±1.12***

[P values: **P<0.01, ***P<0.001 when results of each group were compared with results of toxicant control. P values: [a]P<0.001 when results of toxicant control were compared with results of normal control]

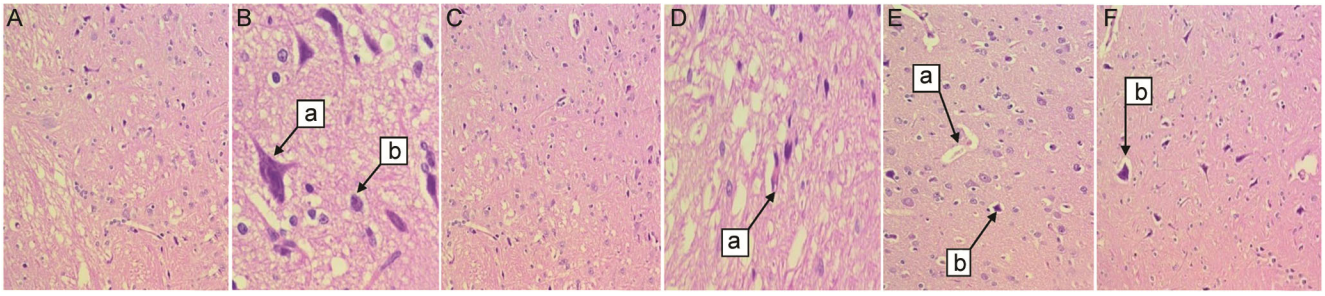

Fig. 7 — Effect of MAD on histopathology of brain in alzhemeric rats. (A) shows brain tissues of normal control group with occasional neurofibrillary tangles and plaque like deposition along with occasional microcyst changes in normal control group of animals. (B) shows the brain tissues of toxicant control with prominent neurofibrillary tangles and significant plaque like deposition possibly neuritic plaques in the hippocampus region. (C) shows the brain tissues of standard group with very mild neurofibrillary tangles and very few plaques like deposition in the hippocampus region. (D) shows the brain tissues of treatment 1 group with moderately neurofibrillary tangles and moderate plaque like deposition in the hippocampus region. (E) shows the brain tissues of treatment 2 group with mildly prominent neurofibrillary tangles and minimal plaque like deposition. (F) shows the brain tissues of treatment group 3 with comparatively more neurofibrillary tangles and plaque like deposition in the hippocampus region.[H&E, ×100]

confirm our hypothesis. MWM, NORT, and OFT were used to assess behavioural changes and cognition, followed by estimation of biochemical, neurochemical, oxidative stress and histological studies. In this study, treatment 1 (MAD supplementation for 4 weeks), treatment 2 (MAD supplementation for 6 weeks) and treatment 3 (MAD for 4 weeks followed by 2 weeks of without treatment) were used to investigate the effect of durations of MAD treatment on AlCl3 induced cognitive, behavioural and neurochemical impairment in rats. When comparing the effects of different treatment durations of MAD, it is clear that although treatment for 6 weeks with MAD is more effective due to better outcomes in studies using animal models and biochemical parameters; treatment for 4 weeks with MAD has also produced outcomes that are almost on par with those of treatment for 6 weeks. Treatment 3 (MAD for 4 weeks + 2 weeks without any treatment) has shown slight improvement in various biochemical, neurochemical, oxidative and tissue parameters in alzheimeric rats thereby confirming relapse phenomena that generally occur with diet intervention studies. The outcomes of behavioural, biochemical and histological investigations validate our research hypothesis.

Aluminium is a well-established neurotoxicant involved in the etiology of AD. It is an abundant metal on earth that easily accessible to the human body through antacids, water, food additives, utensils, deodorants and drugs. In the brain, aluminium predominantly accumulates in the hippocampus and frontal cortex, regions known to be particularly susceptible in AD. It induces misfolding of cytoskeleton proteins which leads to the formation of amyloid beta plaques and tau neurofibrillary tangles in the brain. Aluminium supplementation causes neurodegeneration and apoptotic neuronal loss along with cognitive dysfunction, as it is a potent cholinotoxin[32]. Previous literature indicates that AlCl3 also impairs cognitive processes by reducing glucose absorption and metabolism[33]. MAD, investigated in the present study, has already shown its potential in diseases like drug-resistant epilepsy, cancer, *etc*. Neurobiological evidence suggests that ketone bodies released by the metabolism of MAD can be an effective alternative energy substrate for the brain and may improve cognition and memory in Alzheimer's disease rats[6].

The findings of animal model investigations (MWM, OFT, and NORT) of our study indicate that MAD treatment significantly restored memory (both spatial and working memory) and cognition in treated animals. MAD treatment improved energy metabolism, reduced ROS levels and significantly boosted cognition and memory retention. The cognitive decline in AD rats was associated with impaired glucose metabolism, while ketone bodies (β-hydroxybutyrate and acetoacetate) from MAD metabolism may have contributed to cognitive improvements by serving as an efficient brain energy source, supporting enhanced activity in AD rats[27,33]. In the present study, MAD treatment for 4 and 6 weeks and MAD for 4 weeks followed by 2 weeks without any treatment led to slight increase in body weight and increase in BMI in alzheimeric rats. It could be due to the fact that MAD provides excessive energy that is not fully utilized by the animal and consequently accumulates up in the adipocytes leading to adipocyte hypertrophy and hyperplasia. This primarily increases the white adipose tissue mass and thus showing increase in weight of the animals along with increase in BMI[34]. Further the *ad libitum* availability of the MAD may be a contributing factor for body weight and BMI increment. However, the treatment does not have any deleterious effect on the level of fasting blood sugar in alzheimeric rats. In AD, cognitive impairment is accompanied by decreased glucose absorption and metabolism. However, it can be assumed that alzheimeric rats treated with MAD may have experienced an enhancement in cognition and memory as a result of production of ketone bodies (β-hydroxybutyrate and acetoacetate) from the metabolism of MAD which acts as an alternative source of brain energy, as shown by neurobiological research[6,35].

In the present study, MAD treatment 1 and 2 has resulted in increased HDL and decreased TG, LDL, VLDL, total cholesterol, and atherogenic index in alzheimeric rats. According to earlier research, MAD treatment was known to reduce HMG-CoA reductase activity, which in turn inhibits biosynthetic pathways and, ultimately, lowers total cholesterol and triglyceride levels[36,37]. Due to variations in MAD composition, several studies have found contradictory results regarding lipid profiles; as a result, the content of the diet is crucial in determining how MAD affects lipid profiles. In the present study, MAD was prepared using premium ketogenic fat powder and olive oil in a predetermined ratio, which may have resulted in a favourable effect on lipid profile. MAD

associated increase in fatty acid levels, uncoupling proteins (UCP), and mitochondrial biogenesis may be responsible for its favourable effects on lipid profile and atherogenic index[36].

In the present study, MAD treatments caused a significant decrease in serum total protein and calcium levels in the alzheimeric rats. It can be hypothesized that as MAD reduces insulin signaling and mimic starvation, thereby increases fatty acid oxidation, decreases insulin/IGF-like signaling and promotes a catabolic state by promoting protein degradation, which may lead to the clearing of degradation-sensitive proteins, such as amyloidal peptides[38]. According to research done in the past, the difference between the MAD and the normal diet in terms of macronutrients may also be a reason why serum calcium levels decline in MAD-treated rats which may ultimately lead to a decrease in mineral bone density and deficiencies in vitamin and mineral components[7].

In AD, hypometabolism as a result of impaired glucose transport in the brain and metabolic abnormalities contributes to an elevated LDH level. Neuronal excitation in the brain is governed by energy consumption and can be slowed down by specialised diets like MAD. Thus, inhibition of LDH by MAD, as demonstrated in the present study, suggests a reduction in the level of neurotoxicity in rats with AlCl3-induced AD. Additionally, MAD reduces glucose levels, increasing ATP oxidative phosphorylation and boosting energy reserves. As a result, the LDH, a marker of impaired brain function, shows a significant improvement and the brain is able to get an appropriate supply of ketone bodies, which act as fuel for brain function. Neurons and glia consume more than 99 percent of the ketone bodies that pass through the plasma membrane and enter the cell[5]. It has been found, however, that raising plasma ketones *via* nutritional supplementation while maintaining safe and moderate level of ketonemia enhances the relative impact of ketones to the brain's fuel supply[39]. It is well known that cognitive deficits in Alzheimer's disease are related with cholinergic neuronal system and decline in the ACh level leads to memory and cognitive disturbances[40]. Dearth of enzyme choline acetyltransferase (ChAT), which is critical for acetylcholine production, was linked to impairments in presynaptic neurotransmission in the neocortical cortex and presynaptic neurons causing decline in memory and learning. An increased level of AChE, as found in this study, indicates decrease in the levels of ACh and cognitive impairment. Treatment with the MAD restored the level of AChE and thereby increased the ACh level in alzheimeric rats[41].

It is well known that oxidative stress and mitochondrial dysfunction both significantly contribute to neurodegeneration. In the present study AlCl3 caused significant increase in the level of oxidative and nitrosative stress in the animals indicating neurodegeneration. Treatment of these alzheimeric rats with MAD for 4 weeks and 6 weeks significantly decreased the level of oxidative and nitrosative stress and, increased the level of antioxidant enzymes indicating that MAD provides neuroprotective benefit by its antioxidant property and by improving mitochondrial function through biochemical changes resulting from glycolysis inhibition and increased ketone bodies (KBs) formation[6]. Results from previous literature indicate that metabolic ketosis can increase mitochondrial energy reserves while decreasing production of reactive oxygen species[42]. The antioxidant property of MAD due to increased levels of PUFA may led to increases in $PPAR_{\alpha}$ and uncoupling protein levels which subsequently improves mitochondrial biogenesis and in turn decreases ROS. Moreover, MAD may modulate the ratio between the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD^+/NADH). An increased NAD^+/NADH ratio plays a role in protection against ROS and improves redox reactions, mitochondrial biogenesis, and cellular respiration, which stabilizes synaptic action. MAD treatment also increased the level of superoxide dismutase 2 and other oxidant enzymes as found in the present study and reported in earlier studies indicating antioxidant activity of MAD[6]. AlCl3 administration in rats adversely affected the histo-architecture of rat brain tissue. Accumulation of neurofibrillary tangles and neuritic plaques due to increased ROS resulting from the mitochondrial dysfunction was evident from the histopathology of the AlCl3 treated rats. Decrease in the level of neurofibrillary tangles and neuritic plaques along with improved morphology of neurons by MAD indicate its neuroprotective effect in AD.

Conclusion

The conclusion that can be drawn from the present study is that MAD has protective effect against AlCl3-induced AD in rats and its co-morbidities. The

study shows that MAD improves cognitive, memory, and behavioural impairment, aberrant lipid profile, marker enzyme levels, oxidative stress, and brain histology in alzheimeric rats. On comparing effects of MAD, it is evident that MAD treatment for 6 weeks is more effective as it has shown better results in animal model studies, biochemical parameters and marker enzyme levels, while MAD treatment for 4 weeks has shown comparable results to 6 weeks treatment. Further the effective nature of MAD treatment for 6 weeks (treatment 2) has been confirmed by the decreased oxidative stress level in brain tissues, and histopathology analysis of rat's brain. However, effective and safe time duration of MAD treatment producing optimum results needs to be established by further studies. Future studies should target the key component responsible for MAD's protective effect against AD. Also, it can be investigated whether MAD only has curative potential or can otherwise show preventive effect against development of AD.

Ethical statement

The guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) of Govt. of India were followed and the experimental protocol was approved by Institutional Animal Ethical Committee with the approval number of CPCSEA/IAEC/SBS/2022/01.

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Conflict of interest

The authors have no conflict of interest to declare.

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