

RESEARCH ARTICLE

Evaluation of possible neuroprotective effects of virgin coconut oil on aluminum-induced neurotoxicity in an in vitro Alzheimer's disease model

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Abstract

Alzheimer's disease (AD) is a progressive neurological disorder that affects various cognitive functions, behavior, and personality. AD is thought to be caused by a combination of genetic and environmental factors, including exposure to aluminum (Al). Virgin coconut oil (VCO) may have potential as a natural neuroprotectant against AD. Aim of this study was to determine neuroprotective effects of VCO on Al-induced neurotoxicity in an in vitro AD model. SH-SY5Y cells were initially cultured in normal growth medium and then differentiated by reducing fetal bovine serum content and adding retinoic acid (RA). Later, brain-derived neurotrophic factor (BDNF) was added along with RA. The differentiation process was completed on the seventh day. Study groups ($n = 3$) were designed as control group, VCO group, Al group, Al-VCO group, Alzheimer model (AD) group, AD + Al-exposed group (AD+Al), AD + VCO applied group (AD + VCO) and AD + Al-exposed + VCO applied group (AD + Al + VCO). Specific markers of AD (hyperphosphorylated Tau protein, amyloid beta 1–40 peptide, and amyloid precursor protein) were measured in all groups. In addition, oxidative stress parameters (total antioxidant capacity, lipid peroxidase, protein carbonyl, and reactive oxygen species) and neurotransmitter-related parameters (dopamine, dopamine transporter acetylcholine, and synuclein alpha levels, acetylcholinesterase activity) were measured comparatively in the study groups. VCO reduced amyloid beta and hyperphosphorylated Tau protein levels in the study groups. In addition, oxidative stress levels decreased, and neurotransmitter parameters improved with VCO. Our study shows that VCO may have potential therapeutic effects in Alzheimer's disease and further experiments are needed to determine its efficacy.

KEYWORDS

aluminum, Alzheimer's disease, coconut oil, neurotransmitter

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1 | INTRODUCTION

Alzheimer's disease (AD) is a progressive neurological disorder that affects various cognitive functions, behavior, and personality. The biochemical changes that occur in the brain are interrelated and lead to difficulties in receiving and processing new information, performing previously known tasks, confusion, social withdrawal, memory loss, and changes in personality (Wang et al., 2016). The typical neuropathological indicators of AD are the presence of intracellular neurofibrillary tangles (NFTs; caused by hyperphosphorylation of human microtubule associated protein [Tau]), extracellular senile plaques (caused by accumulation of amyloid beta peptide, A β P), and loss of synapses and neurons in specific brain regions, including the hippocampus and cerebral cortex (Ashrafian et al., 2021; Soria Lopez et al., 2019).

AD is thought to be caused by a combination of genetic and environmental factors, including exposure to aluminum (Al). Evidence of the relationship between Al and AD includes observations of neurofibrillary degeneration after the intracerebral injection of Al in rabbits (Klatzo et al., 1965) and elevated levels of Al in the brains of AD patients in post-mortem studies conducted in 1973 (Crapper et al., 1973). While early-onset AD is linked to genetic mutations that lead to increased secretion of neurotoxic A β P, the late-onset form, which accounts for 85–95% of AD cases, is not associated with any gene mutations (Ferreira et al., 2008).

Al is a metal that is commonly found in nature and is extensively utilized throughout the world. It can react with other elements to create various chemical compounds. Al is mainly used in cooking utensils, construction material, water purification systems, vaccine adjuvants, and cosmetics (Kamali et al., 2019; Sanajou et al., 2021). Humans are highly exposed to Al in workplaces (Klotz et al., 2017). Moreover, Al adjuvants to vaccines have aroused many questions both in the past and particularly after COVID-19 pandemic (Liang et al., 2020). Although the Al ion does not play a role in metabolic processes, it can be harmful to both humans and animals as a metallic toxicant due to its high accumulation in the organism (Exley, 2013). The safety of Al was brought into question after elevated levels of Al were discovered in the brain tissues of individuals with encephalopathy who were exposed to Al through dialysis (Alfrey et al., 1976). The accumulation of Al in mammalian tissues may cause several adverse health effects including AD (Exley, 2017), bone defects (Klein, 2019), impaired immune function (Zhu et al., 2014), multiple sclerosis, and autism (Kawahara & Kato-Negishi, 2011). The presence of high levels of Al was observed in the hippocampus of animal models along with the formation of NFTs although the exact pathogenesis and relationship is not yet understood. Various studies investigated the pathways of Al-induced neurotoxicity, such as oxidative stress injury, DNA damage, and mitochondrial dysfunction (Exley et al., 2012; Sanajou, Erkekoğlu, et al., 2023). However, there is no unified mechanism that directly correlates Al exposure with the formation of NFTs, which are a hallmark lesion in AD. Previous studies suggested that the appearance of NFTs in the central nervous system as a result of Al toxicity was related to the deposition of abnormally phosphorylated Tau protein in the brain (Exley & House, 2011). This connection indicates that Al toxicity is Tau

dependent. However, the precise mechanistic link between the Al exposure and Tau hyperphosphorylation remains unclear.

Virgin coconut oil (VCO) is obtained from fresh coconuts through a process that involves low heat without the presence of any chemicals. This process ensures that the bioactive components of the oil such as polyphenols, fatty acids, sterols, and tocopherols are retained. VCO is suggested to act as antioxidant, anti-inflammatory, antithrombotic, antipyretic, and anti-hypercholesterolemic agent (Yeap et al., 2015). VCO has been studied for its potential as a natural neuroprotectant against AD although there is still some controversy surrounding its efficacy (de la Ortí et al., 2017; Fernando et al., 2015). Additionally, coconut consumption has been identified as a potential cognitive enhancer (Fernando et al., 2015). Despite numerous studies on this topic, further research is required to determine the protective effects of VCO supplementation before the onset of the disease.

The aim of this study was to determine the possible protective effects of VCO against AD and/or Al in Tau-induced AD model in SH-SY5Y cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and kits

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), aluminum hydroxide [Al (OH) $_3$], brain-derived neurotrophic factor (BDNF), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Mannheim, Germany). VCO Unrefined Cold Pressed Chemical Free was purchased from Balen (Ankara, Turkey). Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 w/L-glutamine w/25 mM Hepes (DMEM-F12), penicillin/streptomycin, and fetal bovine serum (FBS) were obtained from Biowest (Riverside, 97 MO). The intracellular reactive oxygen species (ROS) assay kit was from Sigma-Aldrich (Mannheim, Germany). Thiobarbituric acid reactive substances (TBARS), all-trans retinoic acid (RA), total antioxidant (TAOC) assay kit, total protein assay kit, and protein carbonyl assay kit were purchased from Cayman Chemical (Ann Arbor, MI). Human acetylcholinesterase (AChE) kit, acetylcholine (ACh), human dopamine transporter (DAT), dopamine (DA), amyloid beta 1–40 (A β_{1-40}), amyloid precursor protein (APP), human synuclein alpha (SNC α), and human Tau protein (MAP τ) ELISA kits were purchased from Elabscience (Houston, TX). Gas chromatography (GC) capillary column Rt-2560 100 m, 0.25 mm ID, 0.20 μ m purchased from Restek Corporation (Bellefonte, PA), Supelco[®] 37 Component FAME Mix purchased from Sigma-Aldrich (Mannheim, Germany).

2.2 | Cell culture and media

Human neuroblastoma SH-SY5Y cell line (ATCC[®] CRL-2266[™], Manassas, VA) was obtained from The American Type Culture Collection. The cells were grown in 75 cm²-cell culture plates in an incubator maintained at 37°C and 5% CO $_2$ using DMEM-F12 medium, which was supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. For the study, cells from the 14th passage were used.

2.3 | Determination of the fatty acid content of the coconut oil

The fatty acid content of VCO was determined with GC-flame ionization detector (GC-FID, Agilent Technologies 7890 A GC System) with the G4513A Injector. First, 0.1 g of VCO was taken into a test tube (20 ml). Then, 5 ml of a methylation reagent consisting of methanol, hexane, and sulfuric acid (75:25:1, v/v/v) was added. A reflux condenser was used to heat the test tube in a boiling water bath for 30 min, making sure to keep the temperature of the water bath $\geq 90^{\circ}\text{C}$. After, the mixture was transferred into a separating funnel (50 ml) with the help of 10 ml of distilled water and 10 ml of petroleum ether. The funnel was shaken well, and then, we allowed the phases to separate. The aqueous phase was removed, and the ether layer was washed twice with 20 ml of distilled water. Later, some anhydrous sodium sulfate (Na_2SO_4) was added to the separating funnel, shaken, and let settle for a few minutes. The mixture was filtered, and the filtrate was collected in a 15 ml test tube with a conical base. The solvent was evaporated over a water bath with the help of a nitrogen current; 1 ml of CH_2Cl_2 was added, and the resulting mixture was analyzed for fatty acid content.

2.4 | Cells lysis

Following the incubation period with $\text{Al}(\text{OH})_3$ and/or VCO, cells were washed with pre-cooled PBS and trypsinized. Cell

suspensions were then collected and centrifuged at $1,000\times g$ for 5 min. After removing the supernatants, cells were re-suspended with 800 μl of PBS. To ensure complete cell lysis, the freeze-thaw process was repeated multiple times. Once cell lysis was completed, the cells were centrifuged at $1,500\times g$ for 10 min at $2-8^{\circ}\text{C}$, and the supernatants were collected and stored at -80°C until analysis.

2.5 | Cell viability and cell proliferation

To determine a cytotoxic concentration of $\text{Al}(\text{OH})_3$ and non-cytotoxic concentrations of VCO, the MTT cell viability assay was conducted. The assay involved seeding 20,000 SH-SY5Y cells/well into 96-well microplates and incubating them for 24 h. The culture media was then replaced with fresh DMEM-F12 medium containing varying concentrations of $\text{Al}(\text{OH})_3$ (10, 25, 50, 100, 250, 500, 750, 1,000, 1,500, 2,000, and 2,500 μM) and VCO (15.56, 31.12, 62.5, 125, 250, 500, and 1,000 μM), and the plates were further incubated for 24 h. Later, the medium was removed from each well, and 0.5 mg/ml of MTT was carefully added. The plate was then incubated at 37°C for 4 h. The medium was removed again, and 150 μl of DMSO was added to dissolve the formazan crystals. Optical density (OD) of the samples was measured at 570 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The experiments were repeated six times on different days, and the

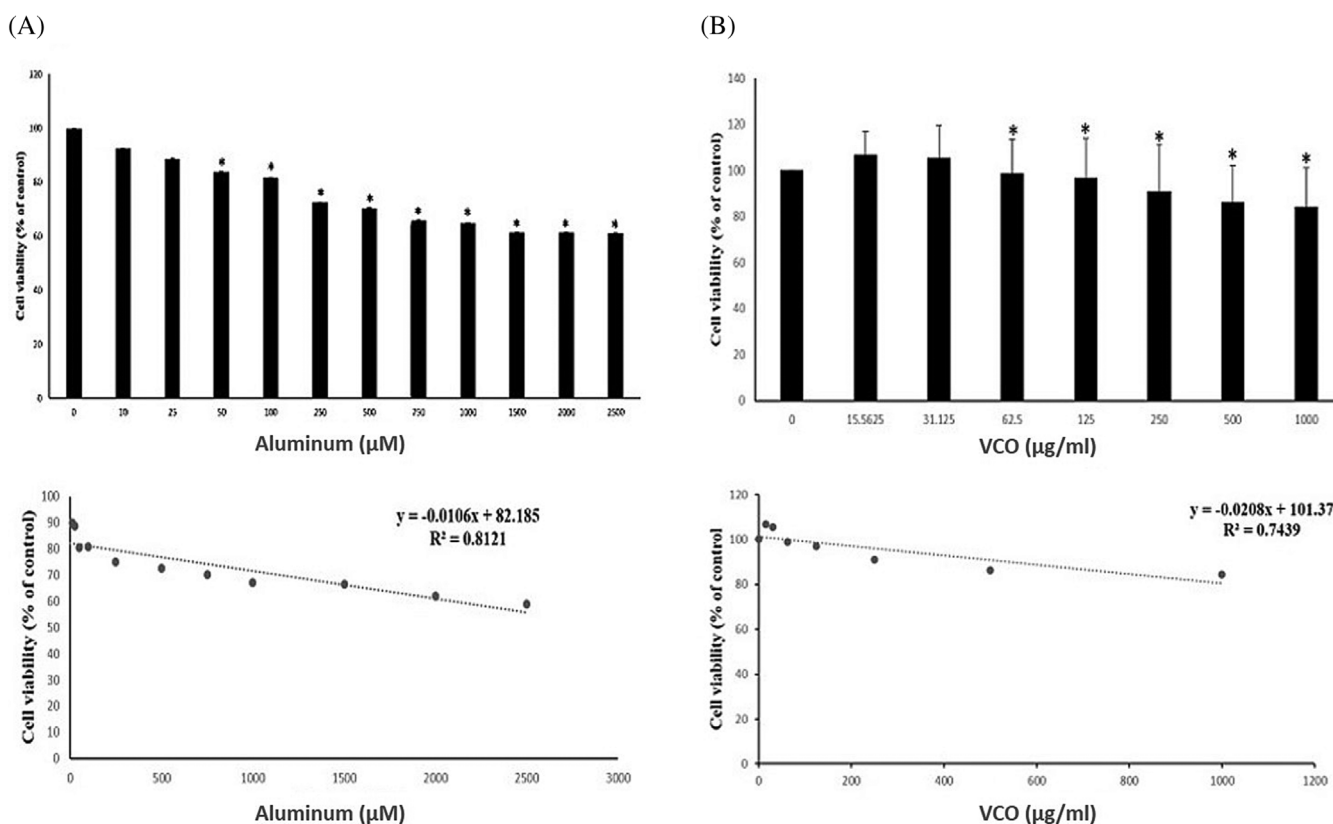


FIGURE 1 Cell viability at different concentrations of aluminum and coconut oil. (A) Cell viability at different concentrations of aluminum. $*p < 0.001$ represents a significant difference between control and individual treatment. Values are given as mean $\pm$ SD. (B) Cell viability at different concentrations of VCO. (C) Cell viability percentage after exposure to aluminum. (D) Cell viability percentage after exposure to VCO. Values are given as mean $\pm$ SD. VCO, virgin coconut oil. $*p < 0.05$

mean results were expressed as percentages relative to the control group (Figure 1). The inhibitory concentration 20 (IC₂₀) value of AI was obtained from MTT test results. For VCO, the highest concentration that did not cause loss in cell viability and provided 100% cellular growth versus control (62.5 µg/ml) was chosen for the experiments.

2.6 | AD model

In this study, a previously developed method for SH-SY5Y cell differentiation by de Medeiros et al. (2019) was used with modifications as described by Yürün et al. (2023) and Sanajou, Erkekoğlu, et al. (2023). When sequentially treated with RA and BDNF, the human neuroblastoma SH-SY5Y differentiates to cholinergic neuron-like cells (de Medeiros et al., 2019). Challenging SH-SY5Y cells with RA + BDNF, without causing considerable decrease of cell viability, provides an in vitro model, which mimics the early-stage pathophysiology of cholinergic neurons affected by AD. It was found that coincident with morphologically evident neurite outgrowth, both the content and phosphorylation state of Tau increased in RA + BDNF differentiated SH-SY5Y cells. Jämsä et al. (2004) suggested that Tau phosphorylation increased at all the examined sites ser-199, ser-202, thr-205, ser-396, and ser-404, all of which were found to be hyperphosphorylated in AD brain.

Briefly, cells were initially cultured in normal growth medium for 24 h and then differentiated by reducing the FBS ratio to 1% and adding RA (10 µM) on the first day after plating. Starting from Day 4, BDNF (2.5 ng/ml) was added along with RA, and the cell medium was replaced every 3 days. The differentiation process was completed on the seventh day, and later, both cells and culture supernatants were collected.

Other than using SH-SY5Y cells, other differentiation protocols by using other cell lines, like LA-N cells, are also used by different researchers. The LA-N cell lines were developed by R.C. Seeger around 1980 (Seeger et al., 1977, 1982). They are immortalized biopsies of neuroblastoma, from a 3-year-old girl (LA-N-2, Seeger et al., 1977) and a 4-months-old boy (LA-N-5, Seeger et al., 1982). In two different studies by Shulman et al. (2023) and Lobentanzer et al. (2020), the researchers used LA-N (LA-N-2 and LA-N-5) cells to create in vitro AD models. The cells were differentiated by using a cholinergic differentiation protocol in which several neurokines (ciliary neurotrophic factor [CNTF], leukemia inhibiting factor [LIF], and interleukin 6 [IL-6]) were used in order to provide differentiation. In this protocol, the elevation of choline acetyltransferase (ChAT), the central cholinergic marker (mRNA, protein, and activity), and its intronic vesicular ACh-transporter gene (vAChT) were observed.

The differentiation modeling and chemical addition timeline is summarized in Table 1.

TABLE 1 Alzheimer model and study design

Groups (n = 3)	Day 1	Day 3	Day 4	Day 7
Control	Fresh DMEM F12 medium (10% FBS)	Fresh DMEM F12 medium (10% FBS)	Observation	Pellet removal
VCO	Fresh DMEM F12 medium (10% FBS)	Fresh DMEM F12 medium (10% FBS) + coconut oil (62.5 µg/ml)	Observation	Pellet removal
AI	Fresh DMEM F12 medium (10% FBS) + AI (362 µM)	Fresh DMEM F12 medium (10% FBS) + AI (362 µM)	Observation	Pellet removal
AI-VCO	Fresh DMEM F12 medium (10% FBS) + AI (362 µM)	Fresh DMEM F12 medium (10% FBS) + AI (362 µM) + coconut oil (62.5 µg/ml)	Observation	Pellet removal
AD	Fresh DMEM F12 medium (1% FBS) + 10 µM RA	Fresh DMEM F12 medium (1% FBS) + 10 µM RA	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + BDNF (2.5 ng/ml)	Pellet removal
AD-VCO	Fresh DMEM F12 medium (1% FBS) + 10 µM RA	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + coconut oil (62.5 µg/ml)	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + BDNF (2.5 ng/ml) + coconut oil (62.5 µg/ml)	Pellet removal
AD-AI	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM)	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM)	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM) + BDNF (2.5 ng/ml)	Pellet removal
AD-AI-VCO	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM)	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM) + coconut oil (62.5 µg/ml)	Fresh DMEM F12 medium (1% FBS) + 10 µM RA + AI (362 µM) + BDNF (2.5 ng/ml) + coconut oil (62.5 µg/ml)	Pellet removal

Abbreviations: BDNF, brain-derived neurotrophic factor; DMEM F12, Dulbecco's modified Eagle's medium-Ham's F12 w/L Glutamine w/15-mM HEPES; FBS, fetal bovine serum; RA, retinoic acid.

2.7 | Study groups

1. Control: only medium was applied.
2. VCO group: VCO group was exposed to VCO (62.5 µg/ml) for 24 h.
3. Al group: Al group was exposed to Al (OH)₃ (362 µM) for 24 h.
4. Al-VCO group: Al (OH)₃ (362 µM) + VCO (62.5 µg/ml).
5. AD group: RA (10 µM) + BDNF (2.5 ng/ml).
6. AD-VCO group: RA (10 µM) + BDNF (2.5 ng/ml) + VCO (62.5 µg/ml).
7. AD-Al group: Al (OH)₃ (362 µM) + RA (10 µM) + BDNF (2.5 ng/ml).
8. AD-Al-VCO group: Al (OH)₃ (362 µM) + RA (10 µM) + BDNF (2.5 ng/ml) + VCO (62.5 µg/ml).

2.8 | Intracellular ROS

All study groups were evaluated for intracellular ROS production using a one-step fluorometric assay kit. SH-SY5Y cells were seeded into a 96-well plate with a black/clear bottom and incubated at 37°C and 5% CO₂ for 24 h. After the incubation period, a master reaction mix was added to each well and incubated in the dark for 1 h. Fluorescence was measured at $\lambda_{\text{excitation}} = 520 \text{ nm}$ and $\lambda_{\text{emission}} = 605 \text{ nm}$. The ROS production of control group was 100%, and the ROS production by other groups was calculated as a percentage of the control (Yirün et al., 2023; Sanajou, Yirün et al., 2023).

2.9 | Oxidative stress assays

To assess oxidative stress, the amount of glutathione (GSH) was measured based on the formation of 5-thio-2-nitrobenzoic acid (TNB) with glutathione reductase (GR). The absorbance of TNB was measured at 414 nm, and the GSH levels were given as µg/mg protein.

Total antioxidant capacity (TAOC) levels were assessed using an assay based on the ability of antioxidants to prevent the oxidation of 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS⁺ caused by metmyoglobin. The absorbance was measured at 405 nm, and the ability of antioxidants to stop ABTS⁺ oxidation was measured in mM Trolox equivalents.

The natural process of lipid peroxidation leads to the formation of malondialdehyde (MDA), which is a by-product of lipid peroxidation (Ayala et al., 2014). Under acidic conditions and high temperatures, MDA reacts with thiobarbituric acid to produce a complex, the amount of which is determined by measuring its colorimetric absorbance at 530 nm. The results were then expressed as nmol/mg protein.

Protein carbonyls indicate protein damage due to oxidation. When ROS like peroxides or superoxide anion are present, several amino acids' side chains convert to carbon (Cai & Yan, 2013). The protein carbonyl levels were measured by spectrophotometric analysis of the hydrazone compound produced by the reaction between the

carbonyls and the 2,4-dinitrophenylhydrazine compound. The results are expressed as nmol/mg protein after measuring the absorbance at 370 nm.

2.10 | Dopamine levels

There is intensive research on the dopaminergic system in AD, as it is the key neurotransmitter system involved with emotion and cognition (Nardone et al., 2014). The dopaminergic system undergoes many changes during the neuropathological aging process. The progression of AD has been associated with these changes in the dopaminergic system. However, previous studies found inconsistent results regarding the relationship between AD, dopamine, and dopamine receptor levels. Some studies suggested that dopamine plays a crucial role in synaptic plasticity (Hagena & Manahan-Vaughan, 2016). The progressive disarrangement of synapses, neurotransmission impairment, and loss of cells would further induce the presence of extracellular deposits of amyloid protein, senile plaques, and intracellular fibrillary tangles. These alterations may lead to the symptoms of pre-dementia, like hypo-activity, gait disturbances, and decline of cognitive functioning (Mattsson et al., 2017).

To determine the concentration of DA in the cell culture media, a competitive ELISA kit was used. First, an antibody was coated onto the 96-well plate, and then, the samples were added to the plate along with an HRP conjugated dopamine and a dopamine-specific antibody. The competitive inhibition reaction between the HRP-labeled dopamine and dopamine without the antibody led to a color change that was inversely proportional to the amount of DA present in the samples. ODs were measured at 450 nm, and the concentration of DA in the samples was expressed in ng/ml (Öztürk et al., 2022).

2.11 | Dopamine transporter levels

Dopamin transporter is a protein that spans across the cell membrane and is responsible for moving DA from the synaptic cleft back into the cell's cytosol. DAT plays a significant role in clearing dopamine from synapses (Carboni et al., 1990). To measure the levels of DAT, a sandwich ELISA kit was used. The wells were coated with a specific antibody for human DAT. After adding samples, avidin-HRP conjugate and biotinylated detection antibody were added to the wells. The reaction was stopped by adding the stop solution. The concentration of DAT in the samples was determined by measuring the absorbance at 450 nm, and DAT levels were expressed as nmol/mg protein.

2.12 | Acetylcholinesterase activity and acetylcholine levels

The activities of AChE and ACh levels of the samples were measured using a sandwich ELISA kit. Both of the kits were pre-coated with an antibody specific to human AChE or ACh. Samples and standards are

added to the plate wells. HRP conjugate was then added to each well. The enzyme-substrate reaction was stopped by adding a stop solution, and the ODs of the samples were measured at 450 nm. The results are calculated using standard curves, and the activity of AChE or the levels of ACh were expressed in U/mg protein and pg/ml, respectively.

2.13 | Synuclein alpha levels

Synuclein alpha levels were measured using a commercially available ELISA kit following the manufacturer's instructions. Briefly, samples were prepared by homogenizing cell lysates in lysis buffer, and the protein concentration was determined using a BCA protein assay kit. Equal amounts of protein were added to the ELISA plate, which was coated with a specific antibody against SNC α . After washing, a biotinylated secondary antibody was added, and later, streptavidin-horseradish peroxidase (HRP) conjugate was added. The reaction was started by adding a TMB substrate, and the ODs were measured at 450 nm. The results were expressed as ng/mg protein.

2.14 | Measurement of amyloid precursor protein and amyloid beta 1–40 and Tau protein levels

The only difference between A β _{1–42} and A β _{1–40} is the two additional C-terminal residues on A β _{1–42}. The concentration of A β _{1–40} in cerebral spinal fluid has been found to be several-fold more than that of A β _{1–42} (Kumar-Singh et al., 2006). Previous studies also suggest that A β _{1–42} and A β _{1–40} affect each other's aggregation rates and toxic activities.

Commercial ELISA kits utilizing the sandwich-ELISA principle were used to measure levels of APP, A β _{1–40}, and Tau protein. The ELISA plates provided in these kits were pre-coated with antibodies specific to the measured parameters. Following incubation, cells are washed with precooled PBS, and trypsin is used to remove them. In the following step, cell suspensions were collected and centrifuged for 5 min at 1,000 $\times$ g. After removing the supernatants, 800 ml of PBS was used to resuspend the cells. Multiple freeze-thaw cycles were performed to fully lyse the cells. The cells were centrifuged for 10 min at 1,500 $\times$ g at 2–8°C after lysis, and the supernatant was collected for experiments and stored at –80°C until analysis.

2.15 | Total protein levels

Total protein levels were determined using a bicinchoninic acid (BCA) assay. The amount of protein levels in samples was determined based on the reduction of Cu²⁺ to Cu⁺ in an alkaline environment. ODs of the samples were measured at 562 nm. The amount of protein in samples was directly related to the reduction, and total protein levels were expressed as mg/ml (Akerboom & Sies, 1981; Miller et al., 1993).

2.16 | Statistical analysis

GraphPad Prism® (Version 8.2.1, GraphPad Software, San Diego, CA) was used to perform the statistical analysis. The differences among the groups were evaluated by one-way analysis of variance (ANOVA), and the Mann–Whitney U test was used for pairwise comparisons. Results are expressed as mean $\pm$ standard deviation (SD). $p < 0.05$ was accepted as statistically significant.

3 | RESULTS

3.1 | Fatty acid content of coconut oil

The fatty acid content of VCO is given in Table 2. The percentages (%) of fatty acids in VCO were 0.67 $\pm$ 0.12% for C6:0 (caproate); 8.13 $\pm$ 0.48% for C8:0 (octanoate); 5.97 $\pm$ 0.70% for C10:0 (decanoate); 47.68 $\pm$ 2.98% for C12:0 (dodecanoate); 18.24 $\pm$ 0.77% for C14:0 (myristate); 8.79 $\pm$ 0.83% for C16:0 (palmitate); and 3.54 $\pm$ 0.50% for C18:0 (stearate).

3.2 | Cell viability and proliferation

The cell viability of SH-SY5Y cells decreased dose-dependently with Al concentrations from 0 to 2,500 μ M (Figure 1A). Inhibitor concentration 20 (IC₂₀) was 362 μ M for Al, and this dose was used throughout the experiments (Table 3).

SH-SY5Y cell viability declined with the increased doses of coconut oil as shown in Figure 1B. For coconut oil, the highest dose that did not cause cytotoxicity was determined as 62.5 μ g/ml. This dose was used throughout the in vitro experiments.

TABLE 2 Fatty acid content of coconut oil

Fatty acids	Percentage (%)
C6:0 (caproate)	0.67 $\pm$ 0.12
C8:0 (octanoate)	8.13 $\pm$ 0.48
C10:0 (decanoate)	5.97 $\pm$ 0.70
C12:0 (dodecanoate)	47.68 $\pm$ 2.98
C14:0 (myristate)	18.24 $\pm$ 0.77
C16:0 (palmitate)	8.79 $\pm$ 0.83
C18:0 (stearate)	3.54 $\pm$ 0.50

TABLE 3 Inhibitory concentrations 50, 30, and 20 for aluminum

	IC ₅₀ (μ M)	IC ₃₀ (μ M)	IC ₂₀ (μ M)
Aluminum	2,812.8	889.8	362

Inhibitory concentration 50: IC₅₀; inhibitory concentration 30: IC₃₀, inhibitory concentration 20: IC₂₀.

3.3 | Intracellular ROS levels

The intracellular ROS levels of the study groups are given in Figure 2. Intracellular ROS levels in all groups were significantly higher compared to the control group. The highest increase was determined in the AI, AD, and AD-AI groups (~200% in AI [$p < 0.01$], ~116% in AD [$p < 0.01$], and ~50% in AD-AI groups [$p < 0.05$]). VCO treatment in AD-AI group provided decreases in ROS formation by 35% compared to AD-AI group. However, only 3% decrease in ROS levels was detected between AD-AI-VCO and control group. Additionally, treatment with VCO has decreased the ROS in AI-VCO group by 50% ($p < 0.01$).

3.4 | Oxidative stress parameters

TAOC levels in the experimental groups are shown in Figure 3A. TAOC levels decreased significantly in AI, AD, and AD-AI groups versus control (72%, 60%, and 78%, respectively; $p < 0.01$). TAOC levels increased approximately sixfold in the AD-AI-VCO group compared to

AD-AI group ($p < 0.01$). In addition, a fivefold increase was observed in AI-VCO compared to AI group.

Protein carbonyl levels of the experimental groups are shown in Figure 3B. Protein oxidation increased by 158% and 194% in AD-AI and AD levels, respectively, compared to control group. Treatment with VCO caused 50% reduction in protein oxidation levels in AD-AI levels. Additionally, protein oxidation decreased significantly in AD-VCO group compared to AD group ($p < 0.05$).

Lipid peroxidation levels are shown in Figure 3C. MDA levels increased by almost threefold in AD group ($p < 0.01$). MDA levels are ~1.5-fold higher in AD-AI group versus control ($p < 0.01$). In addition, lipid peroxidation decreased significantly in AD-VCO group compared to AD group ($p < 0.05$).

3.5 | Dopamine and dopamine transporter levels

Figure 4A displays the dopamine transporter protein (DAT) levels in experimental groups. The results indicate a significant decrease in DAT levels in the AI, AD, and AD-AI groups compared to the

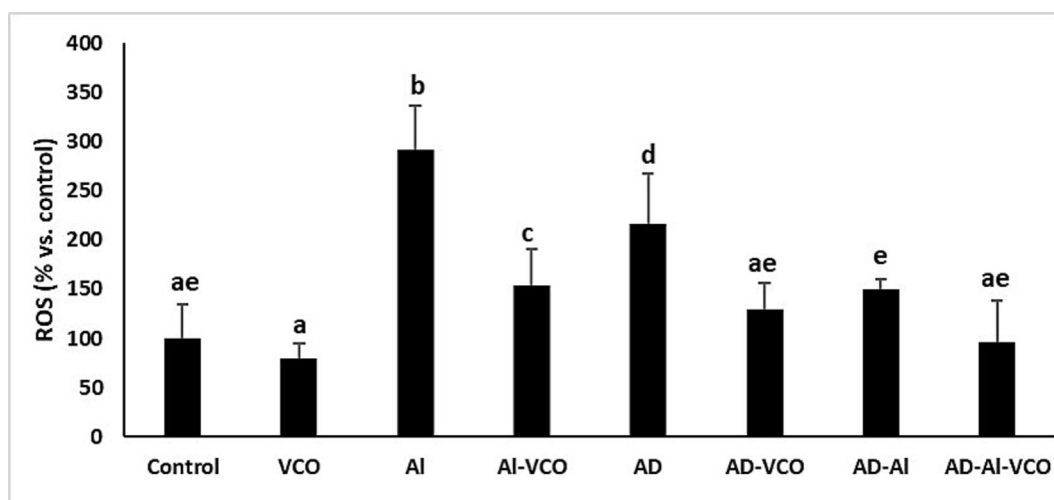


FIGURE 2 Intracellular ROS levels of study groups. The amount of intracellular ROS produced by the control cells was assumed as 100%, and the amount of ROS produced by the other cells was calculated as % compared with the control. ^{a,b,c,d,e}Bars that do not share the same letter (superscripts) are significantly different than each other ($p < 0.05$). ROS, reactive oxygen species

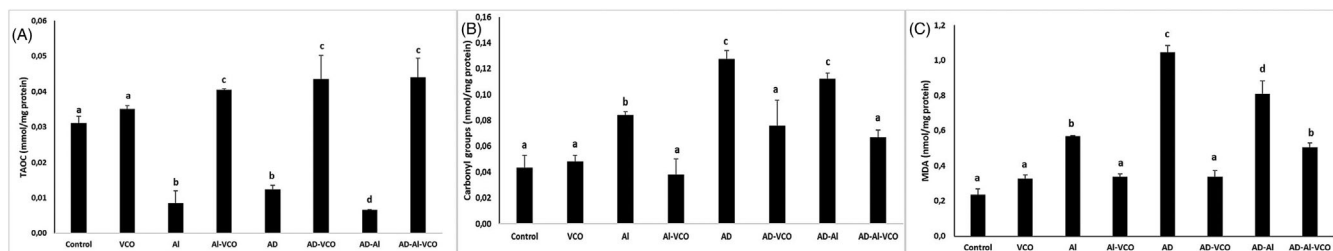


FIGURE 3 Cellular antioxidants and oxidants levels in the study groups. (A) Total antioxidant capacity (TAOC) levels; (B) protein carbonyl levels; (C) MDA levels. ^{a,b,c,d}Bars that do not share the same letter (superscripts) are significantly different than each other ($p < 0.05$). MDA, malondialdehyde; TAOC, total antioxidant capacity

control group (57%, 57%, and 64%, respectively; $p < 0.01$, all). However, the AD-AI-VCO group exhibited a remarkable increase in DAT levels, almost threefold of AD-AI group ($p < 0.05$). Furthermore, DAT levels in the AD-VCO group increased by 100% compared to AD group ($p < 0.01$).

In Figure 4B, dopamine (DA) levels observed in the experimental groups are presented. A significant increase of 134% in DA levels was noted in the AD group ($p < 0.01$), and the increase was almost two-fold in the AD-AI group ($p < 0.01$) compared to the control group. On the other hand, the AD-AI-VCO group exhibited a decrease of 33% in DA levels compared to AD-AI ($p < 0.01$). Additionally, DA levels in the AD-VCO group decreased by 72% compared to AD ($p < 0.01$).

3.6 | Acetylcholinesterase activity and acetylcholine levels

Acetylcholine esterase activity in the experimental groups is shown in Figure 4C. AChE activity increased significantly in AI, AD, and AD-AI groups versus control (~4-, ~8.2-, and ~7.7-fold, respectively; $p < 0.01$ in all). However, AChE activity of AD-AI-VCO group showed almost ~2.3-fold decrease compared to AD-AI group ($p < 0.01$).

Acetylcholine (ACh) levels in the experimental groups are shown in Figure 4D. While ACh levels decreased by 49% in the AD group versus control ($p < 0.01$), the decrease was 73% in the AD-AI group ($p < 0.01$) compared to control group. In the AD-AI-VCO group,

ACh levels increased by approximately threefold versus AD-AI group ($p < 0.01$). Almost twofold increase were recorded in ACh levels in AI-VCO group compared to AI group ($p < 0.01$).

3.7 | Synuclein alpha levels

Synuclein alpha levels were shown in Figure 5. SNC α levels significantly increase AI, AD, AD-AI, and AD-AI-VCO groups significantly compared to control (~74%, ~177%, ~195%, and 77%, respectively). VCO application had reduced SNC α levels in AI-exposed AD-modeled cells ($p < 0.05$).

3.8 | Amyloid precursor protein, amyloid-beta, and Tau levels

The significant increase in APP levels (Figure 6A) in AD and AD-AI groups along with increase in Tau and A β levels in the same groups shows that AD modeling protocol was designed and conducted correctly. On the other hand, in AI-exposed SH-SY5Y cells, there was a significant increase in APP levels versus control, suggesting AI may induce similar changes in neuroblastoma cells similar to AD model.

Tau protein levels of study groups are given in Figure 6B. In AI, AD, and AD-AI groups, Tau protein levels were significantly higher compared to control (~200%, ~200%, and ~227%, respectively).

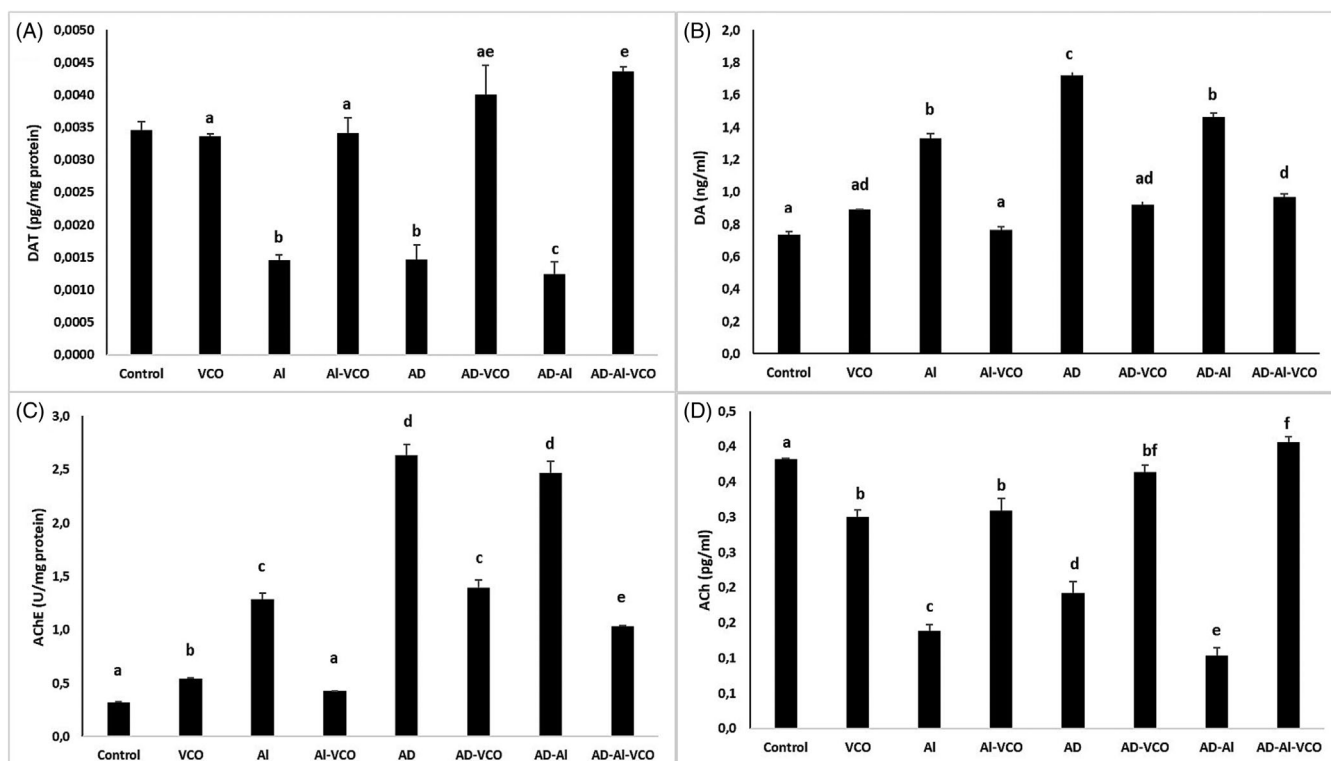


FIGURE 4 DAT levels, DA levels, AChE activity, and ACh levels in the study groups. (A) DAT levels; (B) DA levels; (C) AChE activity; (D) ACh levels. ^{a,b,c,d} Bars that do not share the same letter (superscripts) are significantly different than each other ($p < 0.05$). ACh, acetylcholine; AChE, acetylcholine esterase; DA, dopamine; DAT, dopamine transporter protein

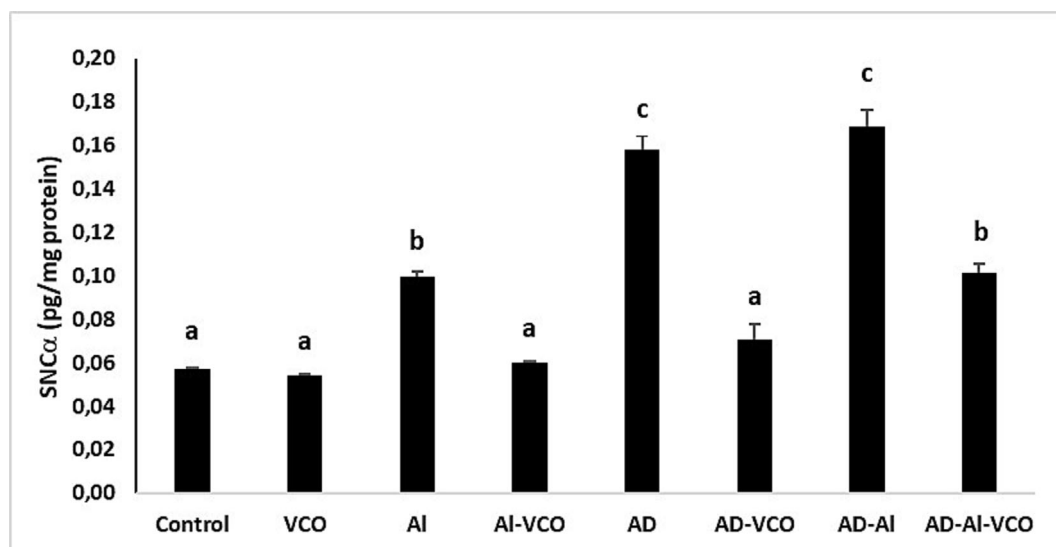


FIGURE 5 SNCα levels in the study groups. ^{a,b,c}Bars that do not share the same letter (superscripts) are significantly different than each other ($p < 0.05$). SNCα, synuclein alpha

Treatment with VCO decreased Tau protein 50% in AD-AI-VCO group versus AD group ($p < 0.05$).

Aβ₁₋₄₀ levels of study groups are given in Figure 6C. A significant increase (~600%) in Aβ₁₋₄₀ was observed in AD-AI group compared to control ($p < 0.01$). When the AD and AI groups were examined, both groups had significantly higher Aβ₁₋₄₀ levels versus control (five-fold and sixfold, respectively). When the AD-AI-VCO group was compared with the AD-AI group, we observed that the Aβ levels were four times lower in the AD-AI-VCO group ($p < 0.01$).

4 | DISCUSSION

AD, the most common cause of dementia, is a growing public health concern with serious consequences for society. AD causes problems with memory, social skills, cognitive, and behavior (Lane et al., 2018). Many factors play a role in the progression of the AD pathology. Due to its multifactorial nature, the etiology of AD has not been fully understood. The drugs used for AD only provide palliative therapy and delay the onset of symptoms (Breijyeh & Karaman, 2020).

Many experts have agreed that cold pressed oils are a healthy alternative to refined cooking oils. Thus, VCO is usually prepared by using “cold pressing,” in which a mechanical extraction is used. This kind of extraction is believed to help retain more nutrients. In addition, the temperature during the production process is also strictly controlled. Using heat can extract more oil from the seed, but heat can degrade the oil's flavor and nutritional quality. Therefore, VCO is undeniably purer than regular coconut oil.

Virgin oils are known to be cholesterol free, are not deodorized, bleached or processed, do not contain harmful solvent residues, and contain natural antioxidants like tocopherols (e.g., vitamin E) and phenolic compounds that are beneficial for health. Virgin oils also have anti-inflammatory properties. VCO helps in healthy functioning of the

thyroid and endocrine system and increases the body's metabolic rate, hence helping in reducing weight. The antioxidants present in VCO reduce inflammation and medium-chained fatty acids (MCFAs) of coconut oil do not need the pancreatic enzymes to be metabolized, so taking VCO eases the strain on the pancreas. The high amount of lauric acid in VCO also helps the heart by reducing total cholesterol and increasing good cholesterol. VCO contains almost negligible amounts of trans-fatty acids (Wallace, 2019; Zeng et al., 2022).

The present study investigated the neuroprotective effects of VCO on AI-induced neurotoxicity in an in vitro AD model. Although the exact cause and genetic origin of AD are not yet known and there is no known prevention treatment, we can suggest that delaying the onset of the disease may be possible with protective agents, like VCO. As VCO contains medium chain saturated fatty acids and higher amounts of polyphenols, it is suggested to be healthier than commercially available oils like copra oil (Marina et al., 2009). Other than polyphenols, VCO contains α-tocopherol, which is a well-known antioxidant (Arlee, 2013).

VCO was suggested to exert different protective effects as an antioxidant (Nevin & Rajamohan, 2006), anti-inflammatory, analgesic, anti-pyretic (Vysakh et al., 2014), anti-hepatosteatotic (Narayanankutty et al., 2016), and chemoprotective (Nair et al., 2016) agent. In addition, VCO and lauric acid treatments are reported to have beneficial effects on lipogenesis, and both can enhance the rate of fatty acid catabolism (Arunima & Rajamohan, 2012; Lekshmi Sheela et al., 2016). According to reports, VCO can also be used as an anti-hypercholesterolemic agent (Intahphuak et al., 2010; Yeap et al., 2015).

AI is potent neurotoxic metal and is suggested to produce oxidative stress both in vitro and in vivo (Laabbar et al., 2021; Zahedi-Amiri et al., 2019).

Several studies have explored different therapeutic interventions to prevent AD progression, and VCO has been studied as a natural neuroprotectant against AD (de la Ortí et al., 2017; DeDea, 2012; Fernando et al., 2015). The active compounds of VCO such as sterols,

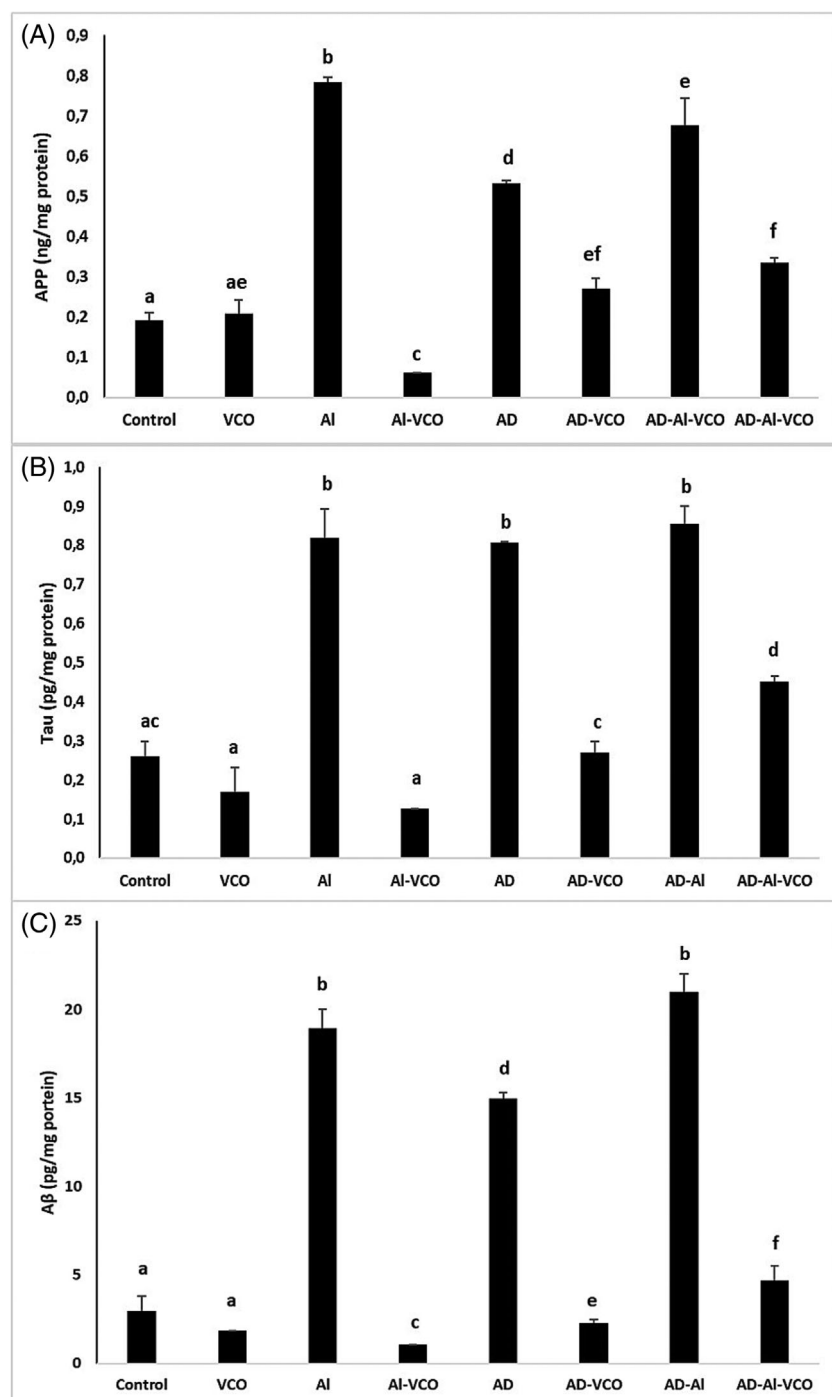


FIGURE 6 APP, Tau protein, and A β levels in the study groups. APP levels; (B) Tau levels; (C) A β levels. ^{a,b,c,d,e,f} Bars that do not share the same letter (superscripts) are significantly different than each other ($p < 0.05$). A β , amyloid beta; APP, amyloid precursor protein

polyphenols, and tocopherols exert strong antioxidant activity and cholinergic activity (Rahim et al., 2017). Additionally, research has shown that VCO can decrease lipid peroxidation levels in the liver, heart, and kidney in vivo (Nevin & Rajamohan, 2006). In addition, VCO lowered lipid peroxidation levels after chemical induced oxidative stress in mice, AD rat models, and in HCT-15 cells (Alghamdi, 2018; Nair et al., 2016). In cell culture studies, pre-treatment with VCO polyphenols reduced oxidant-induced oxidative stress and cell death and restored the levels of glutathione and activities of glutathione reductase, glutathione peroxidase, and catalase. Additionally,

VCO was shown to protect cortical neurons exposed to A β and suppressed the mitochondrial alterations induced by A β (Nafar et al., 2017; Firoozeh Nafar & Mearow, 2014). In accordance with all these studies, our results also show a significant reduction in oxidative stress parameters, including protein oxidation and lipid peroxidation, in both AD and AI-exposed groups. Furthermore, the antioxidant capacity notably increased in these groups after VCO treatment.

Other than antioxidant effects, VCO is able to reduce APP levels, which in turn decreases the production of A β as also shown in the current study. Our results indicate that the in AD model, VCO application

reduced A β by 50% in the AD group and by 100% in the AI-exposed group. Moreover, AD group treated with VCO showed a sixfold decrease in A β compared to the untreated AD group while VOC provided 18-fold decrease in A β_{1-40} levels in cells exposed to AI. These findings suggest that VCO has the potential to reduce the production and accumulation of APP and A β , which are key pathological features of AD. One study also reported similar results and VCO treatment decreased the expression of APP in N2a cells and reduced the secretion of amyloid peptides, A β_{40} and A β_{42} (Bansal et al., 2019).

VCO also provided decreases in Tau protein levels in neuroblastoma cells. We have also demonstrated that exposure to AI aggravated the AD situation and increased the oxidative stress along with increased neuroinflammation and neurotransmitter dysfunction. VCO treatment was also able to decrease the neurotoxic effects of AI in vitro.

Dysfunction of ACh containing neurons in the brain contributes substantially to the cognitive decline observed in those with advanced age and AD (Terry & Buccafusco, 2003). ACh is a crucial neurotransmitter responsible for regulating cognitive functions in the brain. However, increased AChE activity can break down ACh into acetate and choline, leading to the termination of synaptic transmission (Zugno et al., 2014). Previous research indicates that phenolic compounds can decrease AChE activity in rats and VCO may contain phenolic compounds that improve cholinergic activity (Marina et al., 2009; Mirzaei et al., 2019). In this study, we have shown that VCO could regulate the acetylcholine levels in our in vitro AD model by decreasing the AChE activity and increasing the ACh levels.

Dopamine is a crucial neurotransmitter in the brain that belongs to the catecholamine family. It has various essential functions in neuromodulation, including providing benefits for cognitive function, motor control, emotional regulation, motivation, reward processing, and neuroendocrine regulation (Beaulieu & Gainetdinov, 2011). There has been growing evidence indicating that the dopaminergic system is impaired in AD. Autopsy findings of Alzheimer's patients have demonstrated a substantial decline in D1-like and D2-like receptors in the prefrontal cortex and hippocampus (Pan et al., 2019). Despite the unclear mechanism underlying the dysfunction of the dopaminergic system in AD, a hypothesis suggesting its involvement in the pathophysiology of the disease has emerged. As a result, targeting the dopaminergic system through pharmacological intervention may represent a new therapeutic approach for AD (Liu et al., 2023). In our study, we showed that VCO affects the dopaminergic system positively and the dopamine activity is regulated. Our results are consistent with our previous study, which we have shown that AI exposure dysregulated the dopamine neurotransmitter function (Öztürk et al., 2022). Currently, we suggest that treatment by VCO may reverse this phenomenon and regulate the dopaminergic activity in neuroblastoma cells exposed to both AI.

5 | CONCLUSION

Overall, our study provides important insights into the potential therapeutic effects of VCO in AD. Our findings suggest that VCO may be a

promising candidate for further investigation as a natural intervention for reducing Tau protein and A β levels and mitigating oxidative stress parameters in AD. Future research should aim to address the possible protective effects of VCO in animal models. Moreover, studies in human subjects should also be conducted. Elucidating the optimal dose and duration of VCO treatment is needed, and the potential synergistic effects of combining VCO with other interventions like AD drugs currently in use should be investigated. In conclusion, we suggest that our findings may pave the way for the development of novel therapeutic interventions for AD that are based on natural products like VCO.

AUTHOR CONTRIBUTIONS

Göksun Demirel: Conceptualization; methodology; investigation; writing—original draft. **Sonia Sanajou:** Writing—original draft; investigation; validation. **Anıl Yürün:** Formal analysis; methodology. **Deniz Arca Çakır:** Validation; investigation. **Aysel Berkkan:** Investigation. **Terken Baydar:** Supervision; visualization. **Pınar Erkekoğlu:** Project administration; conceptualization; visualization; writing—reviewing and editing.

CONFLICT OF INTEREST STATEMENT

The authors report that there are no competing interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed are available from the corresponding author on reasonable request.

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