Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

Journal of Pharmacology and Toxicology 6 (8): 647-663, 2011
ISSN 1816-496X / DOI: 10.3923/jpt.2011.647.663

The Protective Role of *Coffea arabica* L. and *Crocus sativus* L. Against the Neurotoxicity Induced by Chronic Administration of Aluminium Chloride

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ABSTRACT

Aluminium (Al) affects the antioxidant/oxidant balance and several enzymes relevant to neurotoxicity. Natural products such as saffron and green coffee may have a pharmacological role in ameliorating the neurotoxicity induced by Al as a result of their antioxidant properties. The study aims to investigate the role of saffron and green coffee extracts in ameliorating the toxicity induced by aluminium chloride in the nervous tissues of rats. Sprague dawley rats were used in this study and divided into 6 groups: control group; Green coffee group (600 mg/kg b.w./day for 90 days); Saffron group,(200 mg/kg b.w./day; $AlCl_3$ group,(40mg/kg b.w./day of $AlCl_3$ for 90 days); $AlCl_3$ plus green coffee group, co-administered with 40 mg/kg b.w./day of $AlCl_3$ and green coffee extract at 600 mg/kg b.w./day; $AlCl_3$ plus saffron group,(40 mg/kg b.w./day) of $AlCl_3$ and aqueous saffron extract (200 mg/kg b.w./day). There was a decrease in the AChE activity in rats administered with $AlCl_3$ for 90 days. Additionally, there was a decrease in the enzymatic antioxidant activities in both cerebral hemisphere and cerebellum of rats in $AlCl_3$ group. At the molecular level, the expression of A Disintegrin And Metalloprotease (ADAM10), acetylcholinesterase, P53, Bcl-2 (B cell lymphoma 2) and interleukins (IL-4 and IL-12) genes in $AlCl_3$ group was changed. Both green coffee and saffron extracts could be used to attenuate the neurotoxic effects of $AlCl_3$ as the imbalance in antioxidants/oxidants system and in some genes expression.

Key words: Neurotoxicity, oxidative stress, antioxidants system, real-time PCR, brain, gene expression, saffron, green coffee, rats

INTRODUCTION

Al pots leach aluminium into foods during cooking period with release depending on aluminium quality and food constituents used (Verissimo *et al*., 2006). The typical adult intakes are estimated from 3-12 mg day^{-1} according to dietary Al studies conducted in many countries (Saiyed and Yokel, 2005). Al can accumulate in different body organs such as brain, bone, liver and kidneys (Exley *et al*., 1996) and it is excreted through the kidneys. The markers for cholinergic neurons such as choline acetyltransferase (ChAT) responsible for synthesis of ACh neurotransmitter. AChE plays a key role in cholinergic neurotransmission by rapidly hydrolyzing

ACh at cholinergic synapses and neuromuscular junctions (Soreq and Seidman, 2001). As cholinergic function is required for short-term memory, the cholinergic deficit in AD was also believed to be responsible for much of the short-term memory deficit (Francis *et al*., 1999). *In vitro* and *in vivo* effects of Al on AChE (E.C. 3.1.1.7) activity have been described (El-Demerdash, 2004). However, literature data suggest that Al interacts with the cholinergic system, acting as a cholinotoxin (Gulya *et al*., 1990). As known, clinical drug trials in patients with AD have focused on drugs that augment levels of ACh in the brain to compensate for losses of cholinergic function in the brain. These drugs have included acetylcholine precursors, muscarinic agonists, nicotinic agonists and AChE inhibitors (Livingston and Katona, 2000).

With respect to lipid peroxidation, Al is not a transition metal and does not undergo redox reactions *in vivo*. However, increased the Reactive Oxygen Species (ROS) have been reported during Al exposure (Campbell *et al*., 1999). In fact, Al might very well exert its toxic effects by interfering with pathways involved in normal iron metabolism and homeostasis (Nayak, 2002) and is considered as a pro-oxidant agent (Exley, 2004). Al has the ability to stimulate iron to induce lipid peroxidation (Gutteridge *et al*., 1985; Shati *et al*., 2011). The decreased antioxidant versus oxidant ratio plays an important role in inducing a condition of oxidative stress (Gutteridge and Halliwell, 1990; Shati and Elsaid, 2009; Shati *et al*., 2011). The oxidative status of the cell is a primary factor in the regulation of gene expression and enzymatic activity (Rodriguez *et al*., 2004). Decreases in cellular GSH have been implicated in the causation of several neuro-degenerative disorders, including AD (Bains and Shaw, 1997). It has been reported that Al significantly increased the levels of whole brain Thiobarbituric Acid Reactive Substances (TBARS), CAT and cerebellum GSH-Px and decreased the activity of Glutathione-S-Transferase (GST) indicating that Al induced oxidative stress following free radical generation (Flora *et al*., 2003). Sharma *et al*. (2007) found induction of oxidative stress in brain tissue and serum of rats after $AlCl_3$ exposure (172.5 mg/kg/day orally for 10 weeks). They also found a decrease in the GSH, GSH-Red, GSH-Px, CAT and SOD levels. Shati *et al*. (2011) showed that there was an increase in the TBARS and a decrease in the activities of the antioxidant enzymes such as CAT, SOD and GSH-Px in the mice brain tissues administered with $AlCl_3$ at 40 mg/kg/day for 45 days. In situations, where the generation of free radicals exceeds the capacity of antioxidant activity, oxidative stress may lead to cell membrane degradation, cellular dysfunction and apoptosis (Lucca *et al*., 2009).

Metal dyshomeostasis correlates with the initiation and progress of various human neurodegenerative diseases (Yokel, 2006). The dysregulation of minerals has also been observed in Al-treated cells and Al-induced animal models (Bharathi *et al*., 2008). Although, healthy individuals do not normally accumulate Al in their tissue but its toxicity is enhanced by Ca deficiency (Boudey *et al*., 1997) or disruption of Ca regulation (Nayak, 2002). A disruption of Ca metabolism is cited among the proposed mechanisms to explain Al toxicity (Gandolfi *et al*., 1998). Certain trace elements such as Zinc (Zn) are important in the free radical concern. It is a part of the enzymes SOD and acts as antioxidant in the cells. Lovell *et al*. (1999) suggested that Zn has a double function offering protection against amyloid α-peptides at low concentrations and causing toxicity at high concentrations. A decrease in serum zinc has also been reported, though it is possible that some of the Alzheimer Disease (AD) patients included in one study were malnourished (Baum *et al*., 2010). Besides, the fact that Al is a cholinotoxin and a pro-oxidant agent, its neurotoxic effects could be exerted by additional mechanisms such as the promotion and accumulation of insoluble amyloid α-protein and by interacting directly with the genomic structure (Nayak, 2002). The suppression of gene expression in the AD brain was thought to be mainly due

to the selective loss of neurons (Doyu *et al*., 2001). Expression of some genes encoding antioxidant enzymes (e.g., CAT, SOD and GSH-Px) suggest common mechanisms induced by Al treatment and oxidative stress (Milla *et al*., 2002). Both endogenous (Nicotera *et al*., 1989) and exogenous (Yoo *et al*., 1999) agents act as oxidants and alter cellular oxidative equilibrium and consequently, antioxidant enzyme gene expression (Gomez *et al*., 2005). Numerous studies have shown variations in AChE activity and its molecular forms in tissues involved in AD (Talesa, 2001). The Bcl-2 family consists of three major subgroups (Korsmeyer, 1999). The high expression level of Bcl-2 and/or Bcl-XL in neurons has clearly investigated. In spite of the type of neuronal death induced high expression of anti-apoptotic Bcl-2 and involved in several neurodegenerative disease models (Chong *et al*., 2000). ADAMs are a family of transmembrane and secreted proteins with multiple functions as in cell adhesion and proteolytic processing of the ectodomains of diverse cell surface receptors and signaling molecules (Edwards *et al*., 2008).

Cytokines appear to be involved in the pathogenesis of neurotoxicity. Interleukin-4 (IL-4), an immunosuppressive cytokine, seems to prevent neuronal cell injury (Lugaresi *et al*., 2004). This is supported by the finding that in neuronal hippocampal cell cultures IL-4 significantly enhanced survival; the effects however, were concentration-dependent (Szczepanik *et al*., 2001). IL-4 exerts its neuroprotective effect by the inhibition of IFN-g and the consequent decrease in the concentration of TNF-α and nitric oxide (Chao *et al*., 1993). Interleukin-12 is a cytokine produced by activated blood monocytes, macrophages and glial cells (Rentzos *et al*., 2009). Ahmed *et al*. (2001) concluded that the axonal/myelin changes have seen in IL-12 may be due to a number of factors including humoral immune antibody mediated damage to axons, neurotoxic/excitotoxic mediators and/or proteolytic enzymes, such as tPA which are produced by macrophages and other inflammatory cells.

Coffea arabica L. with its lower bitterness and better flavor, is more appreciated by consumers. Chlorogenic acids (CGA) and caffeine, increase bitterness, the former after degradation into phenol derivatives (Leloup *et al*., 1995). Many health benefits have been accompanied with the consumption of CGA, such as reduction of the risk of cardiovascular disease, diabetes type II and AD (Ranheim and Halvorsen, 2005). Green (or raw) coffee is a major source of CGA in nature (5-12 g 100 g^{-1}) (Farah and Donangelo, 2006). Recent studies demonstrated that the consumption of green coffee extracts produced antihypertensive effect in rats and humans (Kozuma *et al*., 2008), improvement in human visor activity (Ochiai *et al*., 2004), inhibitory effect on fat accumulation and body weight in both mice and humans and modulation of glucose metabolism in humans (Blum *et al*., 2007). Saffron contains three main pharmacologically active metabolites: Saffron-colored compounds are crocins which are unusual water-soluble caroteneoids, Picrocrocin and Safranal. Moreover, saffron contains sugars, flavonoids, mineral matter, gums and other chemical compounds (Winterhalter and Straubinger, 2000). In addition, many studies have shown crocins of saffron to be able to a variety of pharmacological effects, such as neuroprotection (Ahmad *et al*., 2005). The protective mechanism of saffron concerned with its antioxidant activity was hypothesized to be responsible for various pharmacological effects of crocins (Shen and Qian, 2006). Radical scavengers, learning and memory enhancement properties of saffron were recorded (Rios *et al*., 1996; Abe *et al*., 1999).

Therefore, the present work aimed to discuss the effects of aqueous extracts of green coffee and saffron on the induced biochemical and molecular changes resultant in cerebral hemisphere and cerebellum due to the neurotoxicity effect of the Al on rats.

MATERIALS AND METHODS

Natural products extracts: *Coffea arabica* L. (Family Rubiaceae) or common name green coffee (bean) and Iranian *Crocus sativus* L. (Family Iridaceae) or common name saffron (comprised of dried red stigma) were purchased from the local supermarket in Abha, Saudi Arabia. The Green coffee and Saffron were separately immersed in distilled water for 24 h, filtered, stored at -20°C and freshly prepared every three days.

Animal models: Thirty *Sprague dawely* rats (180±10 g) were used in the present experiment and the treatment duration extended for 90 days. Animals were obtained from animal house at faculty of Science, at King Khalid University, Abha; Kingdom of Saudi Arabia. The local committee approved the design of the experiments and the protocol conforms to the guidelines of the National Institutes of Health (NIH). All measures were taken to minimize the number of rats used and their suffering. Rats were divided into six groups. Animals were caged in groups of 5, allowed standard rat chow diet and water *ad libitum* as follows: Control group; rats were daily intraperitoneally (i.p.) administered with normal saline solution (0.9%) for the experimental periods. Green coffee group; rats i.p., administered with 600 mg kg^{-1} body weight of water extract of green coffee. Saffron group, rats were i.p. administered with 200 mg kg^{-1} body weight per day. $AlCl_3$ group; rats were i.p. administered with 40 mg kg^{-1} b.w./day $AlCl_3$ for 90 days, freshly prepared every four days for the experimental periods. $AlCl_3$ plus coffee group; rats were i.p. administered with 40 mg/kg b.w./day $AlCl_3$ and green coffee extract at 600 mg/kg b.w./day for 90 days. $AlCl_3$ plus saffron group; rats were i.p. administered with 40 mg/kg b.w./day $AlCl_3$ and water saffron extract at 200 mg/kg b.w./day for the experimental period. Animals were maintained at 22-25°C and 40-60% relative humidity with 12 h light-dark cycles. At the end of the experimental period rats were sacrificed and sera, cerebellum and cerebral hemisphere tissues were collected from each group. The nervous tissues were homogenized in a phosphate buffer solution pH 7.4, centrifuged at 3000 rpm at 4°C and the supernatant was stored at -80°C.

Enzymatic assay

Acetylcholinesterase activity (AChE): This assay depends on the ability of the enzyme to produce a thiocholine and form a complex with dithio-bis-nitrobenzoate (Weber, 1966). The mean absorbance change was determined per 30 sec at 405 nm.

Superoxide dismutase: This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye (Nishikimi *et al*., 1972).

Catalase: It reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one minute with CAT inhibitor (Aebi, 1984).

GSH-Px: The activity was measured by the method described by Ellman (1959).

GSH-Red enzyme: According to the method of Goldberg and Spooner (1983), GSH-Red catalyzed the reduction of the oxidized glutathione (GSSG) in the presence of NADPH that oxidized into $NADPH^+$. The decrease in absorbance was measured at 340 nm.

GSH content: GSH serves as an antioxidant, reacting with free radicals and organic peroxides, in amino acid transport and as a substrate for the GSH-Px and glutathione-S-transferase in the

detoxification of organic peroxide and metabolism of xenobiotics, respectively. The method based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm (Beutler *et al*., 1963).

Total antioxidant capacity: Determination of the antioxidant capacity was performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H_2O_2). The antioxidants in the sample will eliminate a certain amount of the provided hydrogen peroxide. The residual H_2O_2 is determined colorimetrically by an enzymatic reaction which involves the conversion of 3, 5, dichloro-2-hydroxyl benzensulphonate to a colored product (Koracevic *et al*., 2001).

Lipid peroxidation: The Thiobarbituric Acid Reactive Substances (TBARS) as malondialdehyde were estimated by the method of Ohkawa *et al*. (1979).

Calcium: Calcium ion produces with methyl-thymol blue, in an alkaline medium, a blue color its intensity is in proportion to the calcium concentration. The presence of hydroxy 8-quinoline eliminate the interference due to the magnesium ions as described by Gindler and King (1972).

Zinc: It is chelated by zincon (2-caboxy-2-hydroxy-5-Sulfoformazyl-benzene) at alkaline pH. The formed complex is measured at a wavelength of 610 nm as described by Hayakawa and Jap (1961).

Extraction of total RNA from brain tissue homogenates: Total RNA was isolated from cerebellum and cerebral hemisphere homogenates using RNeasy Mini Kit according to manufacturer's instructions (QIAGEN, Germany) as published (Shati *et al*., 2011).

Real time PCR and gene expression

Neurotoxicity genes (ADAM 10 and AChE): The extracted RNA from different groups was subjected to examine the expression level of ADAM10 and AChE genes using specific primers in the presence of Glyceraldehyde-3-phosphate dehydrogenase as a house keeping gene. The Real time reaction consists of 12.5 μL of 2X Quantitech SYBR® Green RT Mix (Fermentaz, USA), 2 μL of the extracted RNA (50 ng μL^{-1}), 1 μL of 25 pM μL^{-1} forward (F) primer, 1 μL of 25 pM μL^{-1} reverse (R) primer (Table 1), 9.5 μL of RNAase free water for a total of 25 μL. The real time PCR program was performed as follows: Initial denaturation at 95°C for 10 min. 40 cycles of 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene 6000 system (QIAGEN, USA).

DNA repairing genes (P53 and Bcl-2): The extracted RNA was subjected to Real-Time PCR reaction to examine the expression p 53 and Bcl-2 genes in $AlCl_3$, $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups and compared with the control ones. The PCR reaction constituents and conditions as mentioned above except that the annealing temperature as shown in Table 1.

Interleukins (IL-4 and IL-12): Real Time PCR was performed using specific primers for two of cytokines genes, IL-4 and IL-12. The reaction was performed on the total RNA extracted from the cerebral hemisphere and cerebellum of all experimental groups. The PCR reaction constituents and conditions as previously mentioned except that the annealing temperature as shown in Table 1.

Table 1: Primers nucleotides sequence used in this study

Primer name	Primer sequence from 5`-`3	Annealing temp. (°C)
ADAM10	R-CCATGCTCATGGAAGACAGTT	
	F-CCTTCTTCACCATAAATATGTCCA	60
AchE	R-CCACCGATCCTCTGGACGAG	
	F-CGCTCCTGCTTGCTATAGTG	60
P53	F-AGGGATACTATTCAGCCCGAGGTG	
	R-ACTGCCACTCCTTGCCCCATTC	64
Bcl2	F-ATGTGTGTGGAGAGCGTCAACC	
	R-TGAGCAGAGTCTTCAGAGACAGCC	63
IL-4	F-CTATTAATGGGTCTCACCTCCCAACT	
	R-CATAATCGTCTTTAGCCTTTCCAAG	60
IL-12	F-CAGCCTTGCAGAAAAGAGAGC	
	R-CCAGTAAGGCCAGGCAACAT	65
GPDH (Housekeeping gene)	F-ATTGACCACTACCTGGGCAA	
	R-GAGATACACTTCAACACTTTGACCT	60-65

GPDH: Glyceraldehyde 6-phosphate dehydrogenase

Molecular data analysis: Comparative quantitation analysis was performed using Rotor-Gene-6000 Series Software.

Statistical analysis: The recorded biochemical data and gene expression were registered as Mean±SD and statistical and correlation analyses were undertaken using the One-way ANOVA followed by a post-hoc LSD (Least Significant Difference) test. A p-value <0.05 was statistically significant. A statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL, USA).

RESULTS

Biochemical data: Table 2 showed significant decrease in AChE activity, a very high significant decrease ($p<0.001$) in total antioxidant capacity, CAT, SOD and GSH-Px activities in the cerebral hemisphere of $AlCl_3$ treated group when compared with control ones. Also, a decrease in GSH ($p<0.001$) and significant increase in TBARS ($p<0.001$) in $AlCl_3$ treated group in comparison with the control group were observed. There were no significant changes of positive control groups treated with green coffee and saffron extracts when compared with control ones. Both $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups showed significantly higher increase ($p<0.05$) in the enzymatic and non-enzymatic antioxidants and significantly lowered TBARS in the cerebral hemisphere.

Table 3 showed significant decrease in AChE activity, a very high significant decrease ($p<0.001$) in total antioxidant capacity, CAT, SOD and GSH-Px activities in the cerebellum of $AlCl_3$ treated group when compared with control ones. Also, a decrease in GSH ($p<0.001$) and significant increase in TBARS ($p<0.001$) in $AlCl_3$ treated group in comparison with the control group were observed. Both $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups showed significantly increase ($p<0.05$) in the enzymatic and non-enzymatic antioxidants and significantly lowered ($p<0.05$) TBARS in the cerebellum homogenate.

Results presented in Table 4 revealed that the level of both calcium and zinc were decreased in cerebral hemisphere and cerebellums of $AlCl_3$ treated group when compared with control ones. The positive control groups showed non-significant changes ($p>0.05$) in their levels when compared with

Table 2: Cerebral hemisphere biochemical parameters

	Groups					
Parameters	Control	Green coffee	Saffron	$AlCl_3$	$AlCl_3$ and Green coffee	$AlCl_3$ and Saffron
Acetyl cholinesterase (U g^{-1} tissue)	2013.60±225.8	2012.4±104	2143.8±245.1	1654.4±157.9*	1827.6±84.8	1859.8±70.3
T. antioxidants capacity (U g^{-1} tissue)	0.57±0.04	0.59±0.03	0.59±0.04	0.33±0.04***	0.44±0.10^{*b}	0.53±0.05†
CAT (U g^{-1} tissue)	2.9±0.49	2.4±0.51	2.8±0.55	1.3±0.08***	1.9±0.48*	2.2±0.27†
SOD (U g^{-1} tissue)	64.6±4.4	61.7±4.2	62.2±3.7	45.9±5.7***	54.3±8.4^{a†}	56.5±5.2†
GSH-Px (U g^{-1} tissue)	1.3±0.15	1.05±0.17	1.06±0.11	0.72±0.09***	0.96±0.14**†	0.95±0.08**†
GSH-R (U g^{-1} tissue)	75.9±8.5	74.1±2.9	72.7±6.7	49.1±6.1***	62.2±5.4^{*b††}	64.2±5.7^{*a†††}
GSH (U g^{-1} tissue)	62.0±5.1	61.8±5.6	63.5±3.5	45.8±4.7***	55.0±5.4†	56.2±2.9†
TBRAS (nmole g^{-1} tissue)	110.2±15.7	93.2±4.7	98.2±19.3	212.3±13.7***	165.6±21.1^{***c†††}	160.3±12.9^{***d†††}

All groups were compared with the control group $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ $AlCl_3$+green coffee and saffron groups were compared with their matched control groups, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, $AlCl_3$+green coffee and saffron groups were compared with $AlCl_3$ group, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$

Table 3: Cerebellum biochemical parameters

	Groups					
Parameters	Control	Green coffee	Saffron	$AlCl_3$	$AlCl_3$ and Green coffee	$AlCl_3$ and Saffron
Acetyl cholinesterase (U g^{-1} tissue)	1928±165.3	1994.2±149.6	2002.6±192.9	1310±198***	1793.4±107†††	1810.2±93.1†††
T. antioxidants capacity (U g^{-1} tissue)	0.57±0.05	0.60±0.02	0.58±0.04	0.39±0.05***	0.44±0.06^{c}	0.58±0.02†††
Catalase (U g^{-1} tissue)	2.8±0.39	2.7±0.37	2.4±0.21	1.5±0.36***	2.0±0.47	2.1±0.21
SOD (U g^{-1} tissue)	62.7±5.8	63.7±6.7	66.6±5.9	48.1±5.6**	56.3±5.9†	59.7±7.7†
GSH-Px (U g^{-1} tissue)	1.2±0.17	1.0±0.13	1.2±0.14	0.77±0.13***	0.98±0.13^{b}	0.93±0.09†
GSH-R (U g^{-1} tissue)	76.1±8.4	70.7±3.2	78.7±8.5	52.7±4.5***	65.2±3.5**††	64.1±6.8^{**b††}
GSH (U g^{-1} tissue)	55.4±3.9	55.9±3.07	55.5±3.8	40.9±2.6***	51.9±2.9†††	53.1±5.3†††
TBRAS (nmole g^{-1} tissue)	104.2±11.6	92.4±5.9	112.6±8.2	199.3±1.4***	197.5±17.4^{***c}	165.8±7.7^{***c†††}

All groups were compared with the control group $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $AlCl_3$+green coffee and saffron groups were compared with their matched control groups, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, $AlCl_3$+green coffee and saffron groups were compared with $AlCl_3$ group, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$

Table 4: Calcium and zinc levels in different treated groups of rats

	Groups					
Parameters	Control	Green coffee	Saffron	$AlCl_3$	$AlCl_3$ and Green coffee	$AlCl_3$ and Saffron
Cerebral calcium (mg g^{-1} tissue)	78.8±4.6	81.2±2.5	76.6±2.4	64.70±4.7***	73.1±1.9†	70.4±4.6**††
Cerebellum calcium (mg g^{-1} tissue)	80.9±2.7	79.8±4.5	77.8±2.0	66.40±7.0***	72.8±2.9^{*a††}	74.2±3.6
Cerebral zinc (µg g^{-1} tissue)	16.4±2.7	17.3±3.0	17.2±2.8	9.10±1.8**	12.6±2.2	14.3±2.6†
Cerebellum zinc (µg g^{-1} tissue)	14.9±2.1	15.5±2.1	13.8±3.1	8.35±1.6**	10.7±2.7^{a}	11.1±2.7

All groups were compared with the control group $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $AlCl_3$+green coffee and saffron groups were compared with their matched control groups, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$, $AlCl_3$+green coffee and saffron groups were compared with $AlCl_3$ group, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$

the control group. On the other hand, both of $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups showed significant increase ($p<0.05$) in calcium and zinc levels when compared with the $AlCl_3$ group. We assume that the changes in calcium and zinc levels after long term administration of $AlCl_3$ in rat's cerebral hemisphere and cerebellum emphasis the pathophysiological action of Al that alter the functions of these regions in $AlCl_3$ treated group.

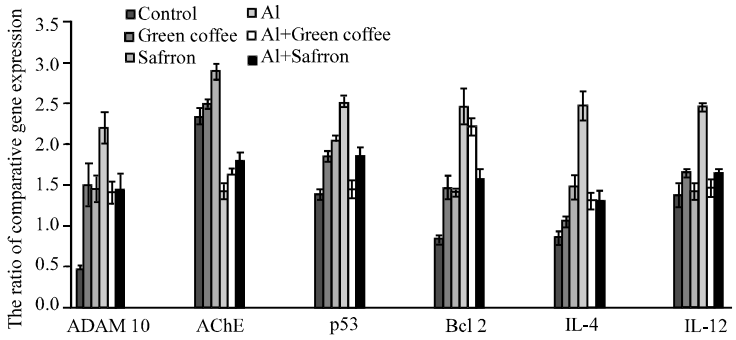

Fig. 1: Comparative gene expression in cerebral hemisphere of different treated groups compared with the control ones. ADAM10 (A Disintegrin And Metalloprotease); AChE (acetylcholinesterase); P53; Bcl-2 (B cell lymphoma 2); IL4 and IL12 (interleukin)

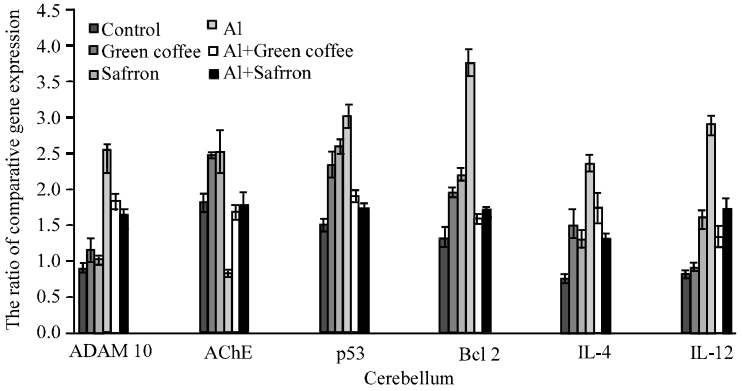

Fig. 2: Comparative gene expression in cerebellum of different treated groups compared with the control ones. ADAM10 (A Disintegrin and Metalloprotease); AChE (acetylcholinesterase); P53; Bcl-2 (B cell lymphoma 2); IL4 and IL12 (interleukin)

Molecular data: The real time PCR results (Fig. 1) revealed that the expression of ADAM10 was increased about three folds in cerebral hemisphere of $AlCl_3$ group when compared with the control ones. On the other hand, the ADAM10 expression decreased in $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups when compared with $AlCl_3$ group and their matched positive control groups. Moreover, AChE gene expression was lower in $AlCl_3$ group when compared with control group. But the expression of AChE gene was increased in $AlCl_3$ groups that treated with green coffee and saffron extracts when compared with $AlCl_3$ group and their matched control groups. The expression of Bcl-2, IL-4 and IL-12 genes in cerebral hemisphere was increased in the $AlCl_3$ group when compared with the control group. As in $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups the expression was decreased when compared with $AlCl_3$ group.

The real time PCR results (Fig. 2) revealed that the expression of ADAM10 was increased about three folds in cerebellum of $AlCl_3$ group when compared with the control ones. On the other hand, the ADAM10 expression decreased in $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups when compared with $AlCl_3$ group and their matched positive control groups. Moreover, AChE gene expression was lower in $AlCl_3$ group when compared with control group. But the expression of AChE gene was increased in $AlCl_3$ groups that treated with green coffee and saffron extracts when

compared with $AlCl_3$ group and their matched control groups. Additionally, the expression of Bcl-2 was increased two folds in the $AlCl_3$ group when compared with the control group. Also, the cytokines IL-4 and -12 genes were high expressed in the $AlCl_3$ group in comparison with the control ones. The aquatic extracts of green coffee and saffron have a promising role in attenuation the changes associated with the $AlCl_3$ treatment as in Fig. 2.

DISCUSSION

The elevation of brain lipid peroxidation suggests participation of free-radical-induced oxidative cell injury in mediating the toxicity of $AlCl_3$ as proposed by other studies (Anane and Creppy, 2001; Dua and Gill, 2001). Also, Kaizer *et al.* (2005) were demonstrated that Al enhanced lipid peroxides, as measured by TBARS formation, mainly in the cortex and hippocampus. Taken together, the results of TBARS indicate that Al can induce oxidative stress in some specific brain regions. TBARS elevation after exposure to Al has also been reported by El-Demerdash (2004). Deloncle *et al.* (1999) reported that the neurotoxicity of $AlCl_3$ was due to the increase in brain lipid peroxidation. Kumar (1999) reported that increased Al concentration and an increased lipid peroxidation rate could affect the neurons, leading to the depletion of AChE (Flora *et al.*, 2003). It was observed that AChE gene expression was decreased, as the increase in TBARS levels in brain cerebellum and cerebral hemisphere as in Fig. 1 and 2. In fact, the pattern of brain regional sensitivity to neurotoxicants can be, in part, explained by the differences in the brain barrier mechanisms to metals like Al (Zheng *et al.*, 2003), Moreover, Al altered AChE activity and enhanced lipid peroxidation, probably affecting the integrity and functionality of the cholinergic system (Kaizer *et al.*, 2005), the decline in brain cholinergic function is a major factor responsible for the memory deficit in AD. Accordingly, the activity inhibition of AChE in the two brain regions could be referred to the effect of $AlCl_3$ on the synaptic transmission (Chinoy and Memon, 2001).

Our previous studies have shown that Al influences the activities of free-radical related enzymes (Shati *et al.*, 2011). The decrease in antioxidant enzymes could be either as a result of a decrease in the substrate levels of H_2O_2 or of a reduced synthesis of the enzyme itself as a result of higher intracellular concentrations of Al as reported by Nehru and Anand (2005). Similar results have been reported by Chainy *et al.* (1996) in the liver and by Verstraeten *et al.* (1997) in the brain myelin of mice. In accordance, the preceding results suggest that Al promotes oxidative stress by decreasing the activity of free radicals scavenging enzymes such as SOD, CAT and GSH-Px Shati *et al.* (2011). Consequently, the increased accumulation of lipoperoxidation products as TBARS in the investigated brain regions was accomplished with the decrease in the enzymatic antioxidant activities such as SOD, CAT, GSH-Px and GSH-Red and non-enzymatic antioxidant as GSH content in the $AlCl_3$ group when compared with the control rats as in Table 1 and 2. The results are in accordance with Dua and Gill (2001) who observed a significant decrease in the activities of SOD and CAT in cerebral hemisphere, cerebellum and brain stem after Al exposure.

The aqueous extracts of green coffee and saffron ameliorate the changes associated with $AlCl_3$ in the investigated brain regions. Moreover, the defense against long-term $AlCl_3$ exposure consists of both antioxidants synthesized in the tissues and exogenous antioxidants supplied with diet. Radical scavenger effects of saffron as well as learning and memory improving properties (Abe *et al.*, 1999) and promote the diffusivity of oxygen in different tissues. Saffron extract is also chemopreventive and showed protective effects on genotoxins-induced oxidative stress in Swiss albino mice (Premkumar *et al.*, 2003). The antioxidant properties of water extract of saffron (Shen and Qian, 2006) lead to the alleviation of the disturbances in all biochemical parameters as

in Table 2-4. The increase in the antioxidant capacity in the distinct brain regions of $AlCl_3$ and saffron group was represented by the improvement of the enzymatic and non-enzymatic antioxidants due to the crocins or crocetins (active ingredients of saffron) (Xiang *et al*., 2006; Shati *et al*., 2011). We assumed that, the phenolic compounds as CGA and caffeine of green coffee, act as antioxidant, sharing in the improvement of the disturbances in the antioxidant/oxidant status in the $AlCl_3$ and green coffee group as in Table 2 and 3.

As known Ca helps in the leakage of acetylcholine at the synapses and their decrement adversely affect the nerve conduction and hence may be lead to a deficit in cognition and memory in $AlCl_3$ administered rats. Several studies have demonstrated that Al, at pharmacological doses, is able to reduce the vitamin D dependent calbindin D-mediated transcellular Ca transport in the small intestine of humans, rodents and chickens (Cox and Dunn, 2001). According to Rao (1992), the direct effect of Al depends on Al binding to different brain cells namely astrocytes, neural cells and synaptosomes, but it interacts more with the former causing inhibition of membrane-bound Na^+, K^+, Ca^{2+} ATPases activity. The decrease in Zn level in the investigated brain regions disturbed with $AlCl_3$ intoxication can explained by the insufficiency of the enzymatic and non-enzymatic antioxidant system as it possesses antioxidant properties. The dysregulation of Zn level may plays a role in AD but conflicting findings of increased and decreased levels of Zn have been reported for AD (Burnet, 1981). This disruption arises from its ability to displace Ca from membrane binding sites either on proteins or on the phospholipids in their vicinity. The protective effect of Zn against Aβ peptides toxicity is due partly to the enhancement of Na^+/K^+ ATPase activity which prevents the disruption of calcium homeostasis and cell death associated with amyloid β-peptides toxicity (Lovell *et al*., 1999).

As reported by Markesbery (1999), Amyloid β-peptides induced neuron death *in vitro* is attenuated by antioxidants such as glutathione. Amyloid β-peptides are capable of spontaneously forming oxygen radicals that damage enzymes. They also generate radicals through interaction with iron and Zn both of which are increased in the brains of AD (Markesbery, 1999). It is clear that Zn not only plays critical roles in the structural and functional integrity of many proteins, but that it also modulates the activity of glutamatergic synapses and indeed may act as a neurotransmitter in its own right. Several of the enzymes involved in processing amyloid precursor protein and amyloid β-peptides are Zn metalloproteases, with an essential requirement for Zn in their catalytic activity (Watt *et al*., 2011).

Many studies have shown that green coffee extract is associated with decreased risk for cognitive decline, dementia and AD in various populations (Eskelinen and Kivipelto, 2010). Green coffee and saffron extracts made induction for ADAM10 gene expression but for limit extent. Green coffee had a capability to increase the ADAM10 expression in $AlCl_3$ administered rats, which may considered as kind of protective for the brain cells in $AlCl_3$ group. ADAM10, a member of A disintegrin and metalloprotease family, is α-secretase capable of anti-amyloidogenic proteolysis of the amyloid precursor protein. The principal function of the prodomain is to uphold the metalloprotease site in an inactive state via a cysteine switch (Lemarie *et al*., 2006). Inactivation prevents ADAMs from auto-catalysis during biosynthesis (Seals and Courtneidge, 2003). Moreover, The increase of the expression of ADAM10 agrees with Stoeck *et al*. (2006), suggesting that ADAM17 displays transient inducible activity mediated by protein kinase C activation, whereas ADAM10 achieves inducible and constitutive activity for substrate processing (Stoeck *et al*., 2006). Kuhn *et al*. (2010) reported that the ADAM10 protein can inhibit the formation of β-amyloid which is responsible for AD. ADAM10 acts like a pair of molecular scissors to cut the protein from which

β-amyloid is formed, effectively preventing the formation of β-amyloid. This makes ADAM10 a key molecule in AD therapy. In this study and based on the previous results, we can reported that both of green coffee and saffron extracts could be used in prevention of neurotoxicity of $AlCl_3$.

Toiber and Soreg (2005) reported that, AD involves normal cellular aging and leading to interrelated changes in gene expression and subsequent neurodegeneration. In addition, AD altered AChE properties which may reflect the changes in the enzymatic and/or non-enzymatic features of the multiple AChE splice variants. Neurotoxic effect of Al suppresses the expression of AChE gene in $AlCl_3$ and green coffee group. In contrast, AChE expression was higher in $AlCl_3$ and saffron group than in the control ones. Other studies showed that $AlCl_3$ group show an initial decrease in choline acetyltransferase (ChAT) and a subsequent reduction in AChE protein levels (Younkin *et al*., 1986); these reflect aberrant acetylcholine signaling and impaired nerve impulse transmission. Under chronic cellular stress, aberrant AChE regulation may thus facilitate apoptotic pathways, promoting plaque formation, cognitive impairments and degeneration of cholinergic nerve cells (Parihar and Hemnani, 2003). This work supposes that saffron resist the suppression effect of $AlCl_3$ for the AChE which protect the damage of the neural cells resulted in the deleterious effect of $AlCl_3$.

On the other side the P53 expression was high in $AlCl_3$ group compared with control group as shown in Fig. 1 and 2. But the P53 gene expression was low in positive control groups. The same observation was notable in $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups. Moreover, the expression of Bcl-2 gene was highly expressed in $AlCl_3$ rats in comparison with both control and positive control groups. Generally, the treated rats with green coffee and saffron extracts showed amelioration expression of the examined genes in cerebellum and cerebral hemisphere as shown in figures (1, 2). $AlCl_3$ leads to high expression in P53 $AlCl_3$ group when compared with the control ones. Saffron had a positive effect for inhibiting the expression of the P53 gene induced by $AlCl_3$ as in $AlCl_3$ and Saffron groups. On the other hand, coffee suppressed the expression of the P53 in $AlCl_3$ and coffee group and resisted the effect of $AlCl_3$ on the expression of P53 in brain cells. Hooper *et al*. (2007) postulated that P53 is associated with neurodegenerative disorders. It is worth noting that in brain cells $AlCl_3$ group, P53 was found to be conformational altered, making these cells less vulnerable to stressors or genotoxic insults. The expression of P53 gene was lower in $AlCl_3$ and coffee group. Thus, P53 seems to play a pivotal role in AD, implying that modulation of cell death pathways might be of therapeutic benefit in AD and indeed in other age related neurological disorders (Hooper *et al*., 2007). Bcl-2 induced in the $AlCl_3$ administered group compared with control ones. But when $AlCl_3$ administered groups treated with aqueous green coffee extract low expression of Bcl-2 was observed as a result, green coffee extract inhibit the expression of the Bcl-2. Also saffron extract make suppression of the Bcl-2 in the $AlCl_3$ administered group compared with their control rats. Another study revealed that apoptosis-related genes Bcl-2 played important roles in the course of neuronal apoptosis in brain of $AlCl_3$ administered group. Here we suggest that green coffee suppressed both of P53 and Bcl-2 in the $AlCl_3$ administered rats treated with coffee which may help in deteriorating of the $AlCl_3$ group's brain cells and prevented the neurotoxic effect of $AlCl_3$ to transfer into the other healthy cells. O' Barr *et al*. (1996) showed that the expression Bcl-2 protein is increased in by more than three-fold in AD compared to nondemented samples as detected by immunoblots. In examination of IL-4 and IL-12 genes; their expression was high in $AlCl_3$ groups when compared with the matched control groups. But in the contrary a low gene expression was observed in $AlCl_3$ and green coffee and $AlCl_3$ and saffron groups in comparison with controls and

positive controls. Both coffee and saffron made induction for the IL-4 in $AlCl_3$ administered rats. Moreover, the increase of IL-4 expression means more resistance effect of the brain cells in the investigated brain regions against the neurotoxicity in $AlCl_3$ group. Afkhami-Goli *et al.* (2007) revealed that increase in the expression of IL-4 and astrocytic activation of pro-inflammatory responses or neuronal injury. A few data in the literature indicate that levels of IL-12 in cerebrospinal fluid (Engelborghs *et al.*, 1999) or serum (Singh and Guthikonda, 1997) of patients with AD may not be significantly different as compared with controls. But a high amount of IL-12 in $AlCl_3$ administered group treated with green coffee and saffron extracts was observed as gene expression compared with control ones. This confirms that both coffee and saffron extracts help in increasing the $AlCl_3$ administered rat's immune system through increasing the interleukins expression as anti-inflammatory. IL-12 is a heterodimeric cytokine produced by activated blood monocytes, macrophages and glial cells (Rentzos *et al.*, 2009). It enhances differentiation and proliferation of T cells and increases production of pro-inflammatory cytokines (Li *et al.*, 2003). To move in the end we suggest that both green coffee and saffron extracts play a vital role as anti-neurotoxic especially in cerebral hemisphere and cerebellum tissues of rats.

CONCLUSION

The disruption of the efficient antioxidant enzymes in cerebellum and cerebral hemisphere was associated with the oxidative stress induced by $AlCl_3$ exposure. The administration of aqueous green coffee and saffron extracts can improve the hazards of $AlCl_3$ and also can modulate the antioxidants system and oxidative stress in balance. Therefore, green coffee and saffron have a vital role in the neurotoxicity at least in rats. Beside to ADAM10, AChE, P53, Bcl-2 and Interleukins 4 and 12 genes were adversely affected within cerebral hemisphere and cerebellum in $AlCl_3$ administered rats. The green coffee and saffron extracts attenuate the changes in the expression of these genes in $AlCl_3$ treated rats.

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