Original Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ROLE OF *DUNALIELLA SALINA* EXTRACT IN COMPETING ALZHEIMER´S DISEASE IN EXPERIMENTAL ANIMALS

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ABSTRACT

The purpose of the current work was to study the role of *Dunaliella salina* extract in ameliorating Alzheimer´s disease (AD) in rat brain. Aluminium chloride ($AlCl_3$) was orally administered to male albino rats (100mg/kg/day for six weeks), while, *D. salina* ethanolic extract (150mg/kg/day for six weeks) was orally administered post $AlCl_3$ treatment. At the end of experiment, $AlCl_3$ treatment markedly increased lipid hydropyroxide (LOOH) and protein carbonyl (PC) levels while, decreased the total protein content (TPC) and total antioxidant capacity (TAC) when compared with normal control rats. As a result of $AlCl_3$ treatment, cerebral cortex showed pyknosis and necrosis of neurons as well as focal cerebral haemorrhage while, hippocampus showed pyknosis and necrosis of pyramidal cells. Treatment of AD rats with *D. salina* extract decreased the levels of lipid hydropyroxide and protein carbonyl, while it increased TPC and antioxidant status when compared with Al-induced rats. On the other hand, *D. salina* administered to neurodegenerative rats declared no histopathological changes in cerebral cortex meanwhile, hippocampus showed necrosis of sporadic pyramidal cells. In addition, the cerebral cortex of rivastigmine treated rats showed necrosis of sporadic neurons while, no histopathological changes were observed in hippocampus. The current research clearly indicates the potential of *D. salina* extract in counteracting the damage inflicted by $AlCl_3$ on rat brain regions.

KEY WORDS: ***Dunaliella salina*, Antioxidant, Oxidative stress, Alzheimer´s disease, Rivastigmine**

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INTRODUCTION

Aluminum (Al) element is considered as one of the widely available elements in the environment. High risk was detected for individuals exposed to all from different origin of environment and interference.[1] It has been involved in various neurodegenerative diseases of human, including Alzheimer's disease (AD), loss of memory, tremor, movements of jerky, and propagated spasms.[1,2] AD is initiated as a result of Al entry to the brain through its high affinity with transferring receptors localized in the blood brain barrier.[3] When AlCl3 enter inside the brain, the slow and fast transports of axon are affected, motivates inflammatory reaction, blocking long-term strength of nerve impulses along pathways, and creates abnormalities in synaptic structure leading to intense loss of memory.[3,4] At the genetic level, it affects topology of DNA, transcriptional of gene and metabolism of cellular energy.[5] It stimulates misfolding and self-accumulation of microtubule-linked proteins, euro-filaments which are highly phosphorylated cytoskeletal proteins and β-amyloid (Aβ) implicated in AD.[6] Impairment of mitochondrial function which leads up to production of uncontrolled free radical ultimately conducting DNA detritions, oxidation of protein residues and lipid peroxidation.[7] The reasonable machinery of AD has been correlated with destruction of cells by elaborated radical anion.[8] The elevation in malondialdehyde (MDA) product is considered as the principle ramification of oxidative stress.[1] A morphological biomarker of lipid peroxidation; Lipofuscin regarded as an end-product of lipids oxidative damage by free radical.[9] This inappropriately age-pigment, contains lipid peroxidation derivatives such as MDA and metal ions such as Cu, Fe, Zn and Al.[1,10] Aggregation of such ions and the competitiveness among Al and such fundamental elements can produce toxicity. In addition, perception of the allocation is critical in the determined of veracity threat for organisms.[1] It is markedly understood that in AD, the pro-oxidant-antioxidant equilibrium is disturbed resulting in perturbation of brain regular biological role and changes in energy pathways (lipids and proteins) accountable for knowledge come down in young animals.[11] Antioxidant and phenolic compound-derived plants and marine algae are being attempt as a chemoprevention and ameliorative agents in epidemiological and experimental researches to control the development of oxidative stress related diseases.[12] Antioxidant medication has been intended to recover the oxidative stress linked with various disorders. Clinically nootropic, agents may be utilized in the cases of learning abilities disorder, memory enhancement, mood and behavior, but side-effects of these agents made their utility limited.[12] Natural products are a source of substances such as carotenoids, with known antioxidant properties and possible benefits on diabetes.[13] Among them, *D. salina* is a microalga with high content in carotenoids. β-carotene is a remarkable antioxidant type, which performs by radical trapping preventing cancer of various organs like lungs, stomach, cervix, pancreas, colon, rectum, breast, prostate and ovary.[14] Additional to antioxidant characteristic, they can affect intracellular intercourse, immune reaction, transformation neoplastic cells and growth control.[14] Moreover, different carotenoid metabolites such as retinal, apocarotenoids, ketones, aldehydes and epoxides have an impact on the biochemical pathways.[15] Carotenoids combat versus radical ions trapping through electron denoting or through the formation of covalent adduct construction with these radicles. In these processes carotenoids with a natural electron rich create the signal to radicles, by which they preserve biomolecules (lipids, proteins and DNA) from radical deterioration effect.[14] Hence, in view of the aforementioned considerations the current study aimed to evaluate for the first time the potential role of *D. salina* in alleviating Al-induced neurotoxicity in experimental rats' model.

MATERIALS AND METHODS

Chemicals

Rivastigmine and all chemicals were purchased from Sigma Co (USA) and $AlCl_3$ from BDH Laboratory Supplies, Poole (UK).

Collection of algae and preparation of D. salina ethanolic extract

Dunaliella salina (Strain No. NIES-2257) was isolated by spreading 0.1ml of water samples collected from the Egyptian Company for Salts and Mineral (EMISAL) effluent ponds using BG11 media for algal isolation with addition of NaCl (100 g/L) into petri dishes containing 1.5% agar for solidification. Single colonies of algae were then recultivated in the specified liquid media as non-axenic batch cultures (50ml) at 25±2°C and 24hr with continuous white fluorescent lamp intensity ≈2500Lux. Cultivation was carried on an open pond with a capacity of 70L containing 55L of growth media. After cultivation, *Dunaliella salina* biomass was harvested using electro flocculation method. 100 g of *D. salina* powder were soaked in ethanol (80%) and shacked on shaker (Heidolph UNIMAX 2010) for 48 hrs at 150 rpm. The extract was filtered using a Buchner funnel and Whatman No. 4 filter paper and the algal residue was re-extracted with the addition of fresh ethanol (80%) for another two times. Combined filtrates were concentrated using Rotary evaporator (Heidolph-Germany) at 40°C under vacuum. The resulting dry extract was evaporated on a rotary vacuum evaporator to dryness. The dry extract was stored at -20˚C in a freeze and kept for further analysis.[16]

Experiment

Animals

Male albino rats (180-200 g) procured from Central Animal House, National Research Centre were used. Animals were acclimatized to the laboratory conditions at room temperature prior to the experimentation. Animals were kept under standard conditions of a 12 h light/dark cycle with food and water ad libitum in plastic cages with soft bedding. The protocol was approved by the National Research Centre Ethics Committee Guidelines for the use and care of animals (dated 29/10/2015).

Drug and treatment schedule

Aluminium chloride (CDH, India) solutions were made freshly at the beginning of each experiment. For oral administration, $AlCl_3$ was dissolved in drinking water and

administered in a dose of 100 mg/kg to rats daily for 6 weeks.[3] Rivastigmine tartrate (5 mg/kg b.w./day) diluted in ultra pure water daily for 6 weeks.[17] Animals were randomized into five groups (fifty adult male albino rats) based on their b.w. Each group had ten animals.

The groups were divided as follows:

Group one: Normal control rats.

Group two: Normal control rats treated with *D. salina* extract.

Group three: Serving as Al-intoxicated rats that were orally administered with $AlCl_3$.

Group four: Al-intoxicated rats treated orally daily with *D. salina* ethanolic extract for 6 weeks in a dose of 150 mg /Kg b.w.[18]

Group five: Al-intoxicated rats orally administered daily with rivastigmine standard drug.

Brain tissue sampling and preparation

At the end of the experiment, the rats were fasted overnight, subjected to anesthesia with diethyl ether and sacrificed. The whole brain of each rat was rapidly dissected, washed with isotonic saline and dried on filter paper. Each brain was divided sagitally into two portions. The first portion was weighed and homogenized in ice-cold medium containing 50 mMTris/HCl and 300mM sucrose at pH 7.4 to give a 10 % (w/v) homogenate.[19] This homogenate was centrifuged at 4000 × g for 10 min at 4 °C. The supernatant was stored at -80 °C and used for biochemical analyses (oxidative stress biomarker; lipid hydroperoxide, protein carbonyl, TPC and TAC). The second portion of the brain was fixed in 10 % formalin for histological investigation. The ethical conditions were applied such that the animals suffered no pain at any stage of the experiment. Animals were disposed of in bags provided by the Committee of Safety and Environmental Health, National Research Center.

Biochemical analyses

Determination of LOOH, protein carbonyl, TPC and TAC

LOOH levels were measured in brain tissue by the method of Haldebrandt and Roots.[20] The protein oxidation was measured in brain tissue by estimating the protein carbonyl levels according to the method of Liu et al.[21] The protein content was measured by the method of Lowry et al.[22] using bovine serum albumin (BSA) as standard. Brain TAC was assayed according to the method of Koracevic et al.[23]

Histological examination

The brain tissue was fixed in 10 % formalin for one week, washed in running tap water for 24 h and dehydrated in ascending series of ethanol (50–90 %), followed by absolute alcohol. The samples were cleared in xylene and immersed in a mixture of xylene and paraffin at 60 ºC. The tissue was then transferred to pure paraffin wax of the melting point 58 ºC and then mounted in blocks and left at 4 ºC. The paraffin blocks were sectioned on a microtome at thickness of 5 µm and mounted on clean glass slides and left in the oven at 40 ºC to dryness. The slides were deparafinized in xylene and then immersed in descending series of ethanol (90–50 %). The ordinary haematoxylin and eosin (H&E) stain was used to stain the slides.[24]

Statistical analyses

Statistical analysis is carried out using SPSS computer program (version 8) combined with co-state computer program, where unshared letters are significant at $P \leq 0.05$.

RESULTS

LOOH and protein carbonyl levels in control and treated rats

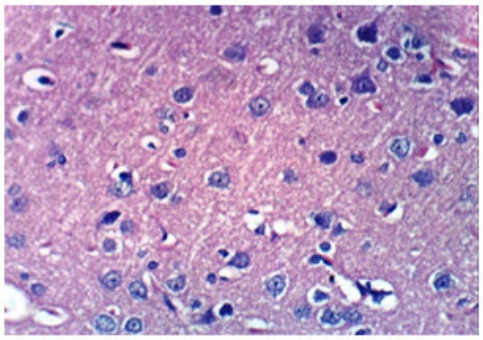
The present results revealed insignificant differences in LOOH and protein carbonyl in *D. salina* extract treated normal rats comparing with untreated one. While, significant increase in lipid hydroperxide and protein carbonyl levels in Al-treated rats were detected with percentages increase 115.82 and 98.75%, respectively. *D. salina* extract treated neurotoxic rats showed marked improvement in lipid hydroperxide and protein carbonyl levels with percentages of amelioration 102.07 and 78.25%, respectively however, they still recorded significant increase as compared to normal control rats (Table 1). Meanwhile, the amelioration percentages reached to 85.19 and 80.00%, for lipid hydroperxide and protein carbonyl levels, respectively upon treated neurotoxic rats with reference drug, rivastigmine.

TPC and TAC in control and treated rats

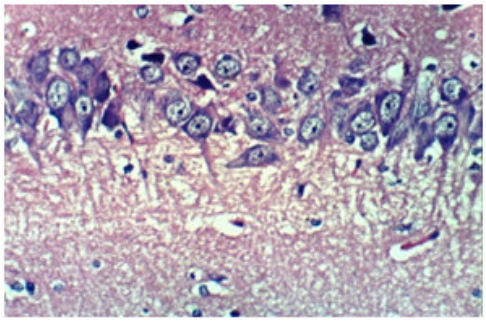
As shown in Table (2), significant difference was detected in TPC in normal rats treated with *D. salina* extract comparing with untreated normal one. Al-treated rats showed significant reduction in TPC with percentage 58.07%. While, Al-treated rats with *D. salina* extract and rivastigmine exhibited significant decrease in TPC as compared to normal control rats with more or less similar improvement percentages 49.56 and 50.93%, respectively (Table 2). Furthermore, significant increase in TAC in normal control treated rats with *D. salina* with percentage increase 42.03 % as compared to untreated normal one (Table 2). Al-exposed rats declared significant decrease in TAC (53.62%), comparing with normal control rats. Marked increase in TAC in Al-treated rats with *D. salina* extract was detected comparing with normal control one with amelioration percent 88.41% which is higher than that was recorded for standard drug (63.77%).

Cerebral cortex and hippocampus architectures of control and treated rats

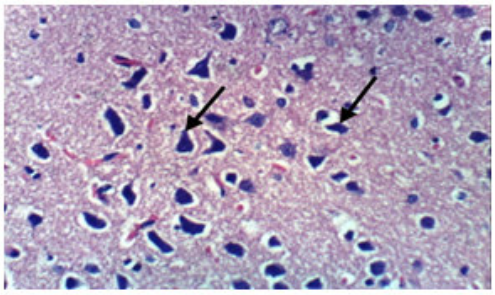
Cerebral cortex of Al-exposed rats showed pyknosis and necrosis of neurons as well as focal cerebral haemorrhage (Micrographs 3-5) as compared to normal rats (Micrographs 1,2). While, the hippocampus of Al-induced AD showed pyknosis and necrosis of pyramidal cells (Micrograph 6) comparing to normal control rats. However, the cerebral cortex of *D. salina* treated neurotoxic rats showed no histopathological changes (Micrograph 7). Meanwhile, hippocampus of *D. salina* extract treated AD induced rats showed necrosis of sporadic pyramidal cells (Micrograph 8). In addition, the cerebral cortex of rivastigmine treated neurotoxic rats showed necrosis of sporadic neurons (Micrograph 9). The hippocampus of rivastigmine treated AD induced rats showed no histopathological changes (Micrograph 10).



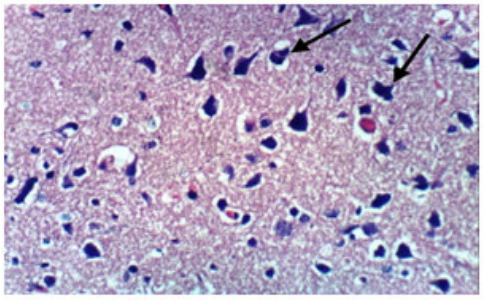
Micrograph 1
Cerebral cortex of rats from control untreated rats showing no histopathological changes (H & E X 400).



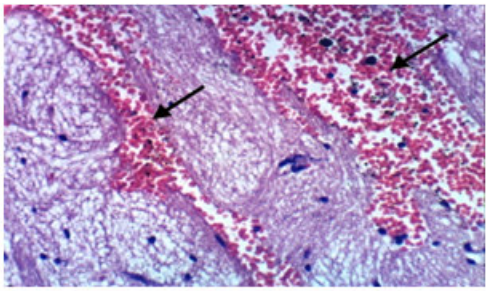
Micrograph 2
Hippocampus of rats from control untreated rats showing no histopathological changes (H & E X 400).



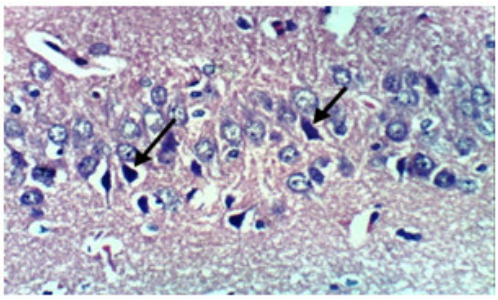
Micrograph 3
Cerebral cortex of Al-induced AD rats showing pyknosis and necrosis of neurons (H & E X 400).



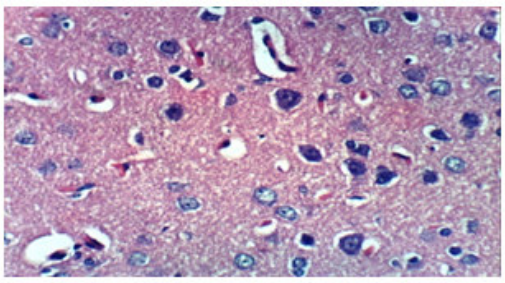
Micrograph 4
Cerebral cortex of AD induced rats showing pyknosis and necrosis of neurons (H & E X 400).



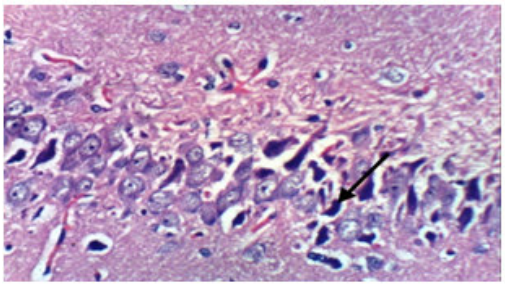
Micrograph 5
Cerebral cortex of Al-induced AD rats showing focal cerebral haemorrhage (H & E X 400).



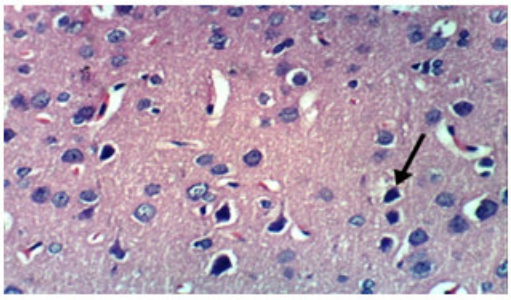
Micrograph 6
Hippocampus of Al-induced AD rats showing pyknosis and necrosis of pyramidal cells (H & E X 400).



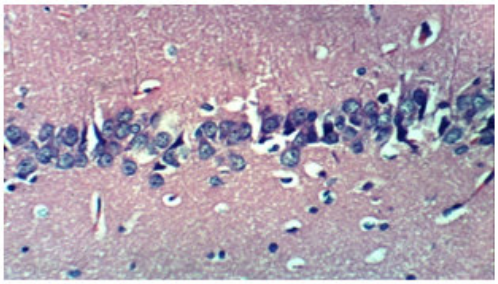
Micrograph 7
Cerebral cortex of D. salina treated Al-induced AD showing no histopathological changes (H & E X 400).



Micrograph 8
Hippocampus of D. salina treated Al-induced AD showing necrosis of sporadic pyramidal cells (H & E X 400).



Micrograph 9
Cerebral cortex of rivastigmine treated Al-induced AD showing necrosis of sporadic neurons (H & E X 400).



Micrograph 10
Hippocampus of rivastigmine treated Al-induced AD showing no histopathological changes (H & E X 400).

Table 1
LOOH and protein carbonyl levels in brain tissue of control and* treated *rats

Biomirkers / Groups	**LOOH** (nmol/mg protein)	**Protein carbonyl** (nmol/mg protein)
Normal control	458.97±9.10[f]	4.00±0.10[a]
Normal treated with *D. salina*	460.25±12.50[f]	3.92±0.13[a]
%change	0.11	2.00
Al–intoxicated rats	992.21±50.10[a]	7.95±1.20[b]
%change	115.82	98.75
D. salina treated Al–intoxicated rats	522.90±11.19[d]	4.82±0.9[d]
%change	13.74	20.50
%of improvement	102.07	78.25
Rivastigmine treated Al–intoxicated rats	600.56±11.95[e]	4.75.066[d]
%change	30.63	18.75
%of improvement	85.19	80.00

Data are means ± SD of ten rats in each group.
Data are expressed as nmol/mg protein for LOOH and protein carbonyl.
Statistical analysis is carried out using SPSS computer program (one way ANOVA, version 8) coupled with Co-stat computer program, where unshared letters between groups are the significance value at P≤ 0.05.

Table 2
***TAC and TPC in brain tissue of control and* treated rats**

Biomirkers / Groups	**TPC** (mg/g of brain tissue)	**TAC** (umol/L)
Normal control	82.10±3.90[b]	0.69±0.01[a]
D. salina	80.10±6.90[b]	0.98±0.05[b]
%change	0.25	42.03
Al–intoxicated rats	33.50±4.60[c]	0.32±0.01[e]
%change	58.07	53.62
D. salina treated Al–intoxicated rats	73.10±5.90[e]	0.93±0.06[b]
%change	8.51	34.78
%of improvement	49.56	88.41
Rivastigmine treated Al–intoxicated rats	74.20±6.10[e]	0.76±0.04
%change	7.13	10.14
%of improvement	50.93	63.77

Data are means ± SD of ten rats in each group.
Data are expressed as mg/gram of brain tissue for TPC and lipid hydroperoxide and umol/L for TAC.
Statistical analysis is carried out using SPSS computer program (one way ANOVA, version 8) coupled with Co-stat computer program, where unshared letters between groups are the significance value at P≤ 0.05.

DISCUSSION

The present study clearly indicated that, AD induced marked elevation in brain LOOH as well as protein carbonyl levels as biomarkers of oxidative stress and protein oxidation associated with significant reduction in TAC and TPC. The present study is in agreement with Tripathi et al.[11] who declared that, AD initiated obvious destruction of biomolecules, impairment in the concentrations of fundamental metals as well as modification in the rat brain architectures. It is possible that, these effects could be attributed to the alteration in cell architectures, DNA sequencing as well as metabolic pathways of neurotoxic rats.[25] Levesque et al.[26] declared that, AD is associated with the suppression in the erythrocyte formation and diminution of their iron content due to Fe combined with Al and receptor of transferring prior passing *via* blood brain barrier and hence enter inside the brain and saved for long time.[27] Although, Zeeca et al.[28] and Tripathi et al.[11] illustrated the neurodegenerative alterations in AD to the brain ions impairment (high level of Al, Fe, Zn in the brain tissue while, low Cu and Se concentrations).The

increase in LOOH and protein carbonyle levels, while reduction in TPC and TAC in AD may be related to the decrease in axonal mitochondria transformation, perturbation of golgi and reduction of synaptic vesicles which results in the release of oxidative products like hydroperoxide and carbonyls as well peroxy nitrites, while decrease in antioxidant enzymes and glutathione within the neurons.[29]The current results also investigated oxidative destruction of brain lipids, as assessed by the high level of LOOH output and protein carbonyl while, decreased in the TPC in brain tissue of AD induced rats. This may be correlated with Fe elicited ROS and due to brain contains high level of polyunsaturated fatty acids which can be easily interact with elaborated radicals and afford oxidative destruction.[25,30] In AD induced rats, high ROS production was detected, stimulating lipids and proteins damage.[11] Also, Aβ plaques which characteristic neurodegenerative brains, can also promote the formation of reactive oxygen species (ROS) *via* ferrous (Fe2+) form impairment which produces OH^- radicals.[31] These radicals attack biomolecules to generate pigment deposition in Alzheimer´s induced rat.[1] Hence, AD enhance proteins oxidative transformation and increase lipofuscin which could be harmfully impact on the neurons, leading to antioxidants and metal ions depletion.[32] It's well known that, brain contains various enzymes responsible for antioxidant defense mechanism such as superoxide dismutase (SOD) which has a principle effect in ROS prevention and tissue perturbation. Also, brain contains reduced glutathione (GSH) and catalase which preserve it versus H_2O_2 mediated destruction of neuron, proteins and lipids oxidation as demonstrated by Kumar et al.[3,32] and Aly et al.[33] So, the decreased level of TAC and TPC in the current study indicating severity of neurotoxicity in the brain tissue and the exhaustion of antioxidant enzymes. The present data are also in harmony with Bihaqi et al.[17] who showed Al conduct noticeable elevation in protein carbonyl level, as an index of protein oxidation by ROS. The increased aggregation of these by product markedly indicated the oxidative damage of aluminium. Histopathological investigation showed cerebral cortex of AD with pyknosis and necrosis of neurons as well as focal cerebral hemorrhage. While, hippocampus showed pyknosis and necrosis of pyramidal cells comparing with normal control rats. The cerebral cortex and hippocampus were selected for many reasons: Al damages these regions more drastically.[34] These brain regions are well known to be specialized admitting in learning and memory. In a good agreement with the present results Bihaqi et al.[17] declared AD periphrasis, command obvious cerebral cortex architectures change inclusive neuronal degradation such as, cytoplasmic vacuolization, hemorrhage, ghost cells and gliosis.On the other hand ,the improvement in lipid hydroperoxide, protein carbonyls, TPC and TAC, beside the cerebral cortex architecture of brain post treatment of AD rats with *D. salina* may be realize on the basis of β-carotene which is the precursor of vitamin A, able to trapping free radicals. Carotenoids are lipophilic in nature, so in lipid environment of biological system are predictable to spend most of their antioxidant action.[35,36] Levy et al.[37] also confirmed the defensive β-carotene role against oxidative destruction. There are few researchers examined the *in vivo* β-carotene effectiveness.[38-40] Hence, Lin et al.[41] declared low-density lipoprotein (LDL) preservation upon using 6.2 moles of β-carotene/day. While, Seddon et al.[42] detected β-carotene useful function in prevention of molecular degeneration. Several studies have investigated that carotenoids in the circulation are essential for regulating cytochrome P450 and controlling metabolizing enzymes.[43] Murthy et al.[14] and Michalak and Chojnacka[44] indicated β-carotene protective function against oxidative destruction which occurs mainly by lipid peroxidation. Moreover, it recovers the antioxidant defense enzymes in liver. In a good agreement with Murthy et al.[14] , the present results indicated high TAC in normal rats treated with *D. salina* extract as compared to untreated normal control one. The authors added that, the protection presented to the animals fed with Dunaliella carotenoids extract is apparent from conservation of liver enzymes even post toxin treatment. The MDA level was recovered three times upon using 125µg Dunaliella carotenoid. Carotenoids of Dunaliella containing both isomers of cis and transform along with other xanthophyll and carotenoids probably act as a potential free radical trapping, lowering hydrogen peroxide and superoxide anion levels leading to recovery of the antioxidant enzymes system. The trans β-carotene bioavailability is demonstrated to be 3 fold higher than cis, similar is present to be higher in cis isomer of lycopene.[45-47] This may also pointed out to the probable de novo synthesis of the antioxidant enzymes stimulated by various carotenoids types of Dunaliella.[14]

CONCLUSION

The results of the present work demonstrated that, *D. salina* ameliorated the cerebral cortex neurotoxic disorders. In addition, it suppressed oxidative destruction and architecture alterations at the cellular level. *D. salina* declared auspicious out come in normalizing the proteins and lipid levels. Thus, *D. salina* is a prospect formularization for ameliorating neurotoxic diseases. Further investigations are guaranteed to investigate the functional compounds of this extract in addition to indicate the accurate technicality by which the natural compounds potentiate either a neuroprophylactic or therapeutic effectiveness. However, several clinical trials will be performed before giving any therapeutic recommendations to community.

ACKNOWLEDGMENT

This work was supported and funded by the project entitled "Biodiesel production from algae as a renewable energy source". Funding organization: Research Development and Innovation program (RDI), Funding Program: EU-Egypt Innovation Fund, 2014-2016.

CONFLICT OF INTEREST

Conflict of interest declared none.

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