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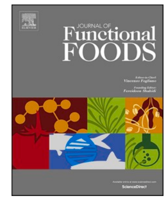

Edible dairy formula fortified with coconut oil for neuroprotection against aluminium chloride-induced Alzheimer's disease in rats

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ABSTRACT

This study investigates the potential protective effects of a dairy formula fortified with virgin coconut oil (VCO) against aluminium chloride ($AlCl_3$)-induced Alzheimer. Forty-two *Wistar* rats were allocated into seven groups which received the fortified formula, VCO, the standard drug (rivastigmine), $AlCl_3$ and a combination of these variants with $AlCl_3$. Different chemical, biochemical, behavioral and histopathological and immunohistochemical evaluations were conducted in this study. Results showed that VCO contains 61.0% medium chain triglycerides, 49.8 mg eq./kg total phenolic compounds and its antioxidant activity (DDPH, IC_{50}) was 53.7 µg/ml. Compared to $AlCl_3$ treated rats, the fortified dairy formula improved the cognitive abilities, increased the serum ketone levels, reduced lipid peroxidation, reduced matrix metalloproteinase (2 and 9) enzyme activity, reversed histological alterations in brain and activate immunohistochemical Nrf2/HO-1 signaling cascade. Overall, the results showed that the developed dairy formula fortified with VCO can induce beneficial effects for cognitive deficits associated with Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is an age related progressive neurodegenerative condition characterized by the presence of intracellular amyloid aggregates and extracellular neurofibrillary tangles (Jack et al., 2018). The earliest stage in AD is characterized mainly by short term memory loss and with the progress of the disease other symptoms arise like confusion, aggression, mood changes, long term memory loss and social withdrawal (Waldemar et al., 2007).

AD was linked to ingestion of some metals like aluminium (Al) which is introduced to the body through occupational exposure (Exley & Vickers, 2014) or ingestion of traces of the metal from drinking water or food cooked in Al utensil (Brian, Stephens, & Jolliff, 2015). This metal can alter the blood brain barrier and ultimately gets accumulated in the brain (Mirza, King, Troakes, & Exley, 2017). Therefore, it is considered as risk factor in neurological disorders (Inan-Eroglu & Ayaz, 2018) and brain Al intoxication (Exley & Mold, 2019). In addition, Al can also inhibit the activities of antioxidant enzyme, altering brain neurochemistry and cause oxidative damage to brain DNA (Liaquat et al., 2019).

Inflammation is thought to be one of the significant reasons in the AD pathogenesis. Therefore, it could be managed through consumption of some natural dietary supplements that have anti-inflammatory properties which can prevent or delay the progression of AD (Muñoz Fernández & Lima Ribeiro, 2018; Szczechowiak, Diniz, & Leszek, 2019). For instance, an early study showed that curcumin, which is a natural plant extract from the rhizomes of turmeric can attenuates Al-induced oxidative stress and mitochondrial dysfunction in rat brain (Sood, Nahar, & Nehru, 2011). The potentials of curcumin to reverse the neurological damage can be significantly increased by formulating this plant extract in solid lipid nanoparticles (Kakkar & Kaur, 2011). More recently, antioxidants from natural resources have been shown to possess anti-Alzheimer activity due to their phenolic content (Taslimi et al., 2020).

Virgin coconut oil (VCO) is among the natural plant extracts that was

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investigated for protection against or suppression of symptoms of AD. The oil showed ability to stop brain cells death and improve cognitive performance in Alzheimer's patients with different possible mechanisms of action (De la Rubia et al., 2017). VCO was found to improve hippocampus histological changes and reduce Aβ plaques in rats' brain (Mirzaei, Khazaei, Komaki, Amiri, & Jalili, 2018). Aβ is the main component of amyloid plaques which is an extracellular deposit found in the brains of patients with AD (Murphy & LeVine, 2010). It can significantly impair the memory and learning via the activation of inflammasome and oxidative stress. The study of Mirzaei et al. (2018) indicated also that VCO can reduce phosphorylated Tau protein which are aggregates found in several neurodegenerative tauopathies including AD (Noble, Hanger, Miller, & Lovestone, 2013). Longer term treatment with VCO can block the negative effects of Aβ on signaling intermediates such as AMPK, Akt, GSK3b and ERK (Nafar, Clarke, & Mearow, 2017). The same study also indicated that shorter term treatment with the oil leads to activation of Akt and ERK and inhibits the effect of Aβ exposure. Therefore, VCO can also be used as an adjuvant supplementary therapy to alleviate the severity of AD (Gandotra, Kour, & Van der Waag, 2014).

Therefore, based on the versatility of VCO in alleviating AD, the current study was dedicated toward incorporation of that oil in a dairy formula which is used as a convenient vehicle for delivering the oil to different sectors of consumers especially AD's patients. That convenience originates from the fact that oral administration of the pure oil at the minimum effective dose (10 g/person/day, Fife, 2005) can be uncomfortable and can cause the "feeling sick" experience to the consumer. However, incorporation of the oil into a dairy formula like pudding is expected to eliminate that uncomfortable feeling and make the formula appealing particularly for senior citizens, who mostly need VCO as brain booster.

In the current study, we used aluminium chloride ($AlCl_3$) as an inducer for neurological damage in rats and the effect of dairy formula fortified with VCO on amelioration of such damage was studied. Different chemical, biochemical, behavioral, histological and immunohistochemical parameters were also evaluated to understand the underlying mechanisms that contribute to the protective effect of fortified dairy formula against AD.

2. Material and method

2.1. Materials

Fresh skimmed milk was obtained from the Animal Production Research Institute, Cairo, Egypt. Its chemical composition was: protein 4.3%, lactose 4.8%, fat 0.1% and ash 0.87%. VCO was obtained from the National Research Centre, Egypt. Glycerol monostearate and kappa-carrageenan were obtained from Dansco, Denmark. Aluminium chloride ($AlCl_3$) and fatty acids methyl esters of different chain lengths were obtained from Sigma-Aldrich Co., (St. Louis, USA). Rivastigmine (Exelon) which is used as a standard Anti-dementia drug was purchased from Novartis Co. (Cairo, Egypt).

2.2. Methods

2.2.1. Extraction of VCO

Coconut oil (VCO) consumed in our study is obtained from the "Oil seeds Expression Unit" at the National Research Center, as indicated in the materials section. The oil was extracted by the cold hydraulic expression of the shredded flesh of the fruit of coconut palm (Cocos nucifera) that were exported from Malaysia. The extract was filtered through a cheese close, then left to stand overnight. The oil was separated from the fine sediments by decantation followed by storing at 4.0 °C till used.

2.2.2. Determination of the total phenolic content (TPC) of VCO

Total phenolic content was determined using Folin-Ciocalteu method according to the method described by Gutfinger (1981). In details, VCO (2.5 g) was dissolved in 5.0 ml hexane and extraction was carried out by methanol/water solution (80:20, v/v). The aqueous phase was collected by centrifugation at 3500 rpm for 5 min, followed by vacuum drying at room temperature. The dried sample was dehydrated in 5.0 ml of the methanol solution, mixed with 2.5 ml of Folin reagent and 10.0 ml of sodium carbonate solution in 50 ml volumetric flask. The absorbance was measured at 765 nm after 30 min. Gallic acid was used for calibration and the results were expressed as mg gallic acid equivalent (GAE) per 100 g of VCO.

2.2.3. Determination of the antioxidant activity of VCO

The antioxidant activities of VCO as well as standard sample of ascorbic acid (as a reference) were evaluated by using 1,1-diphenyl-2-picrylhydrazyl (DPPH˙) radical assay as reported originally by Blois (1958). Two ml of 0.1 mM DPPH˙ of the methanolic solution was added into 20, 40, 60, 80 and 100 μg of the extracts and then 1.0 ml methanol was added. The mixture was thoroughly mixed and kept in a dark place for 30 min. The control was prepared by mixing 1.5 ml of DPPH and 1.0 ml methanol. The absorbance of the mixture was recorded at 517 nm using spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan). The antioxidant activity of the extracts expressed as IC_{50} values, which is calculated from the inhibition percent versus concentration plot. The IC_{50} value indicates concentration (in μg ml^{-1}) of the extract, which is required to scavenge 50.0% of DPPH˙ free radicals.

2.2.4. Manufacture of the dairy formula fortified with VCO

Different batches of fresh buffalo skimmed milk were pasteurized at 72 °C for 15 s, then cooled to 42 °C and used as a base for manufacture of a pudding-like dairy formula containing VCO. The oil was emulsified in the previously pasteurized milk (at 20.0 wt%) using glycerol monostearate as a food-permitted emulsifier (at 4.0 wt%). In addition, *kappa*-carrageenan was used as a thickening agent at different concentrations (0.075%, 0.15% and 0.225%) in order to adjust the viscosity of the dairy formula. Mixing and emulsifying of all the previously mentioned ingredients was conducted using an electric mixer (Brown, Germany) operated at its highest speed. The temperature throughout the whole process was controlled to be in the range of 27 °C ± 1 in order to be far from the solidification point of VCO (at 24 °C). Finally, the dairy formula fortified with VCO was poured into plastic cups (50 ml capacity), caped, and left to cool at room temperature, then transferred into the cooler at 4 °C until used in the oral dosing of rats at the next day.

2.2.5. Analysis of the fatty acid composition of VCO

Virgin coconut oil (VCO) was first derivatized into its corresponding fatty acid methyl esters via trans-esterification process using 3.0% methanol/sulfuric acid mixture according to A.O.A.C. (2000). This process transforms the non-volatile fatty acids of VCO into their corresponding volatile methyl esters which are suitable for gas chromatographic analysis.

After that, 2.0 μl of the derivatized fatty acid methyl ester of VCO were injected (at a split ratio 10:1) into Hewlett Packard HP 6890 gas chromatograph equipped with a flame ionization detector. A 30 m × 0.32 mm i.d. fused silica capillary column coated with DB-5 was used to separate the different methyl esters. The oven temperature was programmed from 150 °C to 240 °C at rate 2.5 °C/min with a 30 min hold at the final temperature. The injector and detector temperatures were 230 °C and 250 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. The fatty acids methyl esters were identified by comparing their retention time with authentic standards. Values of area % of each fatty acid methyl ester were taken as a measure of the abundance of the fatty acid in VCO. The values are mean of two injections from two different analysis ± SD.

2.2.6. Chemical analysis of the dairy formula fortified with VCO

Fat, total solids, total nitrogen, and ash content of the dairy formula fortified with VCO were determined according to A.O.A.C. (2012).

2.2.7. Viscosity of the dairy formula fortified with VCO

The viscosity was measured at room temperature using a Brookfield digital viscometer (Middleboro, MA 02346, U.S.A). The sample was subjected to shear rates ranging from 0.5 to 100 S^{-4} for an upward curve. Viscosity measurements were expressed as centipoise (cP.s) and were performed in triplicate (Salama, Abdelhamid, & Dairouty, 2019).

2.3. Biological evaluations

2.3.1. Animals

Male albino *Wistar* rats weighing 150–200 g were used in the present study. The animals were obtained from private animal house, Cairo, Egypt. They were maintained under controlled conditions of temperature (25 ± 2 °C), humidity (50 ± 5%) and 12 h light/dark cycle. All the animals were acclimatized for seven days before the study, and provided with commercial balanced diet (22.75% protein, 4.63% fats, and 5.35% fibers) and water *ad libitum* throughout the experimental period.

2.3.2. Study design

Forty-two adult male *Wistar* rats were randomly allocated into seven groups of seven rats in each group as follows:

Group I: Serve as the negative control group. Rats in this group were given distilled water orally, daily for 60 days.

Group II: VCO treated group. The rats in this group were administered VCO (1.42 ml/kg b.wt.) daily for 60 days.

Group III: Dairy formula treated group. The rats in this group were administered a dairy formula fortified with equivalent dose of VCO equal to that in group II, daily for 60 days.

Group IV: $AlCl_3$ treated group. The rats were administered $AlCl_3$ orally (100 mg/kg/b.wt., according to Bitra, Rapaka, Mathala, and Akula (2014), daily for 60 days.

Group V: Rivastigmine + $AlCl_3$ treated group. The rats were given Rivastigmine orally (0.3 mg/kg b.wt., according to Lipp, Sharma, Banerjee, and Singh (2020) for 2 weeks followed by co-administration of $AlCl_3$ (100 mg/kg b.wt) for the rest of the 60 days.

Group VI: VCO + $AlCl_3$ treated group. The rats were given VCO for 2 week, followed by co-administration with $AlCl_3$ orally (100 mg/kg b.wt) for the rest of the 60 days.

Group VII: Dairy formula + $AlCl_3$ treated group. The rats were given the dairy formula fortified with VCO for 2 week, followed by co-administration with $AlCl_3$ orally (100 mg/Kg b.wt) for the rest of the 60 days.

During manufacture of the edible dairy formula; as we mentioned before; we used skimmed milk. So we didn't include a group of dairy formula (without VCO) + $AlCl_3$ in our experiment because no traces of medium chain triglyceride (MCT: C8 & C10) is expected to be in that formula. MCT are those fatty acids that are responsible for the improvement of cognitive deficits associated with AD. All groups were gavaged once daily and the dose was adjusted weekly according to their body weight.

Behavioural observation was conducted at the end of the experiment and continued for 10 days. Then; after the last behavioural test; blood samples were collected from the inner canthus of the eye and left to clot in a clear dry centrifuge tubes, then centrifuged at 3500 r.p.m for 15 min. The serum was frozen at −20 °C until subsequent analysis. Then, the rats were euthanized by decapitation and the brain were quickly dissected and fixed in 10% neutral buffered formalin saline for 24 h.

2.3.3. Measurement of rat's body weight

The body weight of rats in all groups was measured at the start of the experiment, and weekly until the end of the experimental period. Also, body weight change was calculated according to the equation (Final body weight of rats – Initial body weight of rats).

2.3.4. Behavioral observations

2.3.4.1. Y-maze test.. The test was conducted according to Wright, Lightner, Harman, Meijer, and Conrad (2006). Y-maze test is used to assess short term memory as well as motor activity (Kokkinidis, Walsh, Lahue, & Anisman, 1976). The Y-Maze is a wooden maze consist of three identical arms (120, 40 cm long and 35 cm height) labelled (A, B and C). The rat was placed at the end of one arm for 5 min and was video tracked using video-tracking system (Anymaze 4.20, Stoelting, USA). The number of arm entries and distance travelled by each animal were recorded. Also, spontaneous alternation behaviour; that is based on the ability of the animal to alternate three arms sequentially (Sarter, Bodewitz, & Stephens, 1988); was calculated as follows

Spontaneous alternation percentage (SAP) = Total alternations/ (Number of arm entries-2) × 100

The maze was cleaned after each rat with 70% alcohol, and fecal pellets were removed.

2.3.4.2. Modified elevated plus-maze test (mEPM). Spatial long-term memory was measured according to Hlinák and Krejcí (2000), simply it depend on the aversion of rats to the open space. We used a wooden maze comprised two open and enclosed arms (50 × 10 × 30 cm), and were connected by central platform. The maze was kept 60 cm above the ground. The rats were handled gently prior the test to decrease the stressful condition, furthermore, the experiment was conducted in a dimly lit semi sound proof room between 10.00 a.m to 12:00 p.m.

The procedure involved two phases 24 h apart, a first acquisition phase where the rat was placed at the end of the open arm and the time required for the rat to move to either of the enclosed arm was measured (transfer latency-1) (TL 1). After entering the enclosed arm, the rats were allowed to explore the entire maze for 30 s. If the rat remained in the open arm during the 90 sec without entering the closed one, the rats was gently pushed to enter the closed arm, allowed for 10 s to explore the arena, and the recorded time was 90 s. After the first acquisition phase, then comes the testing session in which transfer latency-2 (TL2) was measured when the animal reach one of the enclosed arms. The maximum time for the rat was 90 s. After each animal, the maze was cleaned with alcohol spray.

2.3.4.3. Novel object recognition test (NOR):. That evaluation considered the less stressful test for measuring hippocampus dependent memory impairment (Lueptow, 2017). The objects used in this test are two identical Dumbbells, (1.0 kg each), and plastic bottle filled with water. These objects were cleaned using 70% alcohol between sessions. The procedure involved 3 sessions with 24 h interval between each one.

The first session is a habituation phase in which rats are allowed freely to explore the arena (70 × 70 × 35 cm) for 5 min. The second session is an acquisition phase in which rats were allowed to explore two identical objects placed in the arena for 5 min. The objects were 6 cm away from the walls of the arena in opposite corners. The third session is a recall session in which rats were allowed to explore the arena with one familiar abject and a new one for 5 min. Rats were tracked automatically using video-tracking system (Anymaze 4.20, Stoelting, USA), and all exploratory actions were measured automatically and manually to ensure the reliability of the automatic measuring.

Total exploration time in the second session, time spent in exploration of both familiar and new object in third session was measured to calculate the discrimination ratio (DR) which is defined as follows:

$$DR = \frac{\text{New object exploration time} - \text{familar object exploration time}}{\text{New object exploration time} + \text{familar object exploration time}}$$

According to Lueptow (2017), the learning criteria was considered when the DR value was above 0.0.

The recognition index (RI) which measures the ability of the rat to identify the same object at different time points was also measured in our study. It was calculated using the formula of Costa et al. (2008) and Leite, Wilhelm, Jesse, Brandão, and Nogueira (2011) as follows:

$$RI = \frac{\text{New object exploration time} + \text{familar object exploration time}}{\text{New object exploration time}}$$

2.3.5. Biochemical analyses

2.3.5.1. Determination of serum ketone bodies concentration. Serum concentration of ketone bodies was measured by quantitative colorimetric assay, using EnzyChrom^{TM} Ketone Body Assay Kit (EKBD-100) (EKBD-100; Bioassay Systems, Hayward, CA, USA) at 340 nm according to the manufacturer's instructions.

2.3.5.2. Assessment of lipid peroxidation. Serum Malondialdehyde (MDA) concentration was used as the index of lipid peroxidation as described by Halliwell and Chirico (1993). MDA was determined by measuring the thiobarbituric acid reactive species in acidic media giving rise to a red colored pigment that absorbs the ultraviolet (UV) at 532 nm.

2.3.5.3. Assessment of the serum total antioxidant capacity. Serum total antioxidant capacity was measured by quantitative colorimetric assay, using commercial Biodiagnostic kits (Dokki, Giza, Egypt) according to the methods of Koracevic, Koracevic, Djordjevic, Andrejevic, and Cosic (2001) as the antioxidants in the sample eliminate a certain amount of hydrogen peroxide. The residual peroxide is determined by an enzymatic reaction calorimetrically that involves the conversion of 3, 5-dichloro-2-hydroxy benzensulphonate to a colored product which absorbed at 505 nm.

2.3.5.4. Assessment of matrix metalloproteinase (MMP) enzyme activity. The activity of MMP- 2 and 9 was detected in gelatin zymography by a method described by Hawkes et al. (2011). Briefly, serum samples were separated by SDS/PAGE on 7.5% (w/v) gels, containing 1.0 mg/ml gelatin under non-reducing conditions. Then, it was washed twice in 2.5% (v/v) Triton X-100 for 15 min and incubated in development buffer (0.05 M Tris/HCl, pH 8.8, 5 mM CaCl2, 0.02% NaN3) overnight incubation. Gels were stained with 0.1% Coomassie Brilliant Blue R250 in methanol: acetic acid: water (4.5:1:4.5, v/v/v).The zymograms gels were scanned in true colour and then analyzed using commercially available software (my Image Analysis Software; Thermo scientific TM) after conserving to grey scale.

2.3.6. Histopathological investigation

The formalized brain tissues were routinely processed and stained by hematoxylin and eosin (H&E). The detected histological alterations were quantified according to their incidence and severity into (−) absent, (+) mild, (++) moderate and (+++) severe lesions. Some brain sections were stained using Bielschowsky silver stain to visualize the neurofibrillary tangles and plaques (Suvarna & Layton, 2012). The number of amyloid plaques and the cells with tangles were counted within the cerebral cortex and hippocampus (in ten non-overlapping low power microscopic fields). Tissue slides were examined using Olympus (Bx43) light microscope fitted with Olympus (DP-27) digital camera.

2.3.7. Immunohistochemistry investigation

Brain sections were cut (5 μm) from the previously prepared brain tissue paraffin blocks, rehydrated, and subjected to heat induced antigen retrieval followed by protein and endogenous peroxidases blocking steps. Tissue sections were kept with primary mouse monoclonal anti-Nrf2 and anti-HO-1 (at a dilution of 1:100) overnight at 4 °C in humid chamber followed by a washing steps using PBS. Then HRP-labelled secondary antibody was applied (HRP-Goat anti-mouse at a dilution of 1:200), for one hour at room temperature. Afterward, DAB-substrate chromogen kit was used for visualization of the reaction. Control negative slides were obtained by removal of primary antibody step. Positive expression was quantified as area % of expression using Cell-Sens dimensions (Olympus Software).

2.3.8. Statistical analysis

All data were first tested for normality and variance homogeneity, prior to statistical analysis. Having been found to be normally distributed, and variances homogeneous, the data were analyzed by One-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis (SPSS 24.0 software; SPSS Inc., Armonk, NY, USA). GraphPad Prism Version 6.00 (GraphPad Software, San Diego California USA) for Windows was used to drawn histograms. All results were presented as means ± SEM, $P \leq 0.05$ was considered significant.

3. Results

3.1. Characterization of VCO and the dairy formula

The fatty acid composition of VCO which was used to fortify the dairy formula was characterized in the current study by using the gas chromatographic analysis. Results of that approach revealed that the major fatty acids of VCO are: capric ($C_{8:00}$, 5.81%), caproic ($C_{10:0}$, 5.54%), lauric ($C_{12:0}$, 49.52%), myristic ($C_{14:0}$, 20.65%), palmitic ($C_{16:0}$, 8.84%), stearic ($C_{18:0}$, 3.95%), oleic ($C_{18:1}$, 5.22%), linoleic ($C_{18:2}$, 0.83%).

The total phenolic content of VCO was 49.82 mg eq./kg and its antioxidant activity expressed as DPPH (IC_{50}) was 53.71 μg/ml. In addition, the chemical composition of the dairy feeding formula was 20.0% ±0.05 fat (composed entirely of VCO), 3.44 ± 0.1% protein, 3.84% ±0.2 lactose, 28.6 ± 0.6% total solids, 71.4 ± 0.2% moisture and 0.70 ± 0.04% ash.

Results from the viscosity study revealed that an amount of 0.075 wt % k-karrageenan was appropriate for adjusting the viscosity properties of the dairy formula to be suitable for oral dosing of rats (Fig. 1).

3.2. Body weight of rats

As shown in Fig. S1, there was a significant increase in the initial weight of VCO treated rats (group II), $AlCl_3$ treated rats (group IV), and VCO- $AlCl_3$ treated rats (group VI) compared to control rats. While, formula treated rats and formula-$AlCl_3$ treated rats displayed a significant decrease as compared to $AlCl_3$. Conversely, there was no significant difference in the final body weight of rats in all groups. This indicate that the weight of VCO and formula treated rats was markedly increased throughout the experimental period with superiority to the formula group as compared to $AlCl_3$ treated rats (group IV). However, $AlCl_3$ treated rats displayed a marked decrease in their body weights as compared to control group. Moreover, formula-$AlCl_3$ treated rats showed a marked increase in their body weight throughout the experiment as compared to VCO-$AlCl_3$ treated rats.

3.3. Behavioural evaluations

3.3.1. Effect of the dairy formula on the spatial memory of rats in Y-maze task

As depicted in Fig. 2A–C, there was no significant difference in the number of entries into each arm between the control and the rest of the groups, except for group (IV) which comprised $AlCl_3$ treated rats. That group performed less alternation compared to control rats and the other groups; however the difference was non-significant.

Concerning the distance traveled in the Y-maze, it could be noted from Fig. 2A–C that rats treated with $AlCl_3$ significantly traveled less distance in the maze compared to controls. In addition, rats treated with

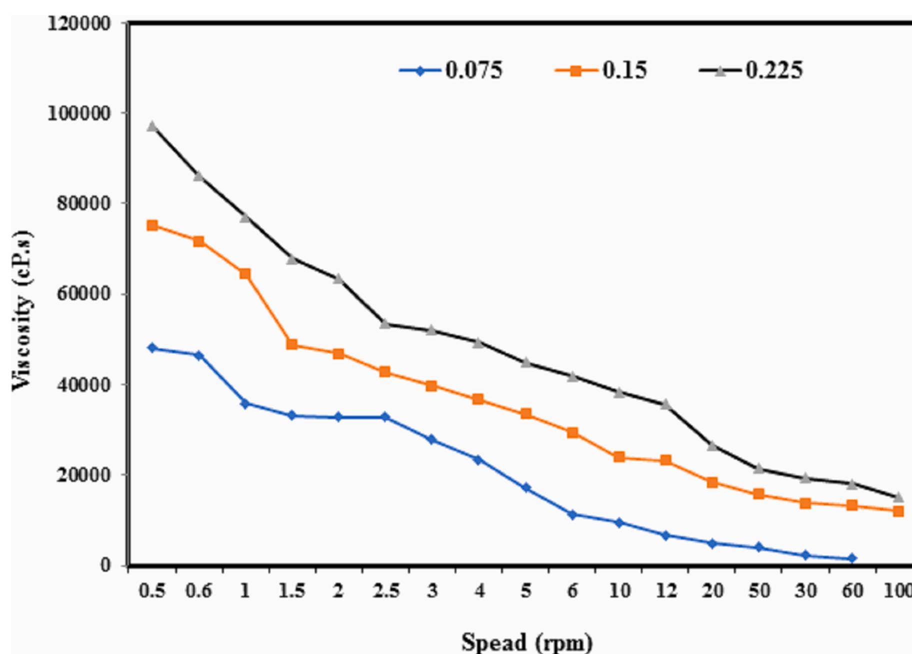

Fig. 1. Viscosity of the dairy formula fortified with virgin coconut oil (VCO) using different concentration (0.075%, 0.15% and 0.225%) of k-carrageenan as viscosity modulating agent.

rivastigmine-$AlCl_3$ (group V) and the dairy formula-$AlCl_3$ (group VII) traveled less distance than rats in the control group (I), rats received VCO (group II), the dairy formula group (III), and VCO-$AlCl_3$ group (VI).

3.3.2. Effect of the dairy formula on the transfer latency (TL) of rats in modified elevated plus maze (mEPM)

As displayed in Fig. 3A, VCO-$AlCl_3$ treated rats (group VI) were significantly spend less time to move to either closed arms (TL1) in the training day 1 (acquisition session) compared to all other treated groups. On the other hand, Fig. 3B showed that in the second day, rats treated with rivastigmine + $AlCl_3$, VCO-$AlCl_3$ and the formula-$AlCl_3$ markedly showed a shortened latency (TL2) (retention session) in the mEPM test compared with $AlCl_3$ treated group.

3.3.3. Effect of the dairy formula on the discrimination ratio and recognition index of rats in novel object recognition test

As shown in Fig. 3C, D, there was a significant decrease of both discrimination ratio (Fig. 3C), and recognition index (Fig. 3D) in $AlCl_3$-treated rats as compared to rats in other groups. On the other hand, there was no statistical difference between controls and all other treated groups.

3.4. Biochemical evaluations

3.4.1. Serum ketone bodies concentration

As depicted in Fig. 4, there was a substantial decrease in the concentration of ketone bodies in rats treated with $AlCl_3$ as compared to controls. However, rats treated with either VCO-$AlCl_3$ or formula-$AlCl_3$ displayed a marked increase in their ketone bodies levels as compared to $AlCl_3$ treated rats and Rivastigmine- $AlCl_3$ treated rats.

3.4.2. Oxidative and antioxidative stress parameters (MDA and TAC)

As shown in Fig. 5A, B, there was a significant increase in lipid peroxidation, expressed as malondialdehyde (MDA) and a significant decrease in total antioxidant capacity (TAC) in $AlCl_3$-treated group (IV) compared with the other groups. In addition, rats received VCO-$AlCl_3$ (group VI) and the formula-$AlCl_3$ (group VII) showed a significant decrease in the MDA levels as compared with $AlCl_3$ group. On the other hand, there was no significant difference in the total antioxidant capacity (TAC) levels between rats received VCO-$AlCl_3$ (group VI) and the dairy formula-$AlCl_3$ (group VII).

3.4.3. Gelatin form of matrix metalloproteinases activity % (MMP-2 and MMP-9)

As depicted in Fig. 5C–E, there was an elevation in the activity of MMP-2 and 9 of $AlCl_3$-treated group compared with the other groups. Also, there was a statistical difference between rats treated with $AlCl_3$ and rats treated with Rivastigmine-$AlCl_3$, VCO-$AlCl_3$, and dairy formula-$AlCl_3$.

3.5. Histopathological investigation

As shown in Fig. 6, the histological examination of brain sections from rats of different groups (I–VII) that were investigated in the current study.

3.5.1. Cerebral cortex

The control group is characterized by a normal histological architecture of cerebral cortex as shown in Fig. 6a. The cerebral cortex is consisted of pyramidal neurons with their characteristic basophilic cytoplasm with some small round nuclei associated with them that belongs to satellite cells. On the other hand, the group received $AlCl_3$ (Group IV) showed a wide range of histological alterations that was appeared in Fig. 6b–h. For instance, blood vessels (especially those of the deep cortex) were surrounded by perivascular hemorrhages (Fig. 6b) and their wall was thickened (cerebral angiopathy) (Fig. 6c). It can be noted that a pink material was deposited with lymphocytes and astrocytes infiltrations around some blood vessels (Fig. 6d).

Perivascular lymphocytic aggregations were frequently detected in the cerebral cortex. Other sections showed focal small aggregations of astrocytes (Fig. 6e). Severely affected individuals exhibited massive aggregates of astrocytes and lymphocytes (Fig. 6f). Pyramidal cells of the cerebral cortex exhibited neuronal degeneration with neuronophagia (Fig. 6g). Abnormally appearing cells with flame shaped processes and faded nuclei were observed (Fig. 6h).

As depicted in Fig. 6i, administration of Rivastigmine (group V) had ameliorative effect against neurotoxicity exerted by $AlCl_3$. The cerebral cortex appeared normal except for few individuals that showed minute

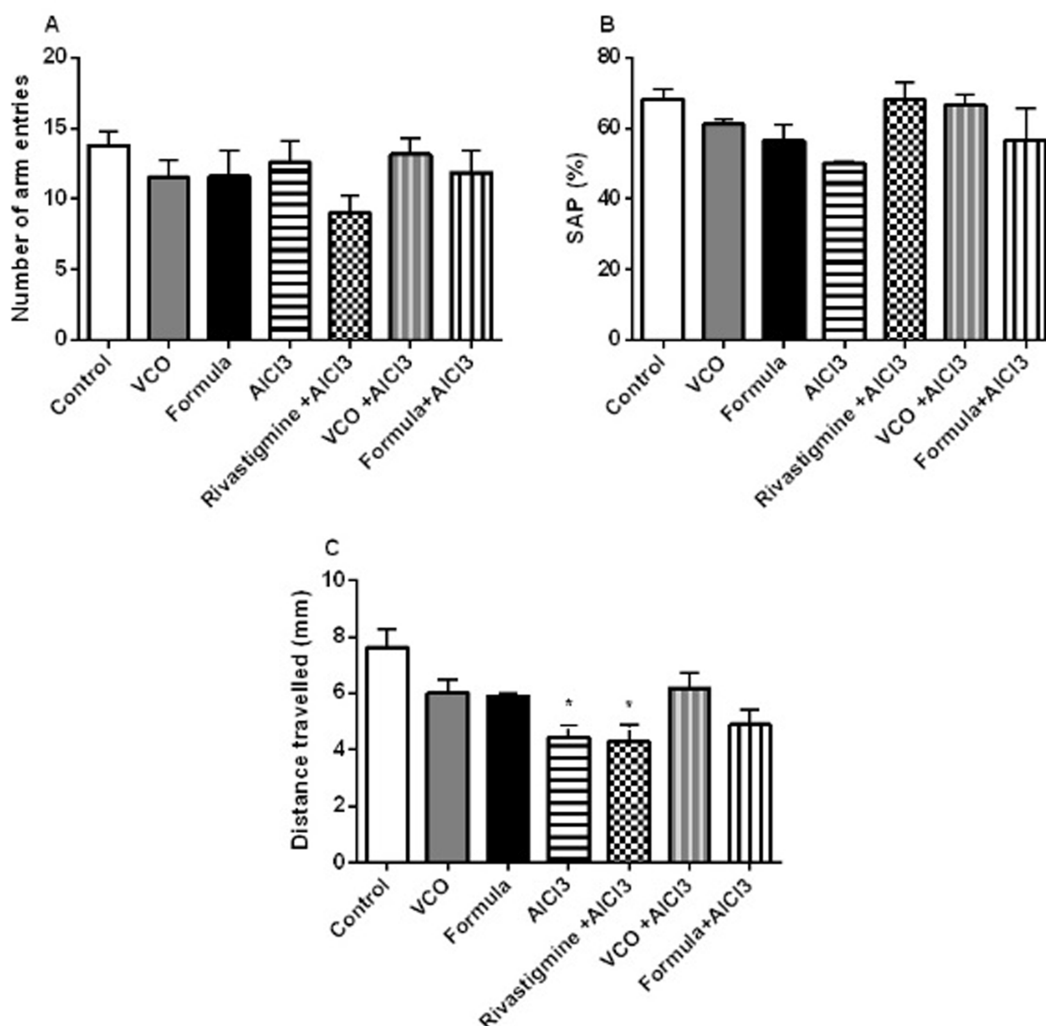

Fig. 2. Effect of edible dairy formula fortified with VCO on the spatial memory in Y maze task of rats. A. Number of arm entries, B. Spontaneous alternation percentage (SAP %) and C. Distance travelled in Y maze. Data are expressed as mean ± standard error (SEM), (one-way analysis of variance (one-way ANOVA) followed by post hoc test Bonferroni test for seven rats in each group. * Significant from control group, $p < 0.05$.

hemorrhages at the deep cortex.

Concerning rats of groups VI and VII which received VCO-$AlCl_3$ and the dairy formula-$AlCl_3$, respectively, exhibited normal histological architecture of different brain regions. In details, VCO in groups (VI) was able to protect the brain tissue against $AlCl_3$ induced toxicity. However, few animals showed focal aggregations of astrocytes and lymphocytes in the deep cerebral cortex (Fig. 6j). All other sections exhibited normal histological architecture of the cerebral cortex (Fig. 6k).

In addition, brains of rats of group VII which received the dairy formula-$AlCl_3$ were apparently normal, congestion of cerebral cortex was a quite common finding (Fig. 6l). Sporadic case showed perivascular hemorrhage and minute perivascular lymphocytic aggregations. Capillary endothelial lining proliferation was noticed in the deep cortex.

3.5.2. *Hippocampus*

Hippocampus region of the control group showed normal structure of cornus Ammonis (CA1 to 4) and dentate gyrus (Fig. 6m). $AlCl_3$ intoxicated group showed numerous degenerating cells within the CA3 and CA4 regions (Fig. 6n) with presence of microglia cells (Fig. 6o), and other sections showed pink fibrillary material in-between degenerated cells (Fig. 6p) at the areas of decreased cell density. The histological evaluation also showed that the hippocampus of rat treated with Rivastigmine appeared normal (Fig. 6q), VCO exerted a protective action as that of Rivastigmine in maintaining the hippocampus structure in rats of groups VI which received VCO-$AlCl_3$ (Fig. 6r). Brains of rats of group VII which received the dairy formula-$AlCl_3$ showed few degenerating cells within CA3 and CA4 regions of hippocampus (Fig. 6s).

3.5.3. *Cerebellum*

Cerebellum of rats from the control group revealed normally appearing molecular layer, Purkinje cell layer and granule cell layer (Fig. 6t). Purkinje cell necrosis (Fig. 6u–v) was frequently detected in the cerebellum of $AlCl_3$ group. No histological alterations were observed in the cerebellum of all protective groups (Fig. 6w–y). A qualitative score of the encountered brain lesions is summarized in Table S1.

Detection of neurofibrillary tangles and amyloid plaques was performed on brain sections stained by Bielschowsky stain (Fig. 7). From the figure it is evident that the control group (I) showed normal dark-

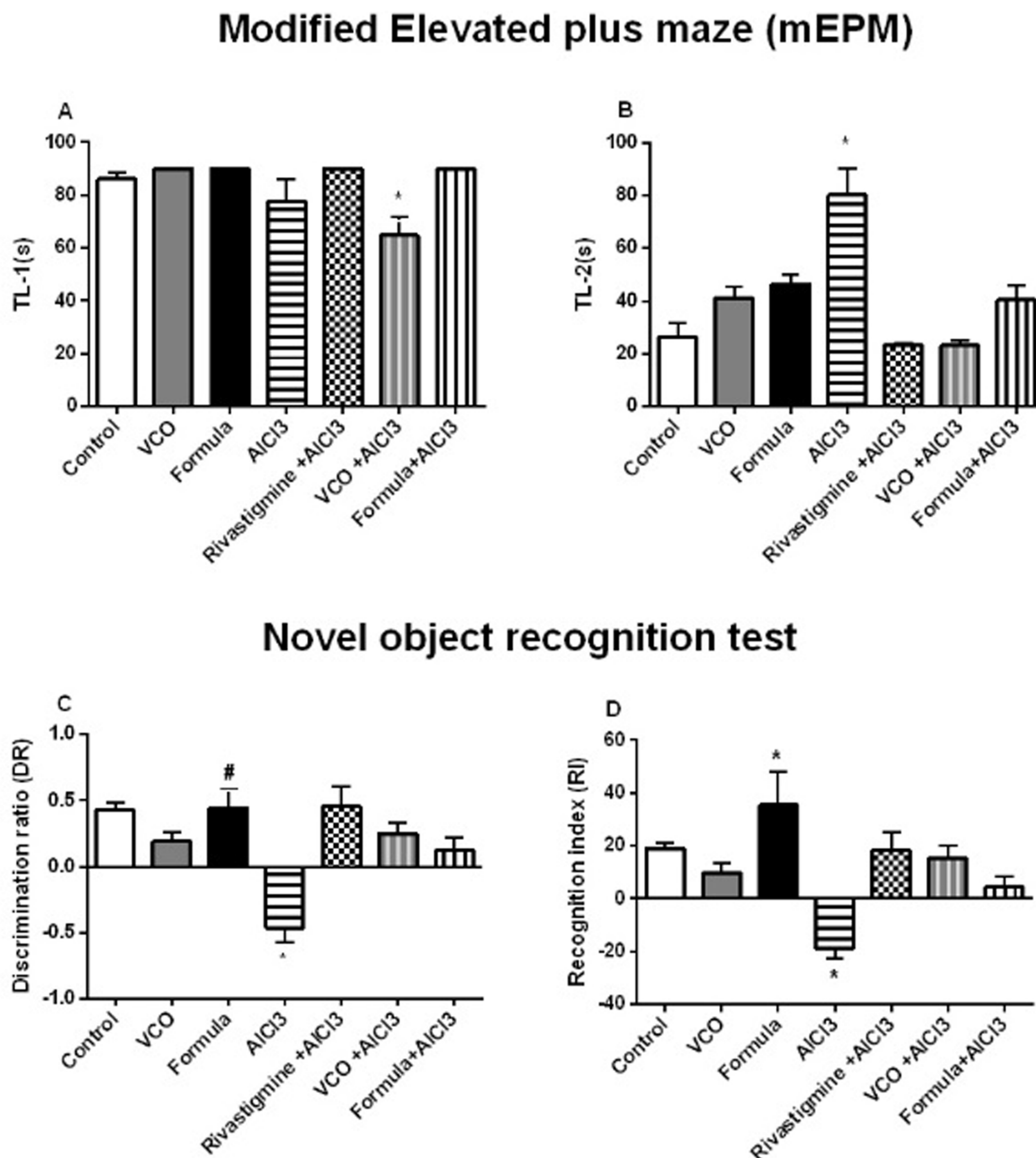

Fig. 3. Effect of edible dairy formula fortified with VCO on the transfer latency (TL) of rats in modified elevated plus maze (mEPM) and on the learning indices in the novel object recognition test. A. transfer latency 1 (TL-1), B. transfer latency 2 (TL-2), C. Discrimination ratio (DR), D. Recognition index (RI). Data are expressed as mean ± SEM, one-way ANOVA followed by post hoc test Bonferroni test for seven rats in each group. * Significant from control group, while # significant from experimental rat groups. $p < 0.05$.

yellow to brown stained cells against yellow background in all brain regions. In resemblance to the control group, brain sections of the groups VCO (group II) and the dairy formula (group III) appeared with normal structure.

On the other hand, the group received $AlCl_3$ (group IV) showed significant increase in the number of the formed primitive plaques compared to the control group (Table S2). Cerebral cortex of VCO treated rats appeared with significantly lower numbers of plaques, the action of VCO was comparable to that of the reference drug Rivastigmine (rats of group V). Brains of rats received the dairy formula-$AlCl_3$ (group VII) also resulted in significant decrease in the number of plaques as well.

Neurofibrillary tangles appeared as black stained filamentous structures extending into the apical dendrite of the pyramidal neuron. They were clearly observed in the group received $AlCl_3$ with significantly higher numbers. Using the protective materials resulted in significant decrease in the number of cells with tangles. No significant difference was observed between the groups received the protective materials within the hippocampus, plaques and cells with neurofibrillary tangles were detected in the areas of decreased cellular density. Co-administration of Rivastigmine (rats of group V), VCO (group VI) and the dairy formula (group VII) with $AlCl_3$ exerted the same protective action in decreasing the numbers of the formed plaques and tangles.

3.6. Immunohistochemistry of Nrf2 and HO-1 in brain tissue

As shown in Fig. 8, brain Nrf2 expression was significantly increased in all treated groups compared to $AlCl_3$ group. There was no significant difference between the Rivastigmine-$AlCl_3$ group and dairy formula-$AlCl_3$ group, also Nrf2 expression was not significant between VCO-$AlCl_3$ group and dairy formula-$AlCl_3$ group. Regarding HO-1 expression in brain tissue, the highest significant value of HO-1 was recorded in $AlCl_3$ group compared to the other experimental groups. All treated groups exhibited significant decrease in HO-1 expression. There was no significant difference between the Rivastigmine-$AlCl_3$ group and dairy formula-$AlCl_3$ group.

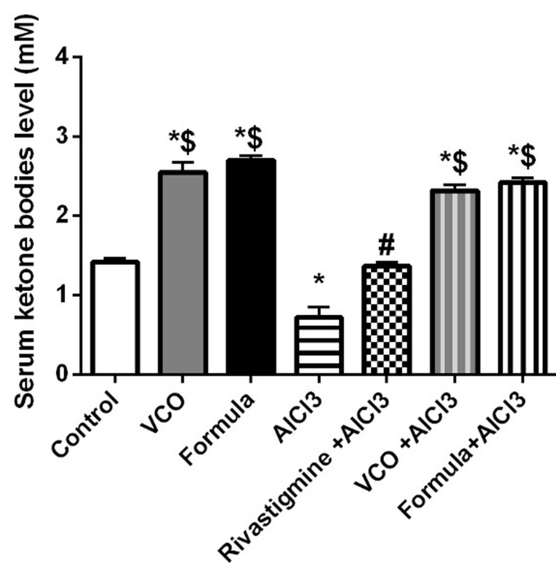

Fig. 4. Effect of edible dairy formula fortified with VCO administration on the serum ketone bodies concentration. Data are expressed as mean ± SEM, one-way ANOVA followed by post hoc test Bonferroni test for seven rats in each group. * Significant from control group, $ significant from $AlCl_3$ groups, and # significant from experimental rat groups. $p < 0.05$.

4. Discussion

Fatty acid composition of VCO was characterized, and the oil was derivatized into its corresponding fatty acid methyl esters. The results revealed that VCO is rich in medium chain triglycerides (MCT) including caproic, capric, and lauric that account all together for ~61.0% of the total composition of fatty acids in VCO. These values are considered as an average values that characterize that oil (Srivastava, Semwal, & Majumdar, 2016). A diet containing pure MCT or its whole peering VCO (such as the dairy formula in the current study) is called a ketogenic diet. That is because it induces mild ketosis, which is a process that links positively with cognitive performance (Avgerinos, Egan, Mattson, & Kapogiannis, 2020; Taylor, Sullivan, Mahnken, Burns, & Swerdlow, 2018). Mild ketosis can also improve cerebrospinal fluid biomarker profile and cerebral perfusion in Alzheimer's patients (Neth et al., 2020). It is important to indicate that the ketosis process lead to production of ketone bodies such as β-hydroxybutyrate and acetoacetate as reported in the earlier study of Drenick, Alvarez, Tamasi, and Brickman (1972). These compounds may provide an alternative energy source to the brain in case of impaired glucose metabolism as the case with AD (Chatterjee et al., 2020). Herein, the levels of serum ketone bodies were markedly decreased in the $AlCl_3$ treated rats. However, the VCO-$AlCl_3$ and formula-$AlCl_3$ treated rats displayed a substantial increase in their ketone bodies levels (Fig. 4). This finding further support the idea of Shimazu et al. (2013) who confirmed that ketone body β-hydroxybutyrate plays an important role in suppression of oxidative stress through inhibition of class I histone deacetylases. Also Kashiwaya et al. (2013) who reported the beneficial effects of a novel ketone ester in ameliorating the behavioural and pathological deficits associated with AD.

In addition, the phenolic compounds of VCO; besides MCT; can contribute to the neuroprotective properties against AD through inhibition of the oxidative stress (Illam, Narayanankutty, & Raghavamenon, 2017). Therefore, the phenolic content of VCO that is used to fortify the dairy formula was determined in the current study to get an approach on its potential contribution to the obtained biological activity. Results showed that the total phenolic content of VCO was 49.82 mg eq./kg oil. This value is considered to be high compared to other reported value such as 22.8–32.7 mg eq./kg (Mulyadi, Schreiner, & Dewi, 2018). On the other hand, it is considered to be low compared with another study which reported 401.2 mg eq./kg (Srivastava et al., 2016). Generally, the variation in the extraction method, the geographical origin of the nuts and the season of harvest are all factors that contribute to the overall phenolic content of VCO.

In addition, the antioxidant activity of that oil in terms of DPPH (IC_{50}) was also evaluated and found to be 53.71 μg/ml. The authors compared this activity to that of a pure sample of ascorbic acid (vitamin C) which is one of the standard antioxidant compounds. Results indicated that the DPPH (IC_{50}) of vitamin C was 5.53 μg/ml. This means that the antioxidant activity of VCO which was used to develop the dairy formula is about 1/10 of a pure standard sample of Vitamin C.

From these results we can presume that both the total phenolic compounds and antioxidant activity of VCO can contribute along with the content of MCT to the overall biological activity of the fortified dairy formula as will be verified in the next relevant sections.

Regarding the chemical composition of the VCO-fortified dairy formula, it is evident that it's fat composition (which was composed entirely of VCO), was deliberately adjusted at 20.0 wt% ± 0.05 of the total formula. This concentration was chosen based on calculations made to deliver 200.0 mg VCO/rat/day by oral administration of 5.0 ml dairy formula/kg rat. It is worth indicating that the applied dose is equivalent to 12.0 g/70 kg human body weight, which satisfy the minimum amount (10 ml per day) of VCO required for human to get the desired biological outcome of that oil (Fife, 2005). The conversion of human body weight into rat equivalent was accomplished using conversion table of animal doses to human equivalent based on body surface area according to Sharma and McNeill (2009).

One should note that it was important to maintain a reproducible dose of VCO (200.0 mg oil/rat/day) for all rats through out the 60 days of feeding period. Therefore, the authors managed to develop a physically stable dairy formula that does not allow VCO to separate during standing for 60 days, leading to inhomogeneous distribution of VCO in the dairy formula. Therefore, two approaches were adopted to achieve that purpose, first: fresh new formulas were manufactured every 15 days for the oral administration till the end of the feeding experiment (60 days). Second, the viscosity of all formulas was modified using a thickening agent like k-karrageenan which chelate with calcium ions of milk to form 3-dimentional net work that thicken the dairy formula and prevents VCO from separation. The concentration of 0.075 g of k-karrageenan/100 g formula was chosen among two other concentrations based on a viscosity study that is illustrated in (Fig. 1). The optimized concentration of k-karrageenan render the dairy formula relatively thick to prevent VCO from separation upon storage, yet, maintain a pour-able characteristic to allow dosing using the gavage tube.

In the current study, chronic administration of $AlCl_3$ (100 mg/kg) for induction of cognitive impairment in rats had a detrimental effect on their behavior as was evident from a behavioral evaluation study, which included the Y-maze test, elevated plus-maze for memory (mEPM) and finally object recognition test.

Y-Maze is used to measure short term working memory which is a hippocampal dependent memory (Kraeuter, Guest, & Sarnyai, 2019), as well as to measure the locomotor activity (Hullmann et al., 2017). $AlCl_3$ treated rats showed reduction in the number of arm entries, spontaneous alternation percentage, though neither one was significant (Fig. 2). This is consistent with a previous finding that showed a reduction of spontaneous alternation percentage in rats received $AlCl_3$ (Khalifa, Safar, Abdelsalam, & Zaki, 2020; Ogunlade, Adelakun, & Agie, 2020). Moreover, $AlCl_3$ treated rats significantly traveled less distance compared to other groups. It is well known that cerebellum is concerned with the locomotor activity (Muzzu, Mitolo, Gava, & Schultz, 2018), therefore, any disturbance in locomotion may indicated cerebellar impairment, which came in agreement with Hritcu et al. (2014).

Behavioral evaluation also included modified elevated plus maze (mEPM) test. It is a simple and rapid test related to long term memory that detect the ability of rats to consolidate information over a long

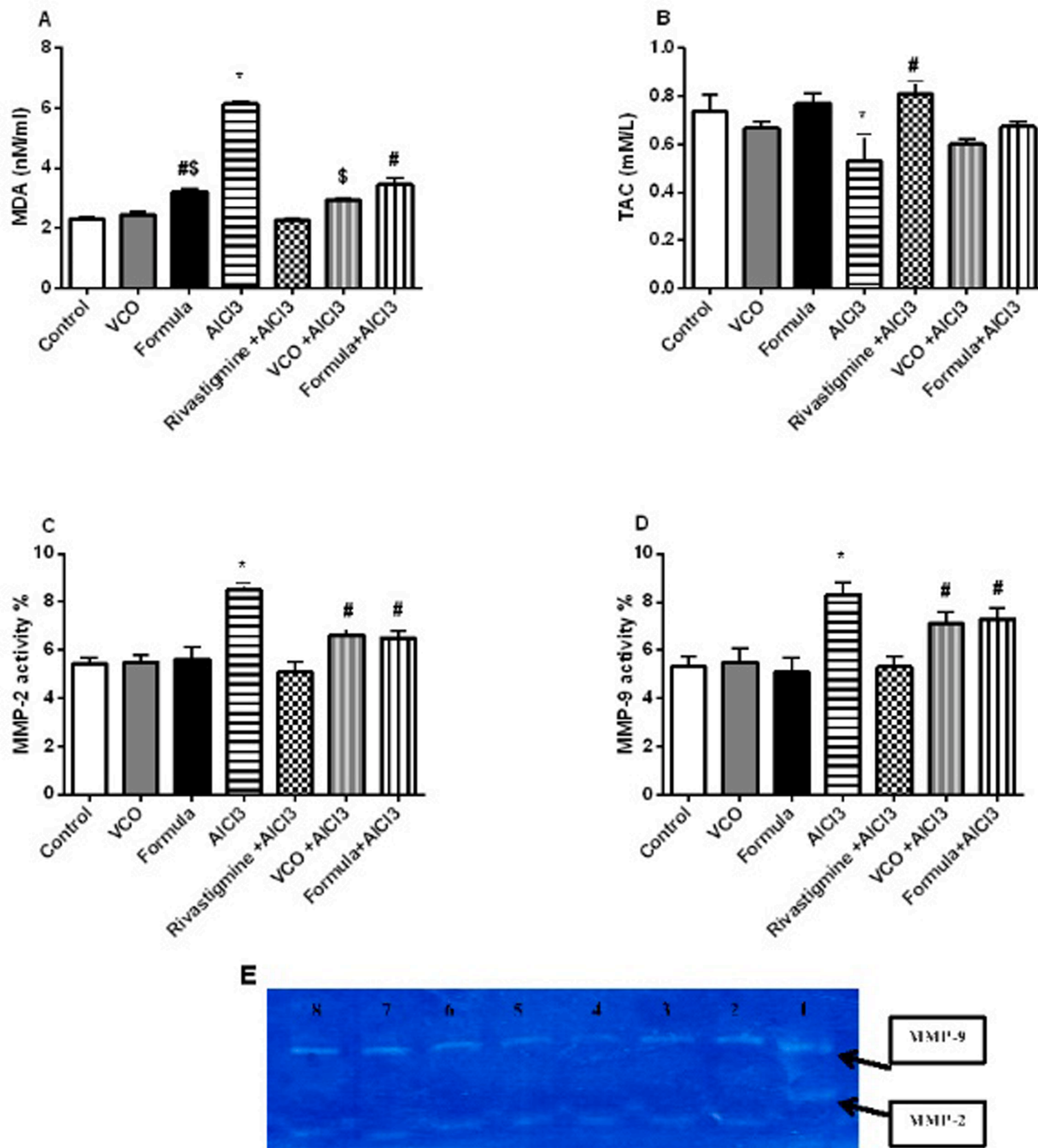

Fig. 5. Effect of edible dairy formula fortified with VCO administration on the oxidative, antioxidative stress parameters (MDA, TAC), and on the gelatin form of matrix metalloproteinases activity percentage % (MMP-2, MMP-9). A. Malondialdehyde (MDA), B. Total antioxidant capacity (TAC), C. MMP-2 activity %, D. MMP-9 activity %. E. Activity of MMPs by gelatin zymography. (Lane 2 = control; Lane 3 = VCO ; Lane 4 = dairy formula ; Lane 5 = $AlCl_3$; Lane 6 = Rivastigmine + $AlCl_3$; Lane 7 = VCO + $AlCl_3$; Lane 8 = Formula + $AlCl_3$), Positive controls shown in lanes1 are from baby hamster kidney cells transfected with active MMP-9 (86 kDa) and MMP-2 (66KDa) that are indicated by arrows. Data are expressed as mean ± SEM, one-way ANOVA followed by post hoc test Bonferroni test for seven rats in each group. * Significant from control group, $ significant from control and $AlCl_3$ groups, and # significant from experimental rat groups. $p < 0.05$.

period of time (Sharma & Kulkarni, 1992). Rats treated with $AlCl_3$ showed reduced transfer latency (to locate the closed arm) in the retention session of mEPM compared to other groups. On the other hand, rats treated with the classical drug rivastigmine-$AlCl_3$, VCO-$AlCl_3$ and the dairy formula-$AlCl_3$ were able to reverse the deficits in mEPM (Fig. 3). That was manifested so clear in the VCO-$AlCl_3$ group which showed reduced transfer latency time in both acquisition and retention session. This result is consistent with a previous finding which showed a reduction in the transfer latency (to locate the closed arm) in the retention session of mEPM of rats receiving $AlCl_3$ (Chiroma et al., 2019) and (Ahmad Rather et al., 2019).

The third behavioral evaluation in the current study was the novel object recognition test. It is also a simple, fast and non-stressful test which is used to evaluate different aspects of memory in rodent. The test depend on the natural tendency of rodent to explore novel object (Lueptow, 2017), therefore it does not require long period of training days. The retention intervals may be short to detect the short-term memory or long to detect the long-term memory (Cowan, 2008). Rats treated with $AlCl_3$ have lower discrimination and recognition values compared to other experimental groups (Fig. 3). That could be due to impaired visual recognition memory reflecting the impairment of the anterior sub-hippocampal cortex as discussed by Didic et al. (2010). On the contrary, rats treated with the dairy formula only (group III) have higher discrimination and recognition values compared to other treated groups. This is consistent with a previous finding that showed lower discrimination and recognition values of rats received $AlCl_3$ (Ji et al.,

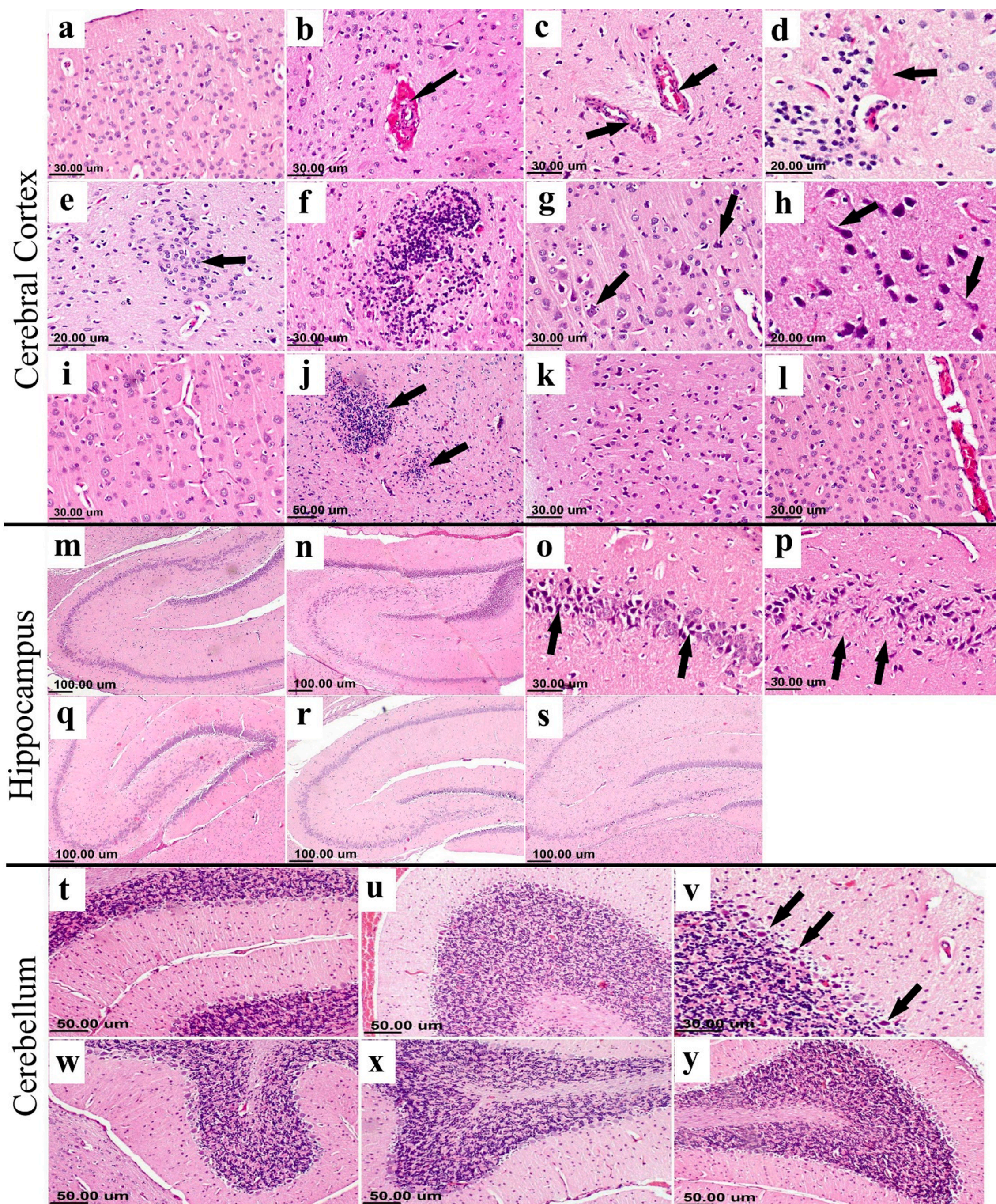

Fig. 6. Photomicrograph of rat brain, Cerebral cortex (H&E) stained; (a) Control group, showing normal cerebral cortex, (b) $AlCl_3$ group, showing perivascular hemorrhage (arrow), (c) $AlCl_3$ group, deep cortex, showing thickened vessels wall (Angiopathy) (arrows), (d) $AlCl_3$ group, showing perivascular deposition of pink material (arrow), note astrocytes infiltrations, (e) $AlCl_3$ group, astrocytes infiltration, (f) $AlCl_3$ group, heavy astrocytes and lymphocytes infiltration, (g) $AlCl_3$ group, showing neuronal degeneration and neuronophagia (arrows), note the presence of microglia, (h) $AlCl_3$ group, presence of abnormal neurons with flame shaped processes and faded nuclei (arrows), (i) Rivastigmine + $AlCl_3$ group, apparently normal cortex with endothelial capillary proliferation, (j) VCO + $AlCl_3$ group, deep cortex, showing astrocytes and lymphocytes aggregations (arrows), (k) VCO + AlCl3 group, apparently normal cerebral cortex and (l) Formula + $AlCl_3$ group, normal cerebral cortex with congested blood vessel. (m-s) Hippocampus region, (H&E) stained; (m) Control group, normal structure of hippocampus, (n) $AlCl_3$ group, showing decreased cell density at CA3 and CA4 regions with presence of degenerating dark neurons, (o) $AlCl_3$ group, higher magnification of degenerating neurons at CA3 region (arrows), (p) $AlCl_3$ group, showing an area of decreased cellular density with presence of pink filamentous deposits (arrows), (q) Rivastigmine + $AlCl_3$ group and (r) VCO + $AlCl_3$ group showing apparently normal structure of hippocampus, and (s) Formula + $AlCl_3$ group, showing few degenerating cells at CA3 and CA4 regions. (t-y) Cerebellum, (H&E) stained;(t) Control group, normal structure of cerebellum, (u) and (v) $AlCl_3$ group, showing Purkinje cell necrosis (arrows), (w) Rivastigmine + AlCl3 group, (x) VCO + $AlCl_3$ group, and (y) Formula + $AlCl_3$ group, showing apparently normal structure of cerebellum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

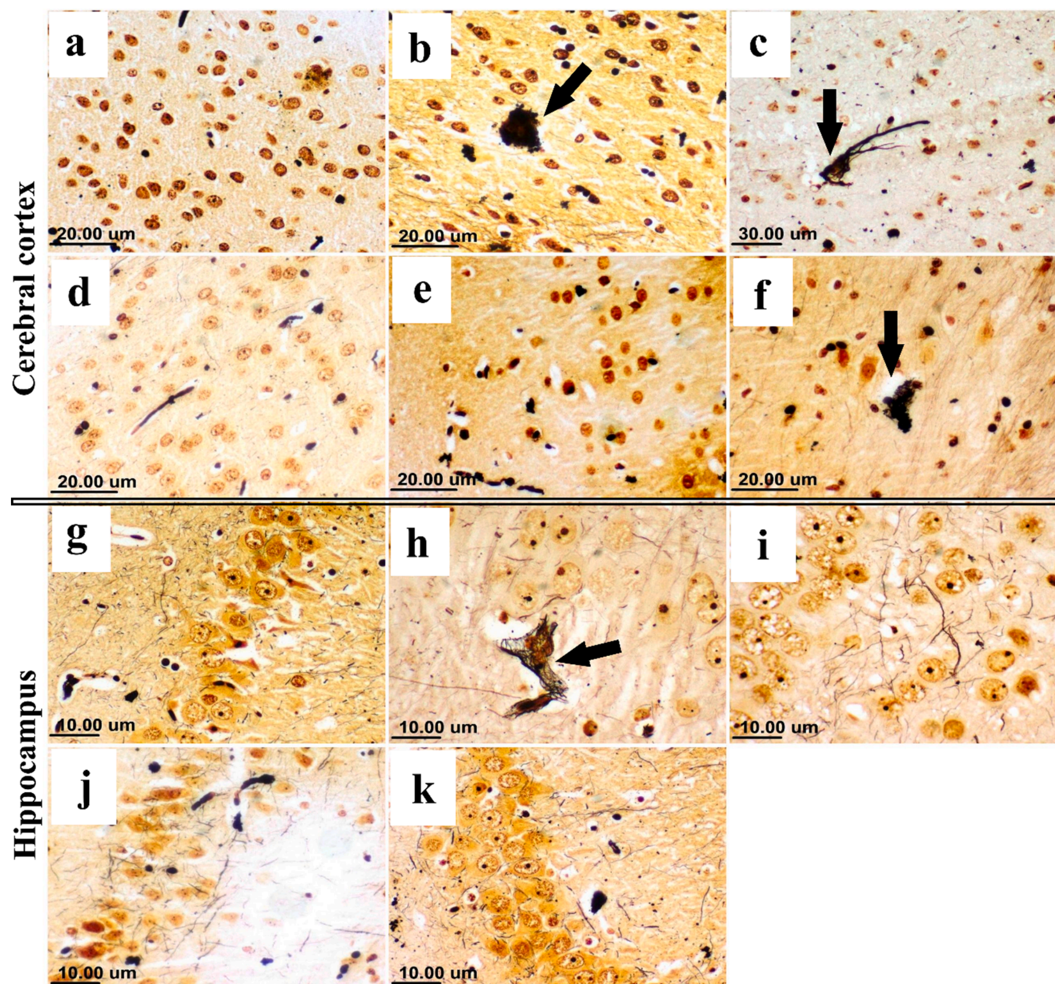

Fig. 7. photomicrograph of rat brain, Bielschowsky stain; (a) Control group, normally stained brown neurons against yellow background (b) $AlCl_3$ group, showing primitive plaque that appeared as black deposits (arrow), (c) $AlCl_3$ group, showing black stained tangles extending into the dendrite of the cell (arrow), (d) Rivastigmine + $AlCl_3$ group, showing normally stained cerebral cortex, (e) VCO + $AlCl_3$ group, showing normal cerebral cortex, (f) Formula + $AlCl_3$ group, showing black stained plaques (arrow), (g) Control group, normally stained hippocampus, (h) $AlCl_3$ group, showing neurofibrillary tangles (arrow), (i) Rivastigmine + $AlCl_3$ group, (j) VCO + AlCl3group and (k) Formula + $AlCl_3$ group showing normally stained hippocampus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2020; Martinez et al., 2019).

There are several possible explanations for the cognitive deficits associated with AD, one of them is oxidative stress, Al is well known to cross the blood brain barrier and accumulate in different areas in brain forming amyloid, also it produce free radicals that may cause brain damage especially in areas concerned with memory and learning (Kumar, Bal, & Gill, 2009). The mechanisms behind oxidative stress in brain still not completely understood, many studies suggested the role of nuclear factor E2-related factor 2 (Nrf2); a transcription factor; in response to stimuli including oxidative stress. Nrf2 translocate to the nucleus and binds to the antioxidant response element (ARE) (Hichor et al., 2018). Nrf2/ARE signaling is responsible for regulation of cellular redox status and modulation of antioxidant defense genes including heme oxygenase 1 (HO-1), CAT, SOD, and GST (Sun, Yang, Leak, Chen, & Zhang, 2017). HO-1 up-regulation is positively linked to neurodegenerative diseases like AD (Nitti et al., 2018). Herein, the upregulation of Nrf2 and down regulation of HO-1 were more prominent in VCO-$AlCl_3$ and formula-$AlCl_3$ compared to $AlCl_3$ treated groups. These findings suggested that the neuroprotective effect of VCO-fortified dairy formula could be due to activation of Nrf2/HO-1 signaling cascade. These findings are consistent with Kerr et al. (2017).

In addition, oxidative stress resulted in increasing the lipid peroxidation products that participate to AD (Kumar & Gill, 2014; Lu et al., 2013). Meanwhile, the total antioxidant capacity is representative of the *in-vivo* balance between oxidizing species and antioxidant compounds (Hadžović-Džuvo et al., 2011). In our study, chronic administration of $AlCl_3$ resulted in significant increase in lipid peroxidation and reduction in the total antioxidant capacity in $AlCl_3$ treated rats compared with other groups (Fig. 5). However, chronic administration of VCO- fortified dairy formula was found to improve not only the memory retention and locomotion but also reduce the oxidative damage associated with AD. That is because oxidative stress and cognitive impairment are strongly correlated (Bitra et al., 2014). As we mentioned before, the antioxidant properties of VCO is due, in part to its content of polyphenols (Illam et al., 2017). One of these phenolic compounds specifically ferulic acid reported to lower cortical Aβ levels in an AD transgenic mouse model (Yan et al., 2013).

Moreover, matrix metalloproteinase (MMPs) especially MMP-2 and MMP-9 are assessed to further support our behavioural and biochemical results. Matrix metalloproteinases (MMPs) constitute a family of at least 28 MMPs, which can all degrade various components of the extracellular matrix (ECM). Matrix metalloproteinases (MMP-2 and MMP-9) are from group called gelatinases which degrade molecules in the basal lamina around capillaries, enable angiogenesis and neurogenesis, participate in inducing cell death, and play a prominent role in injury and repair (Wang, Tan, Yu, & Tan, 2014). In addition, MMPs play an important role

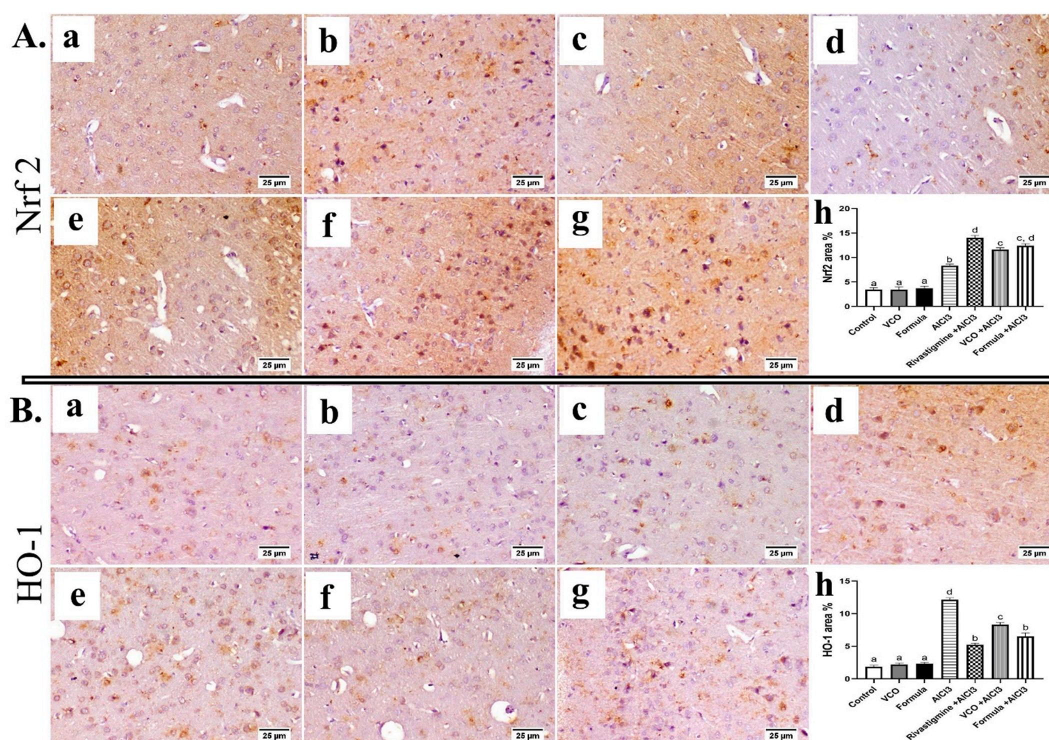

Fig. 8. Photomicrographs of brain, Immune staining (A) Nrf2 expression and (B) HO-1 expression: (a) Control group, (b) VCO, (c) Formula, (d) $AlCl_3$, (e) Rivastigmine + $AlCl_3$, (f) VCO + $AlCl_3$, (g) Formula + $AlCl_3$ group and (h) Data are expressed as mean ± SEM, one-way ANOVA followed by Bonferroni post hoc test, a, b, c and d above bars indicate significant difference. $p < 0.05$.

in the pathogenesis of AD through its ability to degrade amyloid precursor protein (APP) leading to aggregation of Aβ (Ethell & Ethell, 2007). MMP-2 is the major kind of gelatinase which is directly linked to Aβ in the brain and also assumed to have a protective role in AD (Miners, Baig, Tayler, Kehoe, & Love, 2009). As well, MMP-9 are tightly linked to immune activation and thus contribute to matrix restructuring and wound healing during acute and chronic inflammatory events in the brain and throughout the body (Candelario-Jalil, Yang, & Rosenberg, 2009; Del Zoppo, 2010).

Our results reported a significant elevation in the activity of MMP-2 and MMP-9 in $AlCl_3$ treated rats compared with other groups (Fig. 5). These findings are consistent with previous studies (Lorenzl, Buerger, Hampel, & Beal, 2008; Merlo & Sortino, 2012). Furthermore Bruno, Mufson, Wuu, and Cuello (2009) demonstrate an increase in MMP-9 activity during the progression of AD that correlates with cognitive impairment measured by the Global Cognitive Score (GCS) and the Mini-Mental State Examination (MMSE) tests.

Histopathological examination of different brain sections was also validated the deleterious effect of $AlCl_{3.}$ It reveled lymphocytes and astrocytes aggregation besides neuronal degeneration and neuronophagia in the cerebral cortex (Figs. 6 and 7). These results were in agreement with those of Thirunavukkarasu, Upadhyay, and Venkataraman (2012). The hippocampus; which act as one of the major mediators of the spatial learning and memory; showed degenerated neurons with presence of microglia. Decreased cellular density in some areas with presence of pink filamentous deposits was also observed. These findings were consistent with previous studies of Falode, Akinmoladun, Olaleye, and Akindahunsi (2017) and Hajipour et al. (2016).

Furthermore, cerebellar lesions including Purkinje cell necrosis were previously reported by Borai et al. (2017). Interestingly, chronic supplementation of VCO either alone or fabricated in the dairy formula reduced these changes and ameliorated the architectural damage. These findings demonstrate the protective effect of administrating free or formulated VCO in the prevention of oxidative stress damage of brain tissues.

5. Conclusion

The findings in this study demonstrated that administration of a dairy formula fortified with VCO at the described dose can ameliorate the cognitive impairment, activate Nrf2/HO-1 signaling cascade, reduce oxidative stress markers, regulate the metalloproteinases (MMP 2 and MMP 9) and restore the architecture of the brain. The extent of these biological effects is almost the same for the VCO-fortified dairy formula as that of the neat (liquid, unformulated) VCO. Based on that, the fortified dairy formula can be considered as a promising dietary supplement for protection against AD or as a part of a complementary therapy which is based principally on the classical drug Rivastigmine. The formula is also characterized by the ease of ingestion due to its smooth texture and palatable taste which makes it resembles to a pudding dessert. That is expected to encourage the targeted category of people, especially senior citizens, to seek for that VCO-fortified dairy formula on daily basis. That can help them avoiding the discomfort or complains (feeling sick experience) that is associated with direct swallowing “drinking” of the liquid VCO as a complementary supplement.

6. Ethics statement

The experimental protocol was reviewed and approved by the veterinary institutional animal care and use committee, Faculty of Veterinary Medicine, Cairo University, Egypt (VET-IACUC) (Approval Number: vetCU1022019063), and was carried out in accordance with European Council Directive (EU2010/63).

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CRediT authorship contribution statement

Heba M.A. Khalil: Conceptualization, Methodology, Investigation, Writing - review & editing. **Heba H. Salama:** Conceptualization, Investigation. **Asmaa K. Al-Mokaddem:** Investigation, Writing - review & editing. **Samira H. Aljuaydi:** Investigation, Writing - review & editing. **Amr E. Edris:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104296.

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