

ORIGINAL ARTICLES

Effect of some nutraceutical agents on aluminum-induced functional neurotoxicity in senile rats: I. Effect of rosemary aqueous extract and docosahexaenoic acid

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ABSTRACT

Alzheimer's disease (AD) is the most common chronic neurodegenerative disorder associated with aging. Aluminum is a potent neurotoxin that plays a pivotal role in the neuropathology of AD. The current study was designed to investigate the possible neuroprotective effects of rosemary (*Rosmarinus officinalis*) aqueous extract of (RAE) and docosahexaenoic acid (DHA) in ameliorating aluminum chloride (AlCl₃) induced neurotoxicity in male senile rats. Rats were allocated into seven groups and treated daily by oral administration for three months as follows: normal control; olive oil control; RAE (440 mg/kg b.w. /day); DHA dissolved in olive oil (300 mg/kg b.w. /day); AlCl₃ only (100 mg/kg b.w. /day); AlCl₃ plus RAE; AlCl₃ plus DHA. At the end of the experimental period, the two brain areas, cortex and hippocampus as well as blood were taken. Lipid peroxidation, total antioxidant capacity (TAC), nitric oxide (NO), urea, creatinine and calcium levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Na⁺/K⁺-ATPase (ATPase), acetylcholinesterase (AChE) activities were determined using spectrophotometric analysis. Besides, Tumor Necrosis Factor- α (TNF- α) and Interleukin -1 β (IL-1 β) levels were measured in serum using ELISA technique. The results revealed that AlCl₃-induced neurotoxicity caused significant increases in cortical and hippocampal lipid peroxidation, NO, ionized calcium levels as well as AChE activity. Additionally, serum ALT, AST, NO, total calcium, urea, creatinine, TNF- α and IL-1 β values were also elevated significantly due to AlCl₃ intoxication. Moreover, TAC level and Na⁺-K⁺ ATPase activity were significantly reduced in brain cortex and hippocampus of AlCl₃-treated rats. Manipulation with RAE and DHA improved the neurological damages induced by AlCl₃ as indicated by the modulations in most of the biochemical markers. In conclusion, RAE and DHA have neuroprotective effects against AlCl₃-induced cognitive dysfunction and oxidative damage. This may be attributed to its powerful antioxidant activities.

Key words: Aluminum chloride, rosemary, docosahexaenoic acid, brain aging, Alzheimer's disease.

Introduction

Normal aging is accompanied by declines in motor and cognitive performance (Joseph *et al.*, 2005). These declines are amplified in age-related neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), AD, and Parkinson's disease (PD). As the elderly population increases, so will the prevalence of these age-related disorders increase (Nicita-Mauro, 2002). The etiology of these disorders is multifactorial including genetics, head trauma, oxidative stress, inflammation and environmental factors including aluminum toxicity. The potential association of aluminum and AD began at 1965 with the observations which demonstrated the neurofibrillary degeneration in rabbits following aluminum exposure (Klatzo *et al.*, 1965). Aluminum is the most abundant metal on the earth crust, reported to gain access to the body via the gastrointestinal tract and lung tissue. It is a commonly exposed neurotoxin and possesses multiple mechanisms of action on the central nervous system (Miu and Benga, 2006). Experimentally, it has been demonstrated that chronic exposure to aluminum not only causes neurologic signs which mimic progressive neurodegeneration but also results in neurofilamentous changes in the hippocampus, cerebral cortex, brain stem, spinal cord, and biochemical changes which are seen in AD (Bharathi *et al.*, 2006). AD is characterized pathologically by the presence of senile plaques, neurofibrillary tangles (NFT), synapse loss, and neuropil threads. The senile plaque is composed of a core of amyloid beta (A β) surrounded by dying neurites. A β is produced from the proteolytic cleavage of amyloid precursor protein, a ubiquitously expressed transmembrane glycoprotein (Giovannini *et al.*, 2002).

There is substantial evidence that oxidative stress plays an important role in the aging process (Harman, 2001). Oxidative stress, caused by reactive oxygen species, is known to cause the oxidation of biomolecules leading to cellular damage. It is also speculated to be pathologically important in various neurodegenerative

processes including cognitive deficits that occur during normal cerebral aging, Alzheimer's, and Parkinson's diseases (Smith *et al.*, 1996).

Interestingly, intake of polyphenols through diets rich in fruits and vegetables was stated to reduce incidence of certain age-related neurological disorders including macular degeneration and dementia (Commenges *et al.*, 2000; Bastianetto and Quirion, 2002). Therefore, these data suggest that high dietary or supplemental consumption of antioxidants in people may reduce the risk of AD (Orhan *et al.*, 2007).

DHA is the most abundant ω -3 fatty acid in the mammalian central nervous system, and is specifically concentrated in membrane lipids of brain grey matter and the visual elements of the retina. The levels of DHA in the brain increase during development (Martinez, 1992) and decrease with aging (Guisto *et al.*, 2002). Both retina and brain levels of DHA are altered by the dietary ω -3 and ω -6 fatty acid supply (Innis, 1991). Omega-3 fatty acids may play a role in nervous system activity, improve cognitive development and reference memory-related learning, increase neuroplasticity of nerve membranes, contribute to synaptogenesis and are involved in synaptic transmission (Mazza *et al.*, 2007). A number of critical trials have confirmed the benefits of dietary supplementation with omega-3 fatty acids not only in several psychiatric conditions, but also in inflammatory, autoimmune and neurodegenerative diseases (Mazza *et al.*, 2007).

The present work is designed to investigate the neuroprotective effect of one of plant extracts that have potential antioxidant properties in ameliorating aluminum chlorid -induced cognitive dysfunction and oxidative damage in senile rats compared to those treated with DHA, a nutraceutical compound with antioxidant (Ozsoy *et al.*, 2011) and neuroprotective properties (Innis, 1991; Mazza *et al.*, 2007).

Materials and Methods

Animals:

Male senile albino rats weighing 250-300 gm were used in this study. Rats were obtained from Animal House Colony of National Research Centre, Giza, Egypt. Animals were then divided into seven groups (14 rats / group) and housed in stainless steel cages in a temperature-controlled and artificially illuminated room free of any chemical contamination. Fresh tap water and standard rodent food pellets (proteins, lipids, fibers, NaCl, lysine, methionine, vitamins, salts and wheat) were always available. All animals received human care in compliance with the guidelines of the Animals Care and Use Committee of National Research Centre, Egypt.

Material Sources:

The used herbs were collected during the period of January 2010 and purchased to the stores of Abd El-Rahman Harraz (Bab El-Khalk zone, Cairo, Egypt). Omega-3 fatty acid (Omega-3 Plus®) was purchased from SEDICO Pharmaceuticals Co. 6 October City- Egypt. Aluminum chloride anhydrous LR (AlCl_3) was obtained from fine-chem. limited, Mumbai, India.

Herb extraction:

Aqueous extracts from accession of marigold (*Calendula officinalis*), sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), and lemon balm (*Melissa officinalis*) were prepared.

According to the method of Gulcin *et al.* (2006), the aqueous extractions were carried out. 100 g of the powdered herb material were placed in a 1000 ml round-bottom quick fit flask, and 400 ml distilled water were added. The mixture was left for 24 hours. Water fractions were combined and filtered through qualitative No.1 Whatman filter paper (Whatman International Ltd, Maidstone, England). In Aroma and Flavoring Department, National Research Center, the filtrate was subjected to lypholization process through freeze drier (Snijders Scientific-tilburg, Holland) under pressure, 0.1 to 0.5 mbar and temperature -35 to -41°C conditions. The dry extract was stored at -20°C until used.

We examined *in vitro* the antioxidant activities of the aqueous extracts prepared from the tested plants to select the most active one for using in this study.

Determination of total phenolic contents (TPC):

The concentration of phenolics in the herb extracts was determined using the method of Jayaprakasha *et al.* (2003) and the results were expressed as catechin equivalents (CE). 5 mg of the extract was dissolved in a 10 ml mixture of acetone and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of 10-folds diluted Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate solution (7.5%). After 30 min at room temperature, the absorbance was measured at 765 nm using UV – 160 IPC UV-visible spectrophotometer. Estimation of phenolic compounds as catechin equivalents (CE) was carried out using standard curve of catechin.

Determination of Radical Scavenging Activity (RSA) by 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay:

The capacity of antioxidants to quench DPPH radical was determined according to Nogala-Kalucka *et al* (2005) method and calculated according to the following equation:

$$\text{RSA\%} = ([\text{Absorbance of control sample} - \text{absorbance of sample with herb extract}] / \text{absorbance of control sample}) \times 100$$

Certain of crude extract were dissolved in methanol to obtain a concentration of 200 ppm. 0.2 ml of this solution was completed to 4 ml by MeOH and 1 ml of DPPH (6.09×10^{-5} mol/L) solution in the same solvent was then added. The absorption was monitored after 10 min at 516 nm. The reference sample (blank) was 1 ml of DPPH solution and 4 ml MeOH.

Based on the highest free radical scavenging activity, the best extract, rosemary aqueous extract was used in current study.

Animal Grouping:

Animals were divided into seven groups (14 rats / group) and treated orally for three months as follows: Group 1, control; Group 2, normal healthy animals received olive oil only; Group 3, animals received RAE alone (440 mg/kg b.w. /day) (Amin and Hamza 2005); Group 4, animals received DHA (Omega-3 Plus®) alone dissolved in olive oil (300 mg/kg b.w. /day) (Holguin *et al.*, 2008); Group 5, animals received AlCl_3 alone dissolved in distilled water (100 mg/kg b.w. /day) (Sethi *et al.*, 2009); Group 6, animals received combined treatments of AlCl_3 and RAE; Group 7, animals received combined treatments of AlCl_3 and DHA.

Tissue collection:

At the end of the experimental duration, one half of animals of each group were suddenly decapitated (to avoid the biochemical changes which may occur as a result of ischemia). From the decapitated animals, brains were removed and each hippocampus and cerebral cortex were dissected out, weighed and thoroughly washed with isotonic saline. The individual hippocampus and cerebral cortex of each animal were homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 mM tris-HCl and 300 mM sucrose (pH 7.4) (Tsakiris *et al.*, 2004). The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 0°C. The supernatant was examined for the detection of MDA, TAC, AChE, NO and calcium.

The cerebral cortex and hippocampus were chosen for number of reasons: (a) AlCl_3 affects the hippocampus and cortex regions more severely than any other area of the central nervous system. (b) These brain regions are known to be particularly susceptible in Alzheimer's disease, and have an important role in learning and memory functions (Bihaki *et al.*, 2009).

Blood sampling:

From the second part of the animals of each group, blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia (Schermer, 1967) in clean tubes and then centrifuged at 3000 rpm for 10 minutes. The separated sera were stored at -20°C till examined for the detection of AST, ALT activities, IL-1 β , TNF- α , NO, calcium, creatinine and urea.

Analytical Determinations:

MDA as an indirect index for lipid peroxidation was determined in hippocampus and cerebral cortex homogenates following the method described by Ruiz-Larnea *et al.* (1994). Determination of TAC was carried out in hippocampus and cerebral cortex homogenates using Biodiagnostic Kit, Egypt. Colorimetric determination of nitrite as indicator of NO production in biological fluids was carried out using Biodiagnostic Kit, Egypt. Determination of AChE activity in hippocampus and cerebral cortex homogenates was performed according to the manual instruction of AMS Kit, United Kingdom. Na^+/K^+ -ATPase activity in hippocampus and cerebral cortex homogenates was determined according to the modified method of Tsakiris *et al.* (2004). Colorimetric calcium determination was performed using a Kit produced by Biodiagnostic Co., Egypt. AST and ALT activities and creatinine level were determined in serum according to the manual instruction of Quimica Clinical Aplicada S.A.(QCA) kits, Spain, and the quantitative determination of urea was performed in serum using Intermedical Kit, Italy. IL-1 β was determined in serum using Orgenium Laboratories Business Unit ELISA (Enzyme-Linked Immunosorbent Assay) kit, Finland. TNF- α was determined in serum according to the manual instruction of RayBiotech, Inc. ELISA kit, USA.

Statistical analysis:

The obtained data were subjected to one way analysis of variance (ANOVA). The analysis was performed using statistical analysis system (SAS) program software; copyright (c) 1998 by SAS Institute Inc., Cary, NC, USA. Tukey test (Steel and Torrie, 1980) was used to evaluate the significance between the individual groups at $p < 0.05$. The values in this study were expressed as mean \pm standard error.

Results:

The yields, TPC and RSA of the aqueous extracts of tested natural herbs rosemary (*Rosmarinus officinalis*), marigold (*Calendula officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), and lemon balm (*Melissa officinalis*) are illustrated in Table (1). From the obtained data, it can be clearly noticed that RAE possesses the highest RSA (one of the antioxidant mechanism) among the extracts of the other herbs. Therefore, RAE was chosen from all other examined extracts to be one of the selected nutraceuticals beside DHA which were used in this study to investigate their effects in delaying the progression of $AlCl_3$ -related neurodegenerative disorders.

Table 1: Yield, TPC and RSA of aqueous extracts of some natural herbs.

	Marigold	Rosemary	Thyme	Sage	Lemon Balm
Yield (g %)	9.2 \pm 0.3	8.2 \pm 0.3	9.7 \pm 0.3	4.5 \pm 0.3	3.8 \pm 0.3
TPC (mg/g)	1.5 \pm 0.1	2.8 \pm 0.1	3.4 \pm 0.1	2.3 \pm 0.1	1.3 \pm 0.04
RSA (%)	72.4 \pm 2.3	87 \pm 0.8	72.4 \pm 1.6	86 \pm 0.8	73.4 \pm 1.2

All values are represented as means \pm standard error for 3 measurements (M \pm SE).

TPC: Total phenolics content, RSA: Radical scavenging activity.

The effects of RAE and DHA against $AlCl_3$ induced changes in NO, TAC and the level of lipid peroxidation end product MDA are illustrated in Table (2). Animals received RAE or DHA recorded insignificant decreases ($P \geq 0.05$) in MDA, NO and TAC levels in brain cortex and hippocampus compared with the normal control and olive oil control, respectively. On the other hand, animals intoxicated with $AlCl_3$ exhibited a significant increase ($p < 0.05$) in MDA level concomitant with a significant decrease ($p < 0.05$) in TAC level in both brain areas when were compared to normal control. $AlCl_3$ could increase significantly NO level in both brain areas and also in serum. Administration of RAE or DHA to rats succeeded to prevent significantly the $AlCl_3$ -induced changes in the mentioned parameters.

Table 2: Effects of RAE and DHA on oxidative stress markers in $AlCl_3$ -intoxicated senile rats.

	Malondialdehyde		Nitric oxide			Total antioxidant capacity	
	Cortex (nmol/g)	Hippocampus (nmol/g)	Cortex (nmol/g)	Hippocampus (nmol/g)	Serum (nmol/L)	Cortex (μ mol/g)	Hippocampus (μ mol/g)
Control	917 \pm 20.1 ^{de}	726 \pm 9.1 ^c	32.2 \pm 0.3 ^{gh}	29.8 \pm 0.4 ^{fg}	16.1 \pm 0.2 ^{ef}	527 \pm 12.1 ^{ab}	863 \pm 15.7 ^a
Olive oil	889 \pm 19.5 ^e	705 \pm 8.8 ^{cd}	31.8 \pm 0.4 ^h	29.2 \pm 0.4 ^g	15.7 \pm 0.2 ^{ef}	542 \pm 12.4 ^{ab}	890 \pm 16.1 ^a
RAE	875 \pm 12.3 ^e	694 \pm 6.6 ^{cd}	31 \pm 0.3 ^h	27.7 \pm 0.5 ^{gh}	15.6 \pm 0.3 ^f	545 \pm 16.6 ^a	887 \pm 11.5 ^a
DHA	883 \pm 14.9 ^e	701 \pm 11.6 ^{cd}	31 \pm 0.3 ^h	28.9 \pm 0.3 ^{gh}	16 \pm 0.2 ^{ef}	527 \pm 13.6 ^{ab}	866 \pm 8.6 ^a
$AlCl_3$	1129 \pm 24.8 ^a	932 \pm 11.6 ^a	41.1 \pm 0.5 ^a	40.3 \pm 0.5 ^a	20 \pm 0.4 ^a	417 \pm 8.8 ^c	304 \pm 15.9 ^d
$AlCl_3$ +RAE	974 \pm 12.7 ^{cd}	877 \pm 3.5 ^b	34.5 \pm 0.3 ^{de}	33.7 \pm 0.4 ^d	17.4 \pm 0.2 ^{cd}	488 \pm 17.4 ^{bcd}	700 \pm 13.7 ^b
$AlCl_3$ +DHA	1035 \pm 24.7 ^{bc}	888 \pm 7 ^b	35.6 \pm 0.2 ^{cd}	34.7 \pm 0.4 ^{cd}	17.8 \pm 0.1 ^c	467 \pm 10.9 ^{cde}	681 \pm 19.3 ^{bc}

Data are presented as mean \pm S.E. Within each column, means with different letters are significantly different ($P < 0.05$) using one way (Tukey tests) ANOVA test.

The data in Table (3) show that treatment with $AlCl_3$ resulted in significant increases in AChE activity in brain cortex and hippocampus and calcium level in both brain areas and serum, whereas Na^+/K^+ -ATPase activity in brain cortex and hippocampus was decreased significantly by $AlCl_3$ administration. Co-treatment with RAE or DHA with $AlCl_3$ resulted in significant protection of these parameters against the above mentioned effects of $AlCl_3$.

Table (4) comprises the selected specialized serum markers of liver (AST and ALT) and kidney (urea and creatinine) functions among the different groups. It is clearly indicated that RAE and DHA had no effects on these markers indicating the safety of RAE and DHA. Administration of RAE or DHA in combination with $AlCl_3$ showed modulatory positive action on liver and kidney functions as indicated by the improvement of the previously mentioned parameters investigated.

Concerning the effect of $AlCl_3$ on serum TNF- α (Figure 1) and IL-1 β (Figure 2) levels, animals received $AlCl_3$ showed significant ($P < 0.05$) increase in the concentration of serum TNF- α and IL-1 β as compared to normal control. The data in the figures also revealed a moderate protective effect of RAE and DHA on these cytokines against $AlCl_3$ toxicity.

Table 3: Effects of RAE and DHA on the activities of AChE and Na⁺/K⁺-ATPase, and calcium level in AlCl₃-intoxicated senile rats.

	AChE		Calcium			Na ⁺ /K ⁺ -ATPase	
	Cortex (U/g)	Hippoca. (U/g)	Cortex (mg/g)	Hippoca. (mg/g)	Serum (mg/dL)	Cortex (μmol pi/h/g)	Hippoca. (μmol pi/h/g)
Control	36.7±5.5 ^c	65.2±5.7 ^b	7.1±0.3 ^d	8.2±0.3 ^d	10.9±0.4 ^d	5.4±0.3 ^a	9.6±0.5 ^a
Olive oil	35.7±5.3 ^c	63±5.5 ^b	7.4±0.3 ^d	8.3±0.3 ^d	10.6±0.3 ^d	5.6±0.3 ^a	10.1±0.5 ^a
RAE	35.3±1.5 ^c	65±2.1 ^b	7.1±0.2 ^d	8.2±0.5 ^d	10.5±0.2 ^d	5.6±0.1 ^a	9.8±0.4 ^a
DHA	35.7±1.5 ^c	65.1±1.6 ^b	7.2±0.3 ^d	8.3±0.3 ^d	11±0.4 ^d	5.3±0.1 ^{ab}	9.4±0.3 ^{ab}
AlCl ₃	110±5.3 ^a	91.3±5.2 ^a	13.3±0.5 ^a	14.3±0.4 ^a	17.4±0.4 ^a	1.6±0.1 ^e	4.1±0.5 ^d
AlCl ₃ +RAE	64±3 ^b	70.6±3.2 ^b	9.8±0.3 ^c	11.1±0.3 ^c	13.2±0.2 ^c	4.6±0.2 ^{bcd}	7.9±0.2 ^{bc}
AlCl ₃ +DHA	65.3±1.7 ^b	71.5±5.1 ^b	10±0.2 ^c	11.9±0.4 ^{bc}	13.9±0.3 ^{bc}	4.4±0.1 ^{cd}	7.4±0.4 ^c

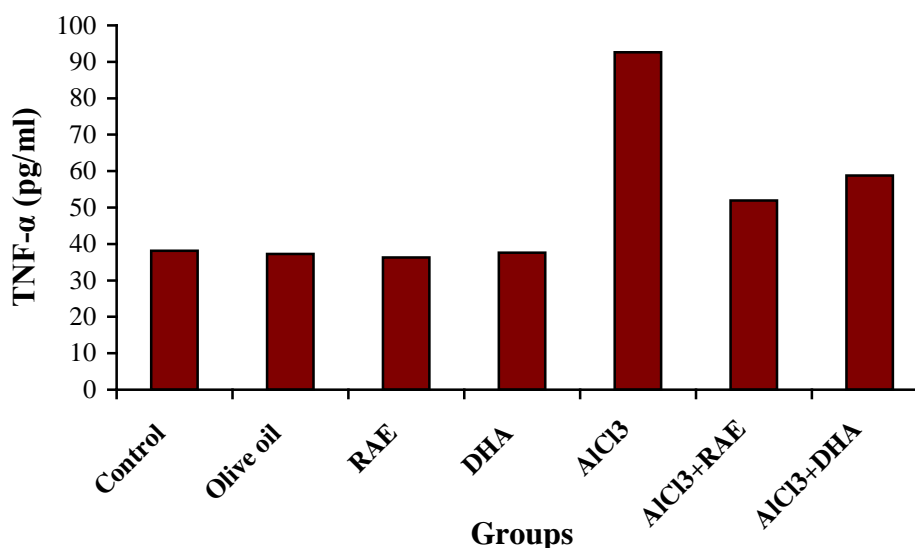
Data are presented as mean ±S.E. Within each column, means with different letters are significantly different ($P < 0.05$) using one way (Tukey tests) ANOVA test.

From the data obtained in the current study, it is worth noting that treatment with RAE was more effective than DHA in alleviating AlCl₃ toxicity.

Table 4: Effects of RAE and DHA on some specific markers of liver and kidney functions in serum of AlCl₃-intoxicated senile rats.

	ALT (U/l)	AST (U/l)	Urea (mg/dL)	Creatinine (mg/dL)
Control	25.8±0.4 ^{cd}	38.3±0.4 ^{de}	38.8±2.4 ^b	1.69±0.3 ^e
Olive oil	25.3±0.3 ^d	37.5±0.4 ^{de}	38.3±2.4 ^b	1.65±0.3 ^e
RAE	24.6±0.4 ^d	36.6±0.3 ^e	36.9±0.6 ^b	1.62±0.3 ^e
DHA	25.1±0.3 ^d	37±0.5 ^e	37.4±0.7 ^b	1.66±0.2 ^e
AlCl ₃	30.4±0.5 ^a	46.3±0.6 ^a	50.6±0.7 ^a	2.52±0.5 ^a
AlCl ₃ + RAE	27.2±0.3 ^{bc}	40.8±0.4 ^c	39±0.5 ^b	1.93±0.1 ^d
AlCl ₃ +DHA	27.5±0.3 ^b	41.7±0.5 ^{bc}	40.5±0.8 ^b	2.01±0.2 ^{cd}

Data are presented as mean ±S.E. Within each column, means with different letters are significantly different ($P < 0.05$) using one way (Tukey tests) ANOVA test.

**Fig. 1:** Effects of RAE and DHA on serum TNF-α level in AlCl₃-intoxicated senile rats.

Discussion:

Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics. Aluminum has been reported to induce LPO, and to alter physiological and biochemical characteristics of biological systems (Dua and Gill, 2001; Sharma *et al.*, 2007). There is a high susceptibility of brain to oxidative insult because it contains a large amount of polyunsaturated fatty acids and consumes 20% of the body's oxygen. Moreover, in spite of the high rate of oxidative metabolism, the brain has a relatively low antioxidant defense system (Dua and Gill, 2001; Sharma *et al.*, 2007).

The present results showed that aluminum promotes oxidative stress by significant reduction of TAC level i.e. decreasing the activity of free radical scavenging enzymes such as superoxide dismutase, catalase and glutathione peroxidase. This biological effect was confirmed by the significant increase in the levels of LPO and NO in brain cortex and hippocampus. Serum NO level was also increased significantly due to the neurotoxicity of AlCl₃. These results suggest participation of free radical-induced oxidative cell injury in

mediating the toxicity of aluminum (El-Demerdash, 2004; Shati *et al.*, 2011). These data are in agreement with those obtained by Yousef (2004) who observed that aluminum administration lowered antioxidant enzymes activities and increased lipid peroxidation in the brains of rabbits.

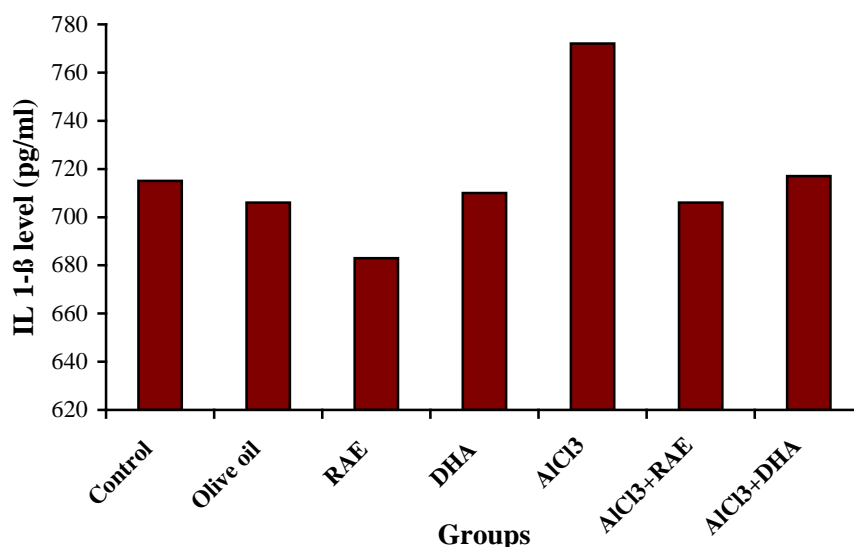


Fig. 2: Effects of RAE and DHA on serum IL 1-β level in AlCl₃-intoxicated senile rats.

Increased reactive oxygen species (ROS) were reported in previous studies during aluminum exposure, which was attributed to electron leakage, enhanced mitochondrial activity and increased electron chain activity. ROS subsequently attack almost all cell components including membrane lipids and producing lipid peroxidation (Flora *et al.*, 2003). Therefore, it can be hypothesized that oxidative stress may be one of the contributing factors for aluminum-induced neurotoxicity (Yousef and Salama, 2009). These effects may also be due to the accumulation of metal in the brain and blood (Sharma and Mishra, 2006). Some studies have shown that aluminum can bind amino acids such as glutamate to form aluminum–glutamate complexes that allow it to reach the blood of the brain (Shrivastava, 2012).

Other studies have shown that aluminum-enhanced peroxidation and neurodegenerative disorders may be related to aluminum-induced nitric oxide synthase (NOS) activity and increased NO products in rat brain tissue and microglial cells (Garrel *et al.*, 1994; Bondy *et al.*, 1998). In biological systems, NO is generated from L-arginine by the enzyme NOS, formed in a variety of tissues and involved in many physiological and pathological processes (Moncada *et al.*, 1991).

NO is important neuromodulator implicated in brain plasticity and disease. The use of NO donors and NOS inhibitors as pharmacological tools revealed that this free radical is probably implicated in the regulation of excitability and firing, in long-term potentiation and long-term depression, as well as in memory processes. Moreover, NO modulates neurotransmitter release (Stevanović *et al.*, 2010). Those authors revealed that the dysregulation of NO was included in AlCl₃-induced neurotoxicity, resulting in both temporal and spatial spreading of damage to the selective vulnerable brain structures with impairment of cognitive functions and cholinergic transmission and deficits in learning and memory.

Our emerging data revealed that administration of AlCl₃ produced significant elevation of AChE activity in brain cortex and hippocampus, whereas the activity of Na⁺/K⁺-ATPase was markedly inhibited in both brain regions as compared to the normal control. These findings are consistent with the previous ones (Mohamd *et al.*, 2011).

AChE is one of the most biological catalysts that play a key role in cholinergic neurotransmission by rapidly hydrolyzing the neurotransmitter acetylcholine (ACh) at cholinergic synapses and neuromuscular junctions. Particularly in hippocampus, it plays an undoubted key role in regulation of learning and memory (El Omri *et al.*, 2010). AlCl₃, a known neurotoxicant (Miu and Benga, 2006), has been reported to alter the blood–brain barrier; as a result of which it gains an easy access to the central nervous system under normal physiological conditions and accumulates in the different regions of brain. It has been reported to be involved in the aetiology of several neurodegenerative diseases (Kawahara *et al.*, 2003). Further, AlCl₃ being a potent cholinotoxin (Gulya *et al.*, 1990) causes apoptotic neuronal loss which is related to high levels of AChE in the brain (Ravi *et al.*, 2000). Moreover, as chronic exposure to AlCl₃ results in over expression to amyloid

precursor protein (APP) and consequently peptide A β production, A β binds to nicotinic receptors leading to increased AChE activity (Arendt *et al.*, 1984; Schliebs *et al.*, 2006).

Unlike AChE, Na⁺/K⁺-ATPase activity was found to be significantly declined in brain cortex and hippocampus of aluminum-intoxicated rats. On the other hand, calcium levels in serum as well as in brain cortex and hippocampus were elevated significantly due to neurotoxic effects of AlCl₃. According to the outcomes of this study, the signaling of oxidative stress, NO and Na⁺/K⁺-ATPase play a role and responsible factor in the underlying mechanisms of the toxicological effects induced by AlCl₃. As increased lipid peroxidation alters the lipid environment; it may also affect membrane's Na⁺/K⁺-ATPase activity. Na⁺/K⁺-ATPase enzyme is involved in maintenance of ionic gradients across the membrane. Therefore, changes in its activity could be associated with alterations in neuronal action potential firing (Riddle *et al.*, 1993; Zhang *et al.*, 2004). This membrane bound enzyme requires phospholipid for its activity and is highly vulnerable to oxidative insult and the mechanism of inactivation under such conditions involves disruption of phospholipid microenvironment of the enzyme or direct damage to enzyme protein by reactive oxygen radicals or lipid peroxidation products (Chakraborty *et al.*, 2003; Stefanello *et al.*, 2011).

The inactivation of Na⁺/K⁺-ATPase could cause the partial membrane depolarization allowing excessive Ca²⁺ entry into neurons with resultant toxic events similar to excitotoxicity and implicated in the pathological and physiological abnormality or neurodegenerative diseases (Huang *et al.*, 2008). Calcium is involved in many facets of neuronal physiology, including activity, growth and differentiation, synaptic plasticity, and learning and memory, as well as pathophysiology including necrosis, apoptosis, and degeneration (Bezprozvanny, 2009). Several evidence have claimed that calcium dysregulation plays a central role in AD pathogenesis (Bezprozvanny and Mattson, 2008; Bojarski *et al.*, 2008).

Regarding the relationship between AlCl₃ and immune function in rats, our results exhibited significant increase in serum IL-1 β and TNF- α level of AlCl₃ intoxicated rats. The obtained data are in agreement with Tarkowski *et al.* (2003), Van-Exel *et al.* (2009) and Passos *et al.* (2010) who mentioned that chronic inflammatory process might contribute to the neurodegeneration associated with AlCl₃, by overexpression of cytokines such as IL-1 β and TNF- α in activated microglia surrounding amyloid plaque. Microglia, the resident macrophage in the brain, are strongly implicated in the pathology and progressively degenerative nature of AD. Microglia is activated in response to both amyloid and neuronal damage, and can become a chronic source of neurotoxic cytokines as well as reactive oxygen species.

IL-1 β determines neuronal dysfunction and death, and can also induce higher production of inflammatory cytokines, leading to self-sustaining and self-amplifying inflammation in the brain (Forlenza *et al.*, 2009). TNF- α is secreted by brain resident astrocytes, microglia, and neurons in response to numerous intrinsic and extrinsic stimuli. The misguided control of inflammatory signaling has been implicated in A β formation (Park and Bowers, 2010). Induction of TNF- α occurs in commensurate with the onset of early AD. TNF- α induces the synthesis of acute phase proteins including APP via reacting with its receptor (Cheng *et al.*, 2010). Conversely, A β itself has been shown to induce the expression of TNF- α and IL-1 β . Thus a direct correlation has been established between A β induced neurotoxicity and cytokines (Lee *et al.*, 2009).

In the present study, the activities of AST and ALT were significantly increased in serum of rats treated with AlCl₃ and this is in agreement with Yousef (2004). This may be due to the leakage of these enzymes from the liver cytosol into the blood stream and/or liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane takes place (Hassoun and Stohs, 1995). Also, Wilhelm *et al.* (1996) reported that aluminum exposure can result in aluminum accumulation in the liver and this metal can be toxic to the hepatic tissue at high concentrations.

Our obtained results showed that the elevation in serum urea and creatinine levels in AlCl₃-treated rats is considered as a significant marker of renal dysfunction, and this is supported by the findings of Szilagyi *et al.* (1994), who reported that alterations in serum urea may be related to metabolic disturbances (e.g. renal function, cation-anion balance). Rudenko *et al.* (1998) reported that AlCl₃ intensifies the acid-secretory function of kidney and changes the transport of sodium. In addition, Katyal *et al.* (1997) reported that AlCl₃ has been implicated in the pathogenesis of several clinical disorders, including renal dysfunction.

Manipulation of aluminum-intoxicated rats with RAE revealed significant reduction in MDA and NO levels in brain cortex and hippocampus. Serum NO level was also decreased significantly by RAE. On the other side, TAC level was significantly elevated in brain cortex as well as hippocampus. These results are in agreement with Posadas *et al.* (2009), suggesting that rosemary may have an antioxidant effect in the brain of aged rats.

Phytochemical studies have shown that rosemary contains essential oils, terpenoids, flavonoids and alkaloids. Some of its constituents such as rosmarinic acid (RA) have been reported as powerful antioxidant protecting against free radicals damage and to reduce hepatotoxicity (Martinez *et al.*, 2012). Other researchers revealed the potential of RA for prevention of neurodegenerative diseases such as stroke, AD and PD, usually caused by an excess of free radicals. Unlike many drugs and natural antioxidants, RA was found to be able to

cross the blood–brain barrier and also to be used in the so called pathologically-activated-therapy (Lipton, 2007).

Rosemary extracts which contains a high amount of total phenolics, is able to donate electrons, and therefore should be able to donate electrons to reactive radicals, converting them into more stable and unreactive species (Sáenz-López *et al.*, 2002; Dorman *et al.*, 2003). Results of Posadas *et al.* (2009) could indicate protective action by rosemary extract in brain tissue through decreases in NOS activity, and subsequently, NO production. Therefore, they suggest that rosemary extract has an antioxidant effect as a free radical scavenger in this organ.

Interestingly, in the present study the increases of AChE activity as well as calcium level and the decrease of Na⁺-K⁺-ATPase activity in the cortex and hippocampus due to AlCl₃ neurotoxicity were attenuated by treatment with RAE. Corroborating our data, Singh *et al.* (2011) and Orhan *et al.* (2012) investigated the AChE inhibitory effect of rosemary and found that rosemary aqueous and methanol extracts inhibited the enzyme at moderate level. RA is most likely the active compound responsible for potent anti- AChE activity of the aqueous extract of the plant (Mata *et al.*, 2007). According Ono *et al.* (2003), the dense and symmetric structure of RA was proposed to be suitable for specific binding of free β -amyloid and inhibition of its polymerization into the fibrillar form. However, RA has been reported to be associated with complement-inhibitory action, another mechanism involved in neuroprotection, by impairing AChE and aggravating Na⁺-K⁺-ATPase activities.

As mentioned before, Na⁺/K⁺-ATPase is particularly sensitive to ROS (Silva *et al.*, 2011). ROS-induced damages were associated with increased Ca²⁺ level and inhibition of Na⁺/K⁺-ATPase activity in mammals (Rodrigo *et al.*, 2007). Thus supplementation of RAE protected against the increase in levels of oxidative stress markers (MDA) and Na⁺/K⁺-ATPase inhibition. This may be attributed to RA, which was found to display potent antioxidant activity. Therefore, RA has been the topic of many studies related to neurotrophic activity, neurobehavioral and neurodegenerative disease (El Omri *et al.*, 2010).

Data presented in this study demonstrated that the hepatotoxic effects as well as renal dysfunctions of AlCl₃, as indicated by significant augmentations of serum ALT, AST, urea and creatinine levels, can be modified by RAE supplementation in combination with AlCl₃. These protective effects of RAE may be attributed to its antioxidant and free radical scavenging activities due to its higher contents of polyphenolic compounds (Sotelo-Felix *et al.*, 2002; Tavafi and Ahmadvand, 2011).

The results of this study represent direct evidence that RAE has a marked anti-inflammatory effect by inhibiting the release of pro-inflammatory mediators, including TNF- α and IL-1 β . In support of our findings, Benincá *et al.* (2011) reported that the anti-inflammatory effect of rosemary extract may be contributed to polyphenolic compounds (RA). These important anti-inflammatory properties may be achieved by decreasing oedema, oxidative stress. Also RA can block nuclear factor kappa beta (NF- κ B) that regulates the expression of various genes encoding pro-inflammatory cytokines, and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) resulting in inhibiting NO, IL-1 β and TNF- α production, thus preventing inflammation (Kuo *et al.*, 2011).

In our present work, administration of DHA in combination with AlCl₃ produced significant reduction in MDA as well as NO levels with concomitant elevation in TAC level of brain cortex and hippocampus. These results are in agreement with Hashimoto *et al.* (2011). One of the mechanisms for the neuroprotective action of DHA is that it exerts antioxidative activity *in vivo*. Indeed, evidence of DHA increasing glutathione reductase activity, decreasing the accumulation of oxidized proteins and levels of lipid peroxide and reactive oxygen species have been published (Calon and Cole, 2007; Ozsoy *et al.*, 2011).

As shown from our results, the activity of AChE as well as calcium level were significantly decreased in brain cortex and hippocampus whereas; Na⁺/K⁺-ATPase activity was markedly increased in both brain regions when AlCl₃-intoxicated rats received DHA. Shahdat *et al.* (2004) reported that the lipids of synaptic plasma membrane (SPM) contain large amounts of polyunsaturated fatty acids, especially DHA and both cholinergic functions and DHA play an important role in formation and/or retention of memory, which deteriorates in age-related pathologies such as AD, which is characterized by a deficit in brain DHA concentration and decrease in membrane fluidity. Shahdat *et al.* (2004) reported also that the decrease in DHA content in the neuronal membrane results in a decrease in membrane disorder or fluidity and synaptic plasma membrane-bound AChE enzyme activity is sensitive to a deficiency of n-3 fatty acids.

Direct polyunsaturated fatty acid (PUFA) interactions with Na⁺/K⁺-ATPase, causing activating effects, have been reported. In addition, Na⁺/K⁺-ATPase may also be regulated by phosphorylation of its α -subunit with protein kinase C (PKC), effects that may be caused by PUFAs (Haag *et al.*, 2003). These results document the importance of the effects of fatty acid composition of membrane phospholipids on the activity of Na⁺/K⁺-ATPase. Deficiency of DHA may influence the functional properties of the Na⁺/K⁺-ATPase and account for these differences (Gerbi *et al.*, 1999). DHA plays a crucial role in membrane order (membrane fluidity), which can influence the function of membrane receptors; regulation of membrane-bound enzymes (Na⁺/K⁺-ATPase); dopaminergic and serotonergic neurotransmission and signal transduction via effects on inositol phosphates,

diacylglycerol, and PKC (Puskás *et al.*, 2003). Our results concerning calcium levels in brain tissues as well as in serum are in accordance with Haag *et al.* (2003) who reported that supplementation with DHA caused an increase Ca-ATPase activity over controls.

The present study demonstrates that DHA can be regarded as good protecting agent against the hepatotoxicity and renal dysfunction of AlCl_3 as it improves AST and ALT activities as well as urea and creatinine levels. These results are in agreement with Parker *et al.* (2012). PUFA deficiency is often observed in patients with advanced liver cirrhosis and a low n-3 PUFA diet increases the development of alcoholic fatty liver and fibrosis. Animal studies show that dietary fish oil or PUFA supplementation has beneficial effects against liver damage caused by thioacetamide, monocrotaline or carbon tetrachloride (Pawlosky and Salem, 2004; Pan *et al.*, 2010; Chen *et al.*, 2012). The hepatoprotective, anti-inflammatory and anti-fibrotic effects of DHA seem to be multifactorial. It is reasonable to propose that resolution of oxidative stress by DHA represents a potential way to attenuate liver injury. Supporting our results concerning the beneficial effects of DHA on kidney functions, Lu *et al.* (2003) proved that detrimental effects of a high-fat diet on early kidney injury, such as increased serum creatinine and serum urea nitrogen levels and decreased creatinine clearance, in rats were ameliorated by omega-3 polyunsaturated fatty acids.

The ability of DHA to influence inflammatory response has been recognized in many recent reports and has generated an increased interest in the design of alternative immunomodulatory strategies based on dietary interventions. In accordance, our findings claimed that DHA has beneficial antiinflammatory effects by inhibiting the release of pro-inflammatory mediators, including $\text{TNF-}\alpha$ and $\text{IL-1}\beta$. These results are consistent with the findings of Mullen *et al.* (2010) and Zue *et al.* (2012). In the same context, Brahmabhatt *et al.* (2013) proposed that DHA may modulate the secretion of proinflammatory cytokines such as $\text{TNF}\alpha$ and $\text{IL-1}\beta$ by innate immune cells. *In vitro* studies also suggest that DHA may reduce neutrophil adhesion molecules such as intercellular adhesion molecule and vascular cell adhesion molecule or by an alteration of cell membrane composition and function and/or a modification of eicosanoid production and/or an alteration of cytokine synthesis and secretion. Thus, via multiple mechanisms, it is likely that in intestinal injury, which is an acute inflammatory situation, an increase in DHA might be beneficial. Studies on human macrophages have showed that DHA had different impacts on the activation of nuclear $\text{NF-}\kappa\text{B}$, which plays key roles in regulating cytokine gene transcription, by altering the expression of the subunits as well as the inhibitory protein kappa B and eventually the cytokine production (Weldon *et al.*, 2007; Gorjão *et al.*, 2009).

In conclusion, RAE and DHA have neuroprotective effects against AlCl_3 -induced cognitive dysfunction and oxidative damage. This may be attributed to its powerful antioxidant activities.

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