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Possible therapeutic role of Grape (*Vitis vinifera*) Leaves Polyphenolic Extract in the regression of Aluminium-induced Alzheimer's disease in rats

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- ✓ Aluminum;
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Abstract

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease. The main histological hallmark of AD-induced brain is neurotoxic amyloid-β (Aβ) plaques, accompanied with oxidative stress and disorders in lipid metabolism. This study demonstrated the therapeutic effect of *Vitis vinifera* (grape) Leaves Polyphenolic (VLP) extract on aluminium chloride ($AlCl_3$)-induced AD in aged male rats. AD- rats showed significant increase in oxidative stress biomarkers (malondialdehyde (MDA), nitric oxide (NO) and protein carbonyls (PC)), amyloid β (Aβ) and lipid profile markers, as well as, sphingomyelins (SM) and phosphatidylcholine (P.Ch.) were elevated. Additionally, brain total protein (TP) content showed significant reduction. The biochemical findings were supported with the histological investigation of brain tissues of AD-rats which demonstrated neurodegeneration in brain cells and amyloid plaques formation. While, treatment with VLP extract has effective role in brain tissue architecture restoration as well as modulating the aforementioned parameters. This work demonstrated that VLP extract has antioxidative, antihypercholesterolemic and antiamyloidogenic activities against $AlCl_3$-induced oxidative stress and dementia in rats, indicating the therapeutic potential of polyphenols in regression of AD.

1. Introduction

Ageing is a major risk factor for the incidence of age-related pathologies such as the acute and chronic cerebrovascular (CBV) and cardiovascular (CV) diseases, which are age-dependentand share common risk factors[1]. Aging is correlated with elevated risk of several oxidative stress and inflammation motivated chronic disease, moreover, there is a role for both cumulative oxidative stress and low grade inflammation in the normal ageing process, independently of disease [2].

Alzheimer's disease (AD) is incurable, disabling progressive neurodegenerative disorder with a steadily growing number of patients and characterized by difficult, time-consuming and challenging diagnosis in the preclinical/presymptomatic phase[3]. A definite diagnosis of AD can only be established by postmortem microscopical examination of the brain of Alzheimer's patient that demonstrated the presence of extracellular amyloid plaques and intracellular tau neurofibrillary tangles, particularly in memory-related brain regions including the entorhinal cortex and hippocampus [4]. Cerebral spinal fluid (CSF) is the most informative sample for monitoring brain pathological processes however, blood sample is "non-invasive, simple to perform, and un expensive"; therefore it is more convenient for routine diagnosis and/or disease monitoring [5]. Current treatment strategy is based upon slow down and/or halt the progression of AD through decrease of cholinergic transmission, by using synthetic acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine), but they have symptomatic adverse effects, with large inter-individual responses[6].

The etiology of AD is not well recognized, except in 1 to 5% of genetic cases [7]. The main hypotheses include: apoptosis of the central cholinergic neurons and reduction of cholinergic neurotransmitters, aggregation and deposition of amyloid-β (Aβ), abnormal phosphorylation of tau protein and formation of neurofibrillary tangles. In addition, theories such as neurotoxicity of the excitatory neurotransmitter, oxidative stress, and mitochondrial

damage can also explain the causes of dementia and provide a new theoretical basis and therapeutic target for the development of novel drugs for AD [8].
It is observed that AD is a protein myopathy featured by specific neuropathological hallmarks: amyloid-β (Aβ) plaques, neurofibrillary au-laden tangles and the neuronal loss and gross atrophy in the cerebral cortex and subcortical regions [9]. Amyloid-β (Aβ) peptide is an output of proteolytic hydrolysis of Amyloid Precursor Protein (APP) [10]. They are two major pathways that determine amount of pathogenic Aβ produced; either non-amyloidogenic (α-secretase) pathway or amyloidogenic (β-secretase) pathway. It is evidenced that lipids are tightly connected with APP metabolism; therefore lipid changes might be correlated with alterations of APP trafficking [11]. ROS generation may activate amyloidogenic β-secretase and further formation of Aβ plaques and finally synaptic and cholinergic dysfunction and neuronal apoptosis in AD [12].
Aluminum chloride ($AlCl_3$) is a neurotoxicant destroying ionic, cholinergic and dopaminergic neurotransmission; it accumulates in the hippocampus, the site of memory and learning, after chronic exposure[13]. High levels of Al and increased risk of neurodegenerative disorders including dialysis encephalopathy, Parkinson's disease (PD) and Alzheimer's disease (AD) are strongly correlated [14].
Progresses in defining the complex etiopathogenesis of AD consider the role of vascular damage that contributes to oxidative stress a core aspect for both AD onset and progression [3]. An antioxidant with the capacity to modulate inflammatory status can thus be beneficial to both normal ageing individuals and those suffering from ageing-related diseases [2]. Recently, a rapidly growing number of polyphenolic compounds with neuroprotective effects have been described; the most interesting effects of polyphenols are the antioxidant properties[15]. The leaves of grape *(Vitis vinifera)* are composed of wide range of polyphenols which have important biological activities including: anthocyanins, flavonoids and also organic acids, mainly malic, oxalic acid and tartaric acid; citric, fumaric and succinic acid can be detected in the leaves only in traces [16].
Theoretically, these activities would have a therapeutic effect for AD. However, there are few studies about the effect of polyphenols on the neuronal activities considering lipid disorders; therefore, the main aim of this work is to study the potential neuroprotective and antioxidative effect of VLP extract against $AlCl_3$-induced neurotoxicity and to further expanding basic findings to humans in an assay to find a simple noninvasive blood-based biomarker to early diagnose AD.

2. Experimental details

1. Plant (*Vitis vinifera*) samples resource

Red grape (*Vitis vinifera* L.), belonging to the family *Vitaceae*, leaves were collected from a local farm in Egypt in May 2015. After washing, *Vitis* leaves were separated from the stems and cleaned, dried at room temperature under shady conditions and coarsely powdered for extraction and stored in polyethylene plastic bags in a dry place.

Preparation of *Vitis vinifera* Leaves Polyphenols (VLP) extract:

Vitis vinifera leaves were defatted by petroleum ether (40°C). The defatted powder (in ratio of 1:10 w/v) were soaked for 30 minutes in dark flasks using acetone/water solvent mixture (80-20, v/v %), the process is repeated thrice and the filtrate was evaporated *in vacuo* at 40°C using a rotary evaporator; the remaining water solution was subsequently lyophilized.

2. Chemicals

- All kits and commercial reagents of analytical grade were purchased from Biodiagnostic Company for diagnostic and research reagents, (Cairo, Egypt).
- Aluminium Chloride anhydrous ($AlCl_3$) was purchased from Sigma Co. USA. Reference commercial drug (Exelon -Rivastigmine- 1.5 mg) was purchased from NOVARTIS Pharmaceuticals, (Cairo, Egypt).
- ELISA kits were provided by OxiSelect (USA) for Protein Carbonyl (PC), IBL (USA) for Amyloid-β.

3. Animals and Ethics statement

The present study was performed on 75 senile male Wistar rats (20-22 months), obtained from the Animal House Colony of the National Research Centre, (NRC); Giza, Egypt. The animals were maintained on standard laboratory diet and water ad libitum. After two weeks of acclimation period, the animals were housed in polypropylene cages in a temperature of (25±1ºC) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received human care and use according to the guide lines for animal experiments which were approved by the National Research Ethics Committee under NRC; license number is **(15-042)**.

Doses and routes of administration:

- **Induction of Alneurotoxicity in rats (*AD-Rat Model*):** Induction of AD in the rat model mimicking AD was carried out by administering $AlCl_3$ orally at a dose 17 mg/kg body weight daily for four successive weeks, according to**Krasovskiĭ*et al.*** [17].
- **VLP Dosage:** The used dose in this study, 100 mg/kg, was based on a study conducted by **Pari and Suresh** [18].

Experimental design

The animals used were classified into five groups (15 rats / group) and treated orally as follows: **Group (1):** Normal aged rats served as negative controls, **Group (2):** Normal aged rats receiving (VLP),**Group (3):** AD-induced rats. **Group (4):** AD-induced rats treated daily orally with (VLP) extract for three mounths at a dose 100 mg/kg body weight, **Group (5):** AD-induced rats treated orally with *Rivastigmine* (RIVA) aqueous infusion (0.3 mg/kg b.wt. /day), daily for three mounths(after stopping $AlCl_3$ administration) for comparison [19].

Blood sampling and brain tissue sample collection

1. *Collection of blood sample*

At the end of the experimental duration, rats were fasted overnight, with free access to water. Under light anesthesia with diethyl ether according to the method of Van Herck et al. [20], rats were sacrificed by cervical decapitation. Blood samples for biochemical estimation were collected just before sacrificing the rats. Blood samples were collected from dorsal aorta of random animals in each group, the blood samples were left to clot in clean dry test tubes for 30 minutes at room temperature and then centrifuged at 4000 rpm for 10 minutes to obtain serum. The clear supernatant serum was then frozen at -20ºC for further biochemical analysis (lipid peroxide, nitric oxide, protein carbonyl, total cholesterol, triacylglycerols, total lipids, phospholipids, sphingomyelins and phosphatidylcholine, Amyloid-β).

2. *Brain tissue homogenate sampling and preparation*

After taking blood samples, the animals were decapitated with the head transferred onto the dry ice, followed by rapid dissection of the whole brain of each animal performed on an ice cooled glass plate, then the brain was thoroughly washed with isotonic saline, dried and then weighed [21]. Then each brain was sagitally divided into two portions. The first portion of each brain was homogenized immediately using an electrical homogenizer to give 10% (w/v) homogenate in ice-cold medium in 9 volumes (1: 9 w/v) of a 50 mM phosphate buffered saline (PBS) pH 7.0 containing 0.1 mmol/L ethylene-diamine-tetra-acetic acid (EDTA). The unbroken cells and cell debris were removed by centrifugation at 4000 rpm for 30 min at 4°C to prepare clear supernatants (10%) to estimate brain total proteins (TP)[22]. Finally, the second portion of the brain was fixed in with 10% buffered-saline formalin for histopathological investigation.

Biochemical assays and analysis:

AD-risk factors: Amyloid-β (Aβ) was performed by ELISA (a sandwich enzyme Immunoassay), according to Selkoe[23]. **Oxidative stress Markers**: Serum Nitric oxide (NO) was determined according to Montgomery &Dymock[24]. Serum malondialdehyde (MDA) levels were determined according to Satoh[25]. Protein Carbonyl (PC) was estimated using the ELISA according to Cadenas*et al.*[26]. **Lipid Profile:** Serum total lipids (TL) levels was determined according to the method ofZöllnerand Kirsch[27], serum triacylglycerols (TAG) levels was determined according to Fossati&Prencipe[28], serum total cholesterol (TC) concentration was valued according to Allain*et al.*[29]. Phospholipids (PL) concentration was valued according toZilversmit and Davis[30]. Phosphatidylcholine (P.Ch.) and Sphingomyelins (SMs) were determined by High Performance Liquid Chromatography (HPLC) according to Jungalwala*et al.*[31]. In addition, quantitative estimation of total protein (TP) in brain homogenate was carried out according to Lowry *et al.*[32].

Histopathological examination:

Brain tissues were fixed in 4% buffered formalin, dehydrated in graded ethanol and embedded in paraffin using standard procedures. Sections of 4μm thickness were stained with hematoxylin and eosin (H&E) for histopathological examination using a light microscope [33].

Statistical analysis:

Data were analyzed using computer software Statistical Package for the Social Sciences (SPSS) version 16 (SPSS Inc. Released 2007, SPSS for Windows, and Version 16.0. Chicago, SPSS Inc.). Simple one way analysis of variance (ANOVA) and Duncan's multiple range tests were used. All data were expressed as Mean±SD for 10 rats of each group.

3. Results and Discussion

Alzheimer's disease (AD) is one of the most important age-associated neurodegenerative disorders. Oxidative stress is involved in the development and progression of AD; therefore using antioxidants is a novel approach to treat AD[34]. Moreover, natural and chemical compounds with anti-oxidative activities have beneficial influences on neuroprotection and neurocognitive performance [35]. This work aimed to explore the potential therapeutic effects of VLP acetone extract in $AlCl_3$-neurotoxicated rats; through the evaluation of specific biomarkers related to oxidative stress and lipid disorders. The Al-exposed AD rat model; is the most commonly applied animal model to mimic AD-like symptoms in humans [36]. Regarding to that both antioxidant defenses and DNA repairing mechanism may decline with age [37].

3.1. Effects of treatment with VLP on Aβ plaque formation in AD-rats:

Estimation of serum Aβ was able to differentiate between AD-induced rats and neurologically normal controls; a significant increase of serum amyloid-β protein was estimated in AD-induced rats reached to 253.99%, as compared to control rats (Figure: 1). This runs in agreement with a previous studies byNayak and Yokel *et al.*[38,39], whichdemonstrated that Al promotes the accumulation of insoluble Aβ (1-42) protein and Aβ plaque formation.Moreover, the study performed by Pesini *et al.*[40] supported the concept that the vascular system is a major player in controlling Aβ levels in the brain; Aβ-plaques appears to be formed if their levels in brain extracellular space surpasses the transport capacity of the clearance mechanism across the blood brain barrier (BBB), or if the vascular transport of the peptide was deteriorated and proved that increased blood Aβ levels is an early event that precedes the onset of cognitive decline and increases the risk of developing AD. The current significant increase in serum Aβ peptide levels in untreated AD-induced rats indicated neuronal cytoskeleton disruption induced by $AlCl_3$ intoxication lead to abnormal accumulation of Aβ peptide in the brain and reflected in its high serum level.Consequently its clearance is considered a primary therapeutic target for managing AD. The neuroprotective potential of polyphenol by reducing neuronal damage and loss induced by neurotoxins or neuroinflammation, altering ROS production, as well as, attenuating the accumulation of neuropathological hallmarks, such as Aβ might takes place through the capacity of polyphenols to interact with molecular signaling pathways and related cellular mechanisms such as inflammation [41,42] or to interact with neuronal and glial signaling [43]. However it is not clear whether this anti-amyloidogenetic activity of polyphenols is attributable to the antioxidant activity and/or to its direct interaction with Aβ[44]. Interestingly, AD-VLP and AD-RIVA showed significant decrease in Aβ levels as compared to AD rats, reflecting the possible role of polyphenols in serum Aβ peptide decrement and clearance.

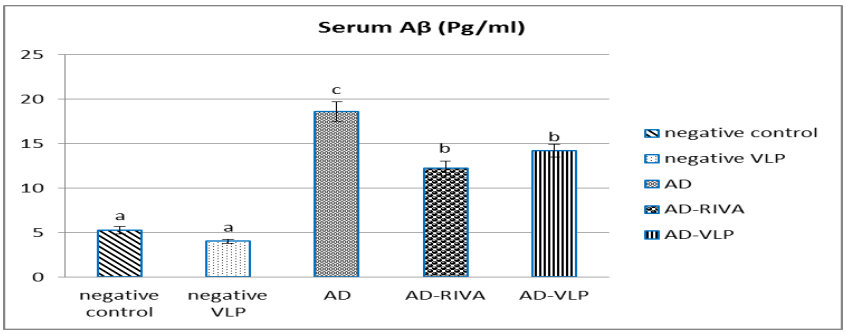


Figure 1:Effects of $AlCl_3$ and $AlCl_3$ +treatments (RIVA or VLP) on serum Amyloid-β (Aβ) levels in different therapeutic groups: Data are presented as mean±SD (n = 10). Mean with different superscripts (a, b, c) are significant at p≤ 0.05. Values not sharing the same superscript letters were significantly different ($P < 0.05$).

3.2. Effects of treatment with VLP on oxidative stress status in AD-rats:

Aluminum is widely defined as an impairment factor of physiological pro-oxidant/anti-oxidant deterioration in favor of ROS formation[45]. Brain, which contains a high percentage of polyunsaturated fatty acids, high rate of oxidative metabolism; consumes 20% of the body's oxygen- and characterized by low enzymatic antioxidant defence mechanism, is unfortunately high susceptible to oxidative trauma[46]. This was corroboratedbyGómez *etal.*[47],who found that Al accumulation and oxidative damage in the brain are strongly correlated, regarding that Al is not a transition metal and cannot initiate peroxidation, *i.e.* inert metal. Virtually, the oxidative damaging effects of Al begins by its accumulation in the brain and blood, Al can bind amino acids such as glutamate to form aluminum-glutamate complexes that allow it to reach the cerebral blood circulation**,** and its ability to form more potent oxidants such as Al-superoxide anions by reacting with superoxide anions [47-49], increased oxy-radicals and loss of cellular homeostasis cause oxidative stress that lead to Al-driven neurotoxicity [50] and further changing physiological and biochemical features of biological systems by

inducing lipid peroxidation through attacking membrane lipids which are essential cellular components.Herein, Al intoxication resulted in evident oxidative/nitrosative stress and the generated ROS subsequently attack almost all cell components as indicated by increases in lipid peroxidation (MDA), PC and endothelial dysfunction (NO) by 132.64, 179.81 and 51.43% respectively in comparison to control rats (Table: 1). Our findings are in accordance with Flora *et al.*[51]who indicated that Al intake produced an oxidative stress-related change and increased ROS contributed to its neurotoxicity and might be attributed to electron leakage, enhanced mitochondrial activity and increased electron chain activity; therefore the correction of oxidative stress could be the first line of therapy for the treatment of dementia.

Table 1: Therapeutic effects of VLP and RIVA on serum levels of PC, MDA and NO in AD-induced rats

Groups		**PC** (nmol/mgprot.)	**MDA** (nmol/ml)	**NO** (µmol/L)
1 Negative control	Mean ±SD	**3.17±0.34** [b]	**8.21±0.26** [a]	**17.5±0.95** [b]
2 Normal VLP	Mean ±SD	**2.51±0.05** [a]	**7.18±0.30** [a]	**13.3±0.66** [a]
	% Change vs. Control	-20.82	-12.55	-24
3 AD-induced	Mean ±SD	**8.87±0.25** [c]	**19.1±0.84** [c]	**26.5±0.58** [c]
	% Change vs. Control	179.81	132.64	51.43
4 AD-RIVA	Mean ±SE	**7.19±0.06** [c]	**9.86±0.64** [b]	**13.5±0.51** [a]
	% Change vs. Control	126.81	20.10	-22.86
	% Change vs. AD	-18.94	-48.38	-49.07
5 AD-VLP	Mean ±SD	**7.91±0.15** [c]	**7.54±0.07** [a]	**11.7±0.50** [a]
	% Change vs. Control	149.53	-8.16	-33.14
	% Change vs. AD	-10.82	-60.52	-55.85

Data are presented as mean±SD (n = 10). Mean with different superscripts (a, b, c, d) are significant at $p \leq 0.05$.

The present results revealed that NO level was increased significantly due to the neurotoxicity of $AlCl_3$. This marker has dual function by being a marker of oxidative stress and endothelial dysfunction, the obtained data is parallel with the results recorded by Stevanović *et al.*[52] who reported that intra-hippocampal injections of $AlCl_3$ in rat induced significant increase in NO and MDA levels. Moreover, Abdel-Salam *et al.*[53], observed increased brain levels of NO following treatment with $AlCl_3$. The mechanism by which NO causes oxidative change to bio-macromolecules (proteins, lipids and DNA) is ascribed to its ability to react with superoxide radical; resulting in the formation of the highly reactive peroxynitrite radical, capable of causing oxidative and/or nitrosative damage to tyrosine residues, thiols, DNA and phospholipids [54]. Furthermore, $AlCl_3$-induced neurotoxicity provokes deterioration of cholinergic transmission leading to impairment in neurocognitive functions and finally cognitive decline through deregulation of NO, the major neuromodulator implicated in brain plasticity and function [55].

As proteins are highly abundant in biological systems and are primarily responsible for most functional processes within cells, they are major targets for ROS/RNS attack, proteins can scavenge the majority (50%–75%) of (ROS/RNS) generated, resulting in oxidative, physical and irreparable changes at every level of protein structure, this can have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity [56].$AlCl_3$-induced ROS generation leads to damage of cellular proteins through carbonylation and results in increased serum PC in AD-rats, this runs in harmony with studies by Choi *et al.* and Conrad *et al.*[57,58], which found an increase in PC (oxidized proteins) levels in AD patients as compared to cognitively intact group [59]; demonstrating the accumulation of PC and impairment of the cell function. Therefore, using PC as a diagnostic biomarker is of a great benefit due to ite early formation, stability, reliablity and long life-span.

The correlations between protein and lipid peroxidation and polyphenol content suggesting that polyphenols could be responsible for the antioxidative activity [60]. Those altered levels of oxidative stress parameters were brought to almost their normal levels; AD-VLP rats showed significant reduction of nitrosative/oxidative stress markers; NO, MDA and PC by (55.85, 60.52 and 10.82%, respectively) as shown in Table (1); demonstrating

the role of this fraction of polyphenols in modulating $AlCl_3$ driven neurodegeneration as a rich source of antioxidants. Even VLP-alone control rats showed more antioxidative activity as compared to control rats and the generation of ROS was significantly decreased, suggesting that polyphenols extract can protect against per-oxidative and free radicals-mediated oxidative injury in those senile rats. This was supported by the previous studies that the *Vitis* derived extracts could reduce lipid peroxidation, suggesting the potential of polyphenols in preventing the oxidative-related physiological alterations. This antioxidative activity of polyphenols is related to their ability to chelate metal ions and acting as ROS scavengers [61,62].

Brain structural and metabolic alterations are represented in changes in blood components [63]. Both AD and cardiovascular disease (CVD) risk factors are common, including; high blood pressure, hypercholesterolemia, obesity, Type 2 diabetes and hyperhomocysteinemia; therefore AD could be considered as a vascular disease. Regarding dyslipidemia, which is largely attributed to accumulation of Al in the liver, causing alteration in lipid metabolism [64,65], furthermore; increased midlife total cholesterol levels is associated with high (two- to three-fold increase) risk of developing AD-dementia [66].

3.3. Effects of treatment with VLP on lipid profile in AD-Rats:

As mentioned above, $AlCl_3$ intake resulted in increased LPO that indicates increased lipid peroxidation of biological membranes that result in the loss of membrane integrity including; alterations in membrane capacity and receptor functions, enhancement in its permeability, loss of its fluidity; affecting membrane-bound enzymes and leading to their leakage from cells[65,67]. In addition, Al has high affinity for phosphate groups and binds to the phospholipid head through electrostatic forces inducing conformational and physical alterations in the lipid bilayer of the plasma membrane [68], that finally leads to impaired brain function. In parallel, our results demonstrated that $AlCl_3$intake provoked a significant increase in serum levels of TC, TL and TAG by 32.76, 65.41 and 42.23%, as compared to controls; thus it could be concluded that, cerebral $AlCl_3$ accumulation may lead to impairment of lipid metabolism and subsequent elevation in lipid profile. These results runs in harmony with the study ofWolozin and Behl[69], who revealed that high TC level represents another important risk factor of AD. Even though the brain cholesterol is independent of the peripheral cholesterol stores, the correlation between elevated peripheral cholesterol and AD was suggested[70]. Moreover, an increase in the membrane cholesterol enhances the lipid raft area, and the Amyloid Protein Precursor (APP) present in the rafts gets into contact with β-secretase very easily leading to increased Aβ production [71].

Table 2: Therapeutic effects of VLP and RIVA on serum levels of TC, TAG and TL in AD-induced rats

Groups		**TC** (mg/dL)	**TAG** (mg/dL)	**TL** (mg/dL)
1 Negative control	Mean ±SD	**81.35±1.19** [b]	**67.78±2.79** [b]	**431±19.29** [a]
2 Normal VLP	Mean ±SD	**63.26±0.99** [a]	**48.11±0.59** [a]	**415±20.45** [a]
	% Change vs. Control	-22.24	-29.02	-3.71
3 AD-induced	Mean ±SD	**108 ±5.31** [c]	**118±4.65** [c]	**613 ±0.86** [c]
	% Change vs. Control	32.76	65.41	42.23
4 AD-RIVA	Mean ±SD	**87.25±1.28** [b]	**71.89±0.21** [b]	**557±15.29** [b]
	% Change vs. Control	7.25	6.06	29.23
	% Change vs. AD	-19.21	-39.08	-9.14
5 AD-VLP group	Mean ±SD	**68.27±1.46** [a]	**67.94±1.90** [b]	**428±6.61** [a]
	% Change vs. Control	-16.08	0.24	-0.70
	% Change vs. AD	-36.79	-42.42	-30.18

Data are presented as mean±SD (n =10). Mean with different superscripts (a, b, c, d) are significant at $p \leq 0.05$.

Treatment with VLP decreased the altered serum levels of TL, TC and TAG, due to Al-induced dyslipidemia, in $AlCl_3$-intoxicated rats, by 36.79, 42.42 and 30.18%, successively. Furthermore, VLP-alone treated control senile rats showed a decrease in their lipid profile proofing the hypolipidemic effect of grape polyphenols. Herein, post-treatment of AD-induced rats with both VLP and RIVA reversed the altered lipid profile to a great extent,

suggesting the ameliorative hypolipidemic role of VLP polyphenols in induced dementia through correction of dyslipidemia. This anti-hyperlipidemic effect of VLP extract might be mostly ascribed to its antioxidant potential, as well as, the membrane integrity is protected from Al-driven ROS attack. Other possible mechanisms for the hypolipidemic impact of this polyphenolic extract include the inhibition of uptake of cholesterol and triacylglycerols by gastrointestinal tract and elevated activity of cholesterol-degrading enzymes that results in cholesterol clearance. We could find that, VLP showed more hypolipidemic effect than RIVA.

3.4. Effects of treatment with VLP on PL, P.Ch. and SM in AD-Rats:

Sphingomyelins (SM) (enriched in lipid rafts) are essential membrane components that are strongly related with cholesterol in construction, metabolism and transport [72]. Central nervous system (CNS) contains large amounts of sphingolipids whose metabolites have important structural roles in cell membranes and function as second messengers for critical intra- and inter- cellular signaling affecting cellular growth, differentiation, proliferation and apoptosis[73]. Abnormalities in sphingolipid metabolism in AD-rains or during normal brain aging, result in accumulation of long-chain ceramides which may contribute to neurotoxic action of Aβ and exacerbate progression of the disease [74].

Although sphingolipids represent only a small fraction of the total cellular lipid pool, even subtle disturbances in their balance may contribute to the development of neurodegenerative diseases [5,75]. For example, levels of sphingomyelin (SM), the most abundant sphingolipid in mammalian cells, are increased exclusively in CSF of prodromal ADpatients [76]. Al-intoxication induced not only dyslipidemia but also, abnormal membrane phospholipid composition. The present results recorded marked increment in sphingomyelins level by 59.10, whereas P.Ch. and PL were significantly depleted in AD-induced rats as compared to control rats by 67.87, and 52.40%. However; study of SM levels are less clear, [77] declared that SM levels have been found to be increased, whileBandaru *et al.*[78], demonstrated decreased in AD as compared to normal control.

Table 3: Therapeutic effects of VLP and RIVA on serum levels of P.CH, PL and SM in AD-induced rats

	Groups	P.Ch. (µg/ml)	SM (µg/ml)	PL (mg/dL)
1 Negative control	Mean ±SD	**2.49±0.17** ^{c}	**4.45±0.31** ^{a}	**108.22±3.61**^{c}
2 NormalVLP	Mean ±SD	**2.80±0.18** ^{c}	**3.72±0.25** ^{b}	**105.27±3.15**^{c}
	% Change vs. Control	12.45	-16.40	-2.73
3 AD-induced	Mean ±SD	**0.80±0.03** ^{a}	**7.08±0.15** ^{c}	**51.51±11.92**^{a}
	% Change vs. Control	67.87-	59.10	-52.40
4 AD-RIVA	Mean ±SD	**1.40±0.03** ^{b}	**5.37 ±0.04** ^{d}	**67.27±5.07**^{b}
	% Change vs. Control	-43.78	20.67	-37.84
	% Change vs. AD	75	-24.15	30.60
5 AD-VLP	Mean ±SD	**1.32±0.02** ^{b}	**5.57 ±0.17** ^{d}	**67.66±3.23**^{b}
	% Change vs. Control	-46.99	25.19	-37.48
	% Change vs. AD	65	-21.33	31.35

Data are presented as mean ± SD (n = 10). Mean with different superscripts (a, b, c, d) are significant at p≤ 0.05.

Furthermore; It was found that Al specifically changes the brain lipid/phospholipid metabolism and/or their transfer to several membrane systems affecting negatively the membrane fluidity/composition [79]. For example; acetylcholine esterase (AChE) is a membrane-bound enzyme, therefore its activity could be declined by impairment in membrane fluidity or alterations of its composition, as well as increased Al-induced ROS generation result in free radicals that attacks membrane lipid bilayer [80]. Our results run in harmony withLütjohann*et al.* [66] who found that Al-exposure resulted in significant decrement in the PL content accompanied by major conformational changes, which is consistent with membrane assumption of AD [81], which revealed that the neurons try to extract choline from choline containing phospholipids to compensate the choline deficiency resulting in the disruption of cell membranes and ultimately in neuronal cell death. Our results showed reduction in SMs and P.Ch., this run in parallel withprevious studies[79,82]. The significant reduction in P.Ch. and PL may be explained regarding the fact thatAD is highly associated with abnormal metabolism of phospholipids from neural membranes [82]. It was suggested that P.Ch.is relatively more

important for membrane function than SM and that choline for neurotransmitter synthesis may be extracted in the first instance from SM component [82].
Treatment of AD-induced rats with VLP normalized the aforementioned parameters; AD-VLP rats exhibited marked increase in both PL and P.Ch.,while SM recorded marked increase. On the other hand, AD-RIVA rats showed decreased percentages of both PL and P.Ch., whereas SM was decreased in both treatments, as compared to AD induced rats, as shown in Table (3).

3.5. Effects of treatment with VLP extract on brain total protein (TP) content in AD-induced Rats:
Virtually, a decreasing total protein reflects a reduction in hepatic protein synthesis, in addition to it confirms the direct damaging effect of $AlCl_3$ [65,83]. Moreover, exposure to free radicals leads to protein fragmentation, protein peroxides generation, enzymatic oxidation and degradation of proteins and ultimately decline in protein content[67]. Brain TP content of AD- rats was significantly decreased by 12.09%, as compared to control rats (Figure: 2). An insignificant change in brain TP content was detected, where returned nearly to its normal levels in both AD-VLP and AD-RIVA rats comparing to normal control. The current results demonstrated that Al exposure caused significant decrease in the brain TP content, this runs in agreement with Chinoy and Memon and Newairy *et al.*[65,84], where TP level was decreased due to $AlCl_3$ administration. TP content assay was performed to evaluate the toxicological nature of aluminium chloride salt. The present study showed that VLP-treatment produced an effective action against the hepatic damages, as shown by a normalization of brain levels of TP in AD-VLP treated rats, which indicated amelioration of impaired liver synthetic function by the treatment with VLP extracts.

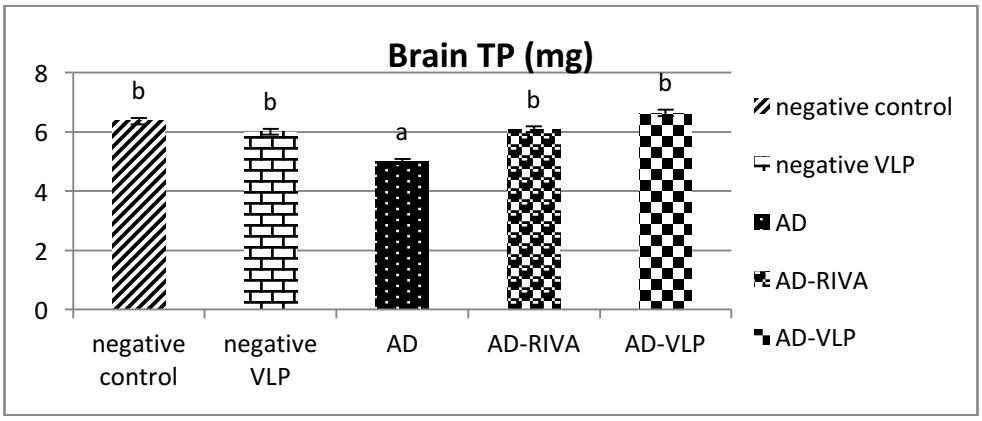
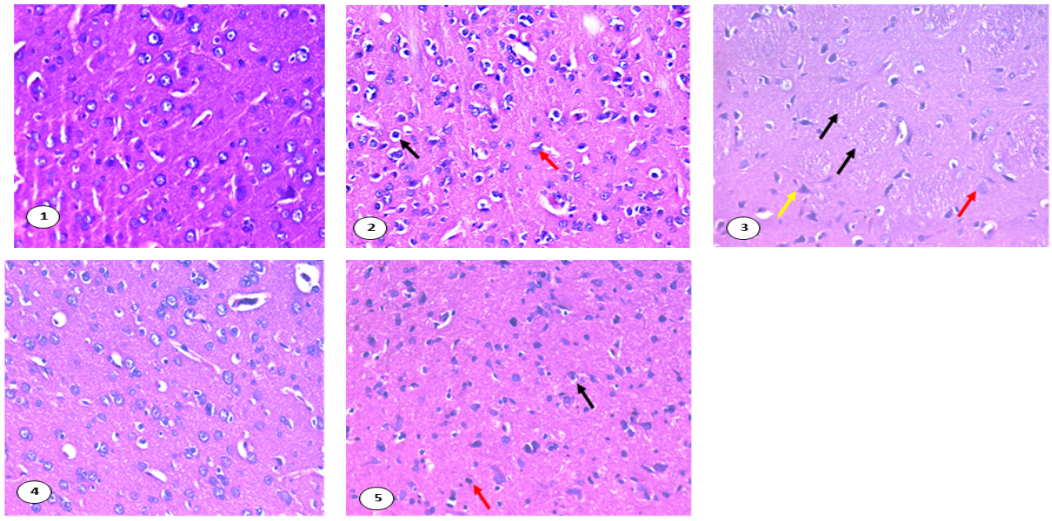


Figure 2:Effects of $AlCl_3$ and $AlCl_3$ + treatments (RIVA or VLP) on brain Total Protein (TP) content in different therapeutic groups: Data are presented as mean±SD (n = 10). Mean with different superscripts (a, b, c) are significant at p≤ 0.05. Values not sharing the same superscript letters were significantly different ($P < 0.05$).

3.6. Histopathological investigations of brain sections:
According to histopathological findings (photomicrographs 1:1 and 2) which corroborated the aforementioned biochemical findings; normal cellular structure of brain observed in normal senile control and normal-VLP groups, indicating normal brain functions. Conversely; AD-group showed spongy necrotic appearance, fatty changes, and loss of normal structure, outlines and nuclei of cells. (photomicrograph 1:3). AD-group proved that $AlCl_3$ mediates progressive alterations and loss of typical cellular structure; which are very well corroborated with the previous reports [53,85,86]. Furthermore, neuropathological examination of the AD-brain sections revealed that appearance of extracellular amyloid β (Aβ) plaques, as well as neurodegeneration, this corroborated with Kaizer *et al.*[87].
On the other hand, remarkable amelioration of brain architecture was observed in both AD-VLP (photomicrograph 1:4) and AD-RIVA (photomicrograph 1:5) groups; the brain cells appeared more or less similar to cells of the control group accompanied by the disappearance of most of amyloid plaques. Concerning RIVA, these results are in agreement with the results of Bihaqi *et al.*[85] who revealed that RIVA reversed/normalized Al-induced histopathological alterations.A study byBailey and Lahiri[88], showed that enhancement of neuronal morphology might be ascribed to AChE inhibition; thereby increasing the neurotransmitter acetylcholine (ACh) level which attenuates plaques and tangles formation[89]. Microscopic examination of brain sections of AD-VLP rats showed remarkable reduction in neurodegeneration with improvement in neuronal features. Moreover, the histopathological investigation revealed that treatment with VLP is more powerful than the RIVA, displayed fewer vacuoles that contained condensed neurons or partially degenerated neurons and fewer dark neurons with hyperchromatic nuclear chromatin, respectively.



Photomicrograph1: Microscopic investigation of brain sections of $AlCl_3$ intoxicated-rats and different therapeutic groups. Photomicrograph (1:1): Microscopic investigation of brain section of **negative control**senile group showing normal histological structure; numerous neurons are present within perineuronal spaces (black arrow),and with large vesicular nuclei. The small cells seen only as nuclei are glial cells (red arrow) (H&E, x200). **Photomicrograph (1:2):** Microscopic investigation of brain sections of **VLP-controls** showed normal brain tissue with dark neurons with dendrites (black arrow) and many degenerated neurons (red arrow), as well as, congestion with perivascular oedema (yellow arrow) (H&E stain, x200)**Photomicrograph (1:3):** Microscopic investigation of brain sections of **AD-group** showed dark neurons with dendrites (yellow arrow) and many other degenerated neurons (red arrow), revealed various sizes of amyloid plaques (black arrow) and diffuse gliosis (H&E stain, x200).**Photomicrograph (1:4):** Section of the brain of **AD-RIVA** rats, showed almost normal architecture showing neurons that appear more or less like normal one (H&E stain, x200). **Photomicrograph(1:5):** Section of the brain of **AD-VLP** rats showed normal brain tissue with several healthy neurons within perineuronal spaces (black arrow), with large vesicular nuclei. The small cells are glial cells (red arrow) (H&E stain, x200) Note: some vacuoles that contain condensed neurons or partially degenerated neurons.

Antioxidative and neuroprotective activities, as well as, other health-promoting properties of the active compounds in grapes were reported [90,91], several studies showed that grapes (leaves, seeds or juices) originated products are able to inhibit the oxidative stress-induced neurodegenerative disorders [92,93]. Polyphenols are excellent source of multiple profitable effects on the nervous system and could represent an important resource for the development of new drugs for management of neurodegenerative diseases [94].

Conclusion

It could be concluded that, VLP extract exhibited *in vivo* neuroprotective and antioxidant effects against the neurotoxin ($AlCl_3$)-induced brain injury in AD-rats. The possible underlying mechanisms are protecting neuronal cells through quenching free radicals; thereby reducing oxidative/nitrosative stress-mediated inflammation, and inhibiting Aβ-induced neurocytotoxicity in brain cells. The neurorestorative potential of VLP was displayed, not only in AD-induced rats, but also in senile control rats (might be cognitively deteriorated) which showed much more amelioration in biochemical parameters of oxidative stress, endothelial dysfunction and inflammation. Furthermore, Al-mediated and/or ageing-related biochemical alterations were reversed, where VLP extract was able to correct hyperlipidemia, oxidative stress, and neuroinflammation in the brain, which was confirmed by the histopathological findings of the brain tissues, wherein the amyloid plaques formed in Al-exposed rats had disappeared in treated rats as compared to AD-group. Altogether; polyphenols have not only a therapeutic impact on AD-induced rats but also a protective potential on age-modified biology as they reversed age-related declines. Besides being a neurodegenerative disease; AD could be regarded as a type of vascular diseases that is characterized by endothelial dysfunction and dyslipidemia.

Future studies

Altogether, this study showed the therapeutic neurorestorative impact of grape-derived polyphenols in neurodegenerative diseases, it is essential to recognize their active components and their underlying mechanisms. Further several clinical investigations must be performed for using VLP as nutraceutical for AD therapeutic interventions. Furthermore, this work aims to find a simple, reproducible and less-invasive set of blood-based biomarkers that could easily help in early diagnosis of AD and quick prediction of subjects prone to dementia.

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