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Original Research

Aluminum-Induced Toxicity and Its Response to Combined Treatment of HEDTA and Propolis in Rats

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Abstract

Our study evaluates the protective effect of the chelating agent N-(2-hydroxy ethyl ethylene diamine triacetic acid) [HEDTA] with and without propolis against $Al(NO_3)_3$-induced toxicity in liver, kidney, and brain. Toxicity was induced by a single administration of $Al(NO_3)_3$ at a dose of 6.5 mg/kg, intraperitoneally. HEDTA, propolis, and a combination of HEDTA+propolis were administered for 3 days after 24 h of Al exposure. Significant enhancements in AST, ALT, ALP, cholesterol, triglycerides, urea, uric acid, protein, and blood sugar were found, whereas albumin was decreased after Al exposure. The fall in GSH contents and increase in LPO were significant in hepatic, renal, and neuronal tissues. $Al(NO_3)_3$ caused significant inhibition in the activity of adenosine triphosphatase, superoxide dismutase, and catalase. It inhibited AChE activity in forebrain, midbrain, and hindbrain. Individual treatment of HEDTA and propolis restored biochemical parameters moderately toward control, but combined treatment of HEDTA+propolis showed better results than monotherapy. Combined treatment of HEDTA+propolis reduced oxidative stress and regained histological features and metabolic enzymes of liver, kidney, and brain toward control.

Keywords: aluminum, HEDTA, propolis, liver, kidney, brain

Introduction

Aluminum (Al) is a trivalent atom found in its ionic form in most kinds of animal and plant tissues and in natural water [1]. It is the third most prevalent element and the most abundant metal in the earth's crust [2]. Dietary Al is ubiquitous and urban water supplies contain a greater concentration of Al ions because water is usually treated with the element before supply. Al causes oxidative stress in brain, liver, and kidney tissues. Since the elimination half-life of Al from the human brain is 7 years, this can result in cumulative damage via the element's interference with neurofilament axonal transport and neurofilament assembly. Metals may cause disease through excess, deficiency, or imbalance in concentration [3]. Al exposure can result in its accumulation in liver and hence can be toxic to hepatic tissue at higher concentrations [4, 5]. Exposure to Al can disrupt/inhibit the action of acid and alkaline phosphatases, phosphodiesterase, and phosphooxydase, and can also affect the triglyceride metabolism and triglyceride concentrations in the body [5-7]. Al is one of the most studied neurotoxicants affecting the nervous system involving various regions of brain [8].

Herbal/natural products represent one of the most common forms of complementary and alternative medicines as these products can be considered safer for human health [9-11]. Propolis is one of the natural products that has a vast array of pharmacological actions. It is collected by honey

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bees from sprouts, exudates of trees and other parts of plants, and is modified in the beehive by the addition of salivated secretions and wax [12]. Hundreds of compounds of different groups, mainly phenolic acids and their esters, aldehydes, ketons, and other components have been identified from propolis [13]. Flavonoids and esters of phenolic acids in propolis have been recognized as antifungal [14], antimicrobial [15], antiparasitic [16], antiproliferative [17], chemopreventive [18], immunomodulatory [19], neuroprotective [20], hepatoprotective [21-27], and for adjuvant effects [28-31].

N-(2-hydroxy ethyl ethylene diamine triacetic acid) [HEDTA] is a polyamino carboxylic acid that has successfully been tried in removing various toxic metal ions from biological systems like manganese [32], lead [33], and beryllium [34] due to its multidentate polyfunctional chelating behavior. Thus, a study was planned to evaluate HEDTA and propolis either alone or in combination against aluminum-induced toxic manifestations.

Material and Methods

Chemicals and Animals

Chemicals used in the study were of analytical reagent grade. Aluminum nitrate [$Al(NO_3)_3$] and HEDTA were procured from the Sigma Aldrich Company (St Louis, MO, USA). Crude propolis was generously obtained from Prof. O.P. Agrawal of Jiwaji University, who collected it locally. Adult male Wistar rats (150±10) were obtained from the departmental animal facility, where they were maintained under uniform husbandry conditions (14 h light and 10 h dark with temperature 25º±2ºC and relative humidity 60-70%). Animals were fed on dry pellets of standard animal diet and drinking water *ad libitum*. Prior approval on study design was taken from the institutional animal ethics committee.

Preparation of Doses, Administration, and Study Design

$Al(NO_3)_3$ was dissolved in triple distilled water to prepare its doses of 6.5 mg/kg ($_{1/10^{th}}$ of LD_{50}) and administered intraperitoneally [35]. Propolis extract was prepared as described earlier [36] with the final yield of 65.4% (w/w). Its aqueous suspension (200 mg/kg) was prepared in gum acacia and administered through gavage. HEDTA doses (20 mg/kg) were prepared in normal saline; pH was adjusted to 7.4 and administered to the animals intraperitoneally. The selection of doses of therapeutic agents was based on previous reports [24, 37]. Thirty animals were randomly assigned to five groups of six each. Group 1 served as control. Single doses of $Al(NO_3)_3$ were administered to the animals of groups 2-5 for the induction of toxicity, and group 2 was treated as experimental control. After 24 h of $Al(NO_3)_3$ intoxication, animals of groups 3, 4, and 5 were administered HEDTA, propolis, and a combination of HEDTA+propolis respectively for three consecutive days as follows:

Group 1: Control (received sodium nitrate 4.41 mg/kg, i.p.).

Group 2: $Al(NO_3)_3$ (6.5 mg/kg i.p., only once)

Group 3: $Al(NO_3)_3$ (as in group 2) + HEDTA (20 mg/kg, i.p. for 3 days)

Group 4: $Al(NO_3)_3$ (as in group 2) + propolis extract (200 mg/kg, p.o. for 3 days)

Group 5: $Al(NO_3)_3$ (as in group 2) + HEDTA+propolis extract (as in groups 3 and 4).

Animals were euthanized under mild ether after 48 h of the last administration. Blood was drawn by puncturing retro-orbital venous sinus to isolate serum. Liver, kidney, and brain were promptly excised, washed in chilled saline, blotted, and processed for tissue biochemistry and histology.

Liver and Kidney Function Tests

Aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (ALP), total cholesterol, triglycerides, protein, albumin, blood sugar, urea, and uric acid were quantified by commercially available diagnostic kits.

Brain Function Test

The activity of acetylcholinesterase (AChE) was determined in different parts of the brain, i.e. forebrain, midbrain, and hindbrain [38].

Markers of Oxidative Stress

Toxicant-induced oxidative stress was assessed in liver, kidney and forebrain by reaction of thiobarbituric acid (TBA) and used as an index of lipid peroxidation (LPO) [39].

Status of Antioxidant Pool

Reduced glutathione (GSH) was assessed in homogenates of liver, kidney, and forebrain using dithio nitrobenzoic acid [40] for the nonenzymatic antioxidant pool. Activity of SOD [41] and CAT [42] were determined in liver, kidney, and forebrain for the enzymatic antioxidant pool.

Adenosine Triphosphatase

Activity of adenosine triphosphatase (ATPase) was determined in liver, kidney, and forebrain according to Seth and Tangari [43].

Histological Study

Small pieces of liver, kidney, and brain were washed in saline and fixed in Bouin's fixative, embedded in paraffin, sectioned at 5 µm, and stained with haemotoxylin and eosin for light microscopic examinations.

Table 1. $Al(NO_3)_3$-induced deviation in blood/serum biochemical indices and its response to combination therapy.

Variables/Units	Control	Al	Al+H	Al+P	Al+H+P	ANOVA
Blood sugar (mg/100 ml)	98.0±5.41	146±8.07*	122±6.74	112±6.19***	108±5.97**	9.34***
Cholesterol (mg/dl)	59.0±3.26	75.7±4.18*	63.1±3.48	67.7±3.74	61.9±3.42**	3.85***
Triglycerides (mg/dl)	80.3±4.43	169±9.34*	126±6.96**	99.9±5.52**	91.6±5.06**	35.4***
Protein (mg/100 ml)	38.1±2.11	61.9±3.42*	47.5±2.62**	47.3±2.61**	45.7±2.52**	12.3***
Albumin (g/dl)	4.21±0.23	3.80±0.21*	4.01±0.22	3.92±0.21	4.11±0.22**	0.61
AST (IU/L)	66.0±3.64	123±6.79*	97.0±5.36**	100±5.52**	82.0±4.53**	19.5***
ALT (IU/L)	41.0±2.26	97.6±5.39*	79.1±4.37**	74.9±4.14**	61.2±3.38**	32.6***
ALP (IU/L)	284±15.7	574±31.7*	374±20.7**	369±20.4**	312±17.2**	32.3***
Uric acid (mg/dl)	3.18±0.17	4.89±0.27*	4.22±0.23	4.21±0.23	3.85±0.21**	16.9***
Urea (mg/dl)	19.6±1.08	58.5±3.23*	44.6±2.46**	42.8±2.36**	38.2±2.11**	42.5***

Values are mean±SE of six animals in each group, *Significant Al *vs* Control, **Significant treatments *vs* Al, ***significant ANOVA at $P \leq 0.05$.
Abbreviations: Al – $Al(NO_3)_3$, H – HEDTA, P – Propolis

Statistics Analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Student's T-test, taking significance at $P \leq 0.05$ [44].

Results

Table 1 presents the toxic manifestation of $Al(NO_3)_3$ and its response to HEDTA and propolis administration in various blood biochemical indices that include liver and kidney function tests. Exposure to $Al(NO_3)_3$ significantly increased triglycerides, cholesterol, blood sugar, and protein, whereas it decreased the albumin. Treatment with HEDTA, propolis individually, and their combination decreased serum triglycerides and protein significantly toward control ($P \leq 0.05$). Significant fall was noted in blood sugar content with therapy of propolis and combination of HEDTA+propolis, whereas cholesterol and albumin were found to be very near control only with a combination of HEDTA+propolis ($P \leq 0.05$). Elevated leakage of AST, ALT, and ALP were noted after $Al(NO_3)_3$ intoxication. Treatment with HEDTA, propolis, and HEDTA+propolis prevented leakage of serum AST, ALT, and ALP toward control ($P \leq 0.05$). $Al(NO_3)_3$ exposure increased urea and uric acid significantly ($P \leq 0.05$). Deviated value of serum urea was significantly regained toward control by individual and combined administration of test drugs, whereas only combination therapy showed significant recovery in uric acid ($P \leq 0.05$).

Fig. 1. presents toxic manifestation of $Al(NO_3)_3$ and its response to HEDTA and propolis administration in various markers of oxidative stress, status of the antioxidant pool, and brain function test in liver, kidney, and brain. Significant increases in LPO and falls in GSH levels were observed in liver, kidney, and forebrain after $Al(NO_3)_3$ administration ($P \leq 0.05$). Therapy with HEDTA, propolis and HEDTA+propolis inhibited hepatic, renal, and neuronal LPO ($P \leq 0.05$). Neuronal GSH level was found toward control only after treatment of HEDTA+propolis ($P \leq 0.05$). Hepatic GSH was not recovered by individual and combination therapy, whereas renal GSH level was significantly increased with all the treatments ($P \leq 0.05$). $Al(NO_3)_3$ exposure significantly decreased the activity of SOD and CAT ($P < 0.05$). Therapy with HEDTA, propolis, and HEDTA+propolis increased the activity of neuronal SOD and CAT toward control ($P \leq 0.05$). Hepatic and renal SOD was maintained toward control only with combination therapy ($P \leq 0.05$). Administration of $Al(NO_3)_3$ caused significant decreases in AChE activity of forebrain, midbrain and hindbrain, indicating alterations in brain function ($P \leq 0.05$). Hepatic, renal, and neuronal ATPase was decreased after $Al(NO_3)_3$ exposure. ATPase, ALPase, and AChE activities were found to be restored toward control after treatment of HEDTA, propolis, and HEDTA+propolis in liver, kidney, and brain ($P \leq 0.05$).

Fig. 2 (A-F) exhibit hepatic histological observations. $Al(NO_3)_3$ exposure caused loss of hepatic cord arrangement, plasma membranes, degenerated nuclei, and loss of sinusoidal spaces. HEDTA treatment exhibited improvement in central vein, and sinusoidal spaces and showed binucleated hepatocytes. Propolis treatment exhibit improved central vein, and cordially arranged hepatocytes. Combined treatment of HEDTA+propolis depicted a well-formed central vein with well arrayed hepatocytes and proper sinusoidal spaces around the CV. Fig. 2 (G-L) exhibits renal histological observations. $Al(NO_3)_3$ exposure caused shrunken and broken glomeruli of kidney. HEDTA treatment improved glomerulus in renal corpuscle. Propolis treatment depicted regeneration in tissues. Combined treatment of HEDTA+propolis exhibit well formed glomerulus in renal

corpuscle, wider lumen, and prominent nuclei in tubules. Fig. 2 (M-R) depicts $Al(NO_3)_3$-exposed forebrain exhibiting degenerated cerebral cortex region. HEDTA treatment improved the histological features of forebrain. Propolis treatment exhibit better histological features when compared to the Al-intoxicated group. The cerebral cortex region showed excellent recovery pattern after combined treatment of HEDTA+propolis.

Discussion of Results

Earlier studies have shown that ingested $Al(NO_3)_3$ elevates its concentration in various regions of the brain [45, 46] and induces oxidative stress [47]. Evidence indicates $Al(NO_3)_3$-induced LPO [45, 48], and inhibition in Na^+, K^+ ATPase in the brain [49]. Its toxicity may be mediated by free radical generation and alterations in antioxidant enzymes, which also cause nephrotoxicity [50] and hepatotoxicity [51, 52]. The results of the present study clearly showed that $Al(NO_3)_3$ exposure elevated LPO and decreased GSH level as well as inhibited the activities of SOD and CAT in forebrain, liver, and kidney, which was an indication of oxidative stress in the animals even with acute exposure to $Al(NO_3)_3$ [53, 54]. Treatment with a combination of HEDTA and propolis reversed $Al(NO_3)_3$-induced changes in different variables. Retardation in $Al(NO_3)_3$-induced oxidative stress indicated the antioxidative potential of propolis, which is known for its antioxidant and antilipidperoxidative capability against a variety of oxidative stresses [21, 22, 29, 55] and thus could suppress $Al(NO_3)_3$-induced oxidative damage. GSH plays an important role in the detoxification and metabolism of many xenobiotics. Combined therapy of the HEDTA+propolis augmented the GSH pool, which could help in countering $Al(NO_3)_3$ toxicity.

Serum AST and ALT were found to be increased after $Al_2(NO_3)_3$ exposure, which might be due to alterations in integrity or permeability of hepatic cell membranes [56]. These results are in agreement with other reports [5]. Combined treatment of HEDTA and propolis significantly reduced the leakage of AST and ALT, indicating the membrane stabilizing potential of this combination. Increased levels of triglycerides, cholesterol, urea, and uric acid were found after $Al(NO_3)_3$ exposure, whereas serum albumin was decreased. Serum triglyceride was increased, possibly

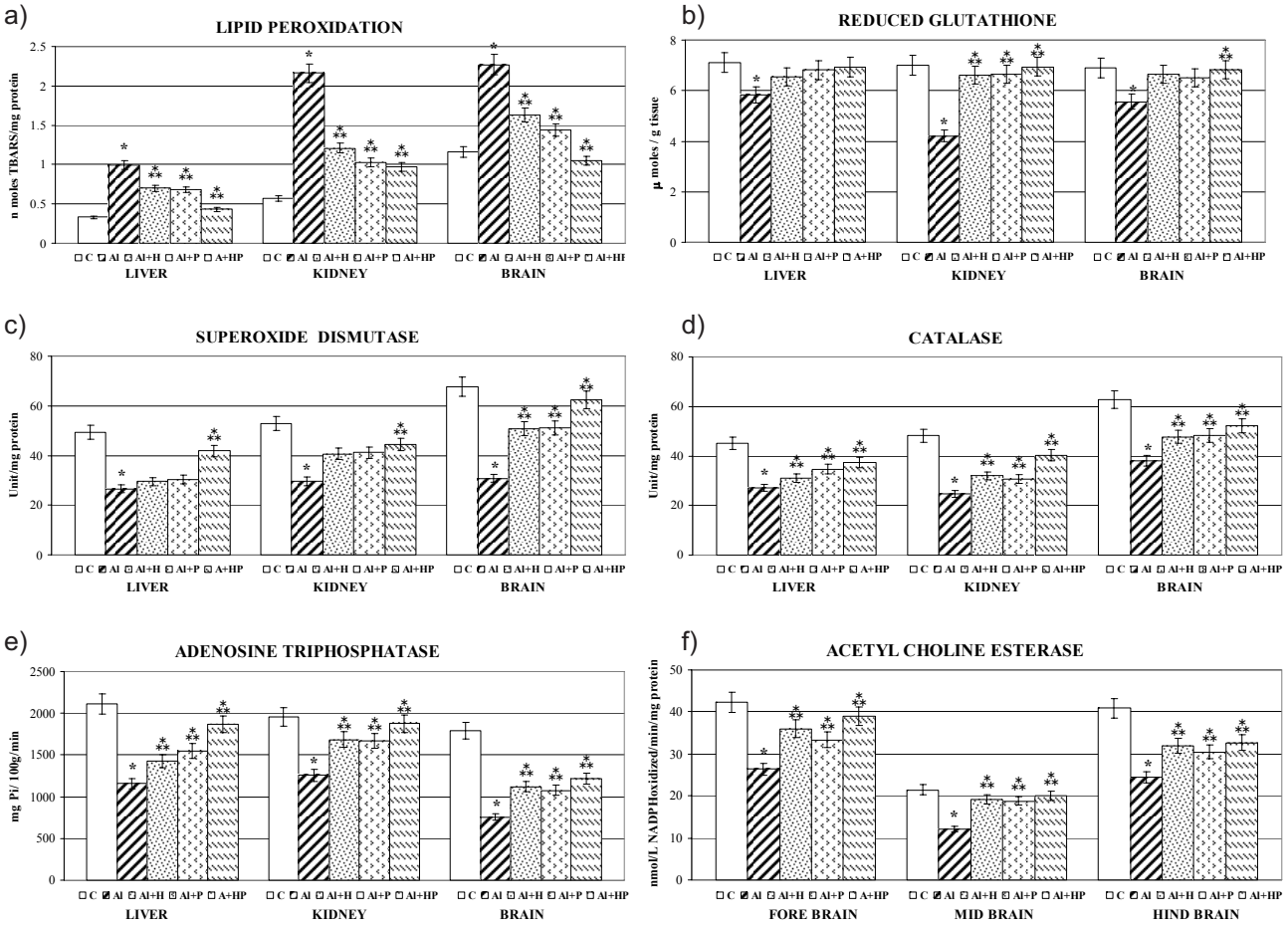

Fig. 1. (A-F). Toxic effects of $Al(NO_3)_3$ on LPO, GSH level, SOD, CAT, ATPase, and AChE activity and therapeutic influence of HEDTA and propolis.

*Significant Al vs Control, ***Significant treatments *vs* Al,

Abbreviations: C– Control, Al – $Al(NO_3)_3$, H – HEDTA, P – Propolis

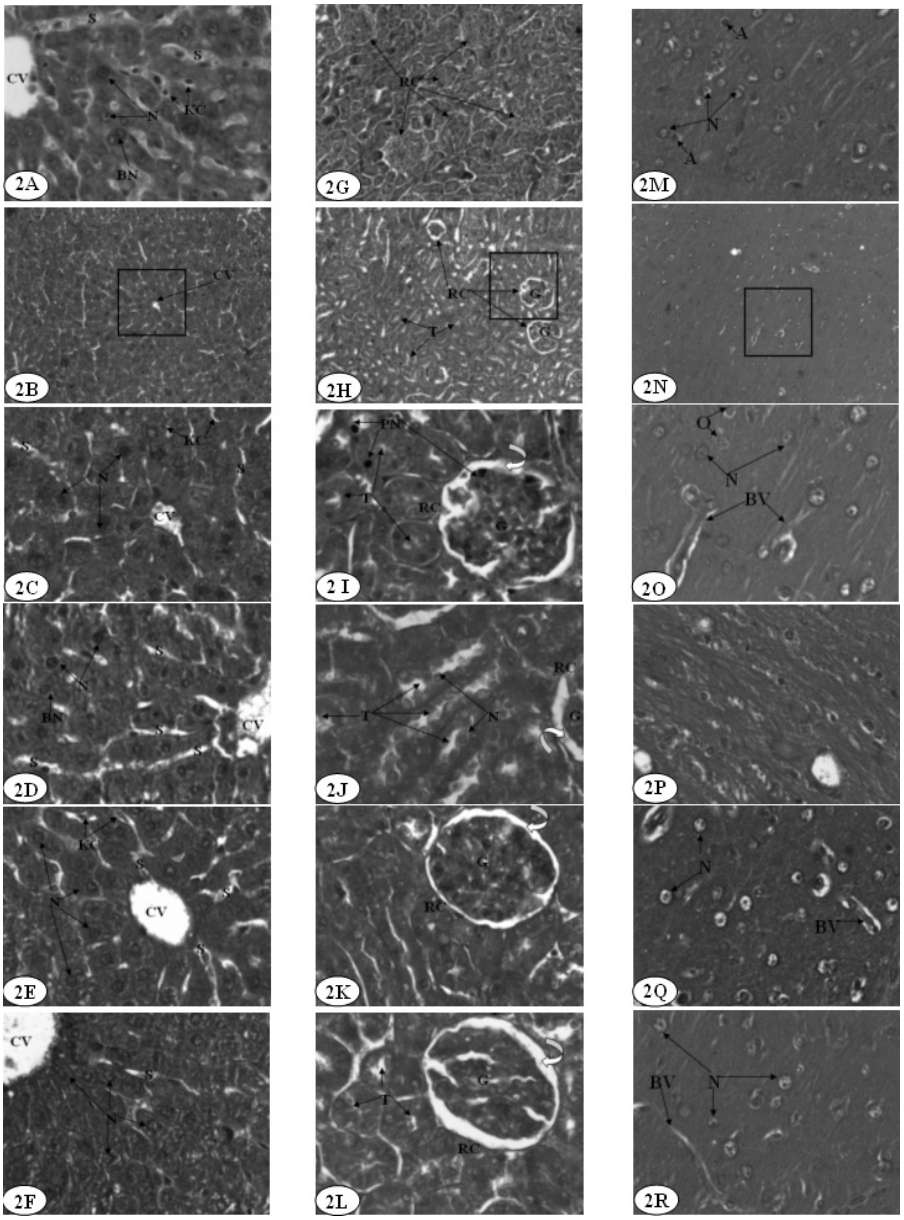

Fig. 2. (A) Histological features of control liver exhibited well formed central vein (CV), hepatic cord arrangement around the CV with prominent cell membranes, nuclei, sinusoidal spaces (S), Kuppfer cells (KC) and binucleated hepatocytes (BN) (400 X). (B) $Al(NO_3)_3$ exposed liver exhibit several irregularities (100 X). (C) Enlarged view of selected area of fig B exhibit irregular central vein (CV), loss of hepatic cord arrangement and cell membranes, degenerated nuclei (N), and loss of sinusoidal spaces (S) (400 X). (D) Liver after HEDTA treatment exhibit central vein (CV) filled with debris, hepatocytes with better formed nuclei (N), binucleated hepatocytes (BN) indicating regeneration, and better formed sinusoidal spaces (S) (400 X). (E) Liver after propolis treatment exhibit improved central vein (CV), hepatocytes arranged in cord like fashion with prominent nuclei (N), and cell membranes. However, sinusoidal spaces (S) were regained only in a few places (400 X). (F) Liver after combined treatment of HEDTA and propolis exhibit well-formed central vein (CV), hepatic cord arrangement with prominent nuclei (N), cell membranes and well arrayed sinusoidal spaces (S) around the CV (400 X). (G) Histological features of control kidney exhibit well-formed renal corpuscles (RC) having regular glomerulus and uniform Bowman's space around it, renal tubules with basal prominent nuclei (100 X). (H) $Al(NO_3)_3$-exposed kidney exhibit shrunken and broken glomeruli (G) (100 X). (I) Enlarged view of selected area of Fig. H exhibit broken glomerulus (G) in renal corpuscle (RC), pyknotic nuclei (PN), obstruction in renal tubules (T) and irregular Bowman's space around glomerulus (curved arrow) (400 X). (J) Kidney after HEDTA treatment exhibit improved glomerulus (G) in renal corpuscle (RC), better Bowman's space around glomerulus (curved arrow), improved renal tubules (T) with wider lumen, prominent nuclei in tubules. However, degeneration in tubular membrane still persists (400 X). (K) Kidney after propolis treatment exhibit better glomerulus (G) in renal corpuscle (RC) (400 X). (L) Kidney after combined treatment of HEDTA and propolis exhibit well-formed glomerulus (G) in renal corpuscle (RC), uniform Bowman's space around glomerulus (curved arrow), well-formed renal tubules (T) demarcated with well preserved plasma membrane, wider lumen, and prominent nuclei in tubules (400 X). (M) Histological features of cerebral cortex region of brain of control group exhibit well-formed neurons (N) of stellate type, blood vessels, and neuroglial cells, including astrocytes (400 X). (N) $Al(NO_3)_3$-exposed brain exhibit cerebral cortex region (100 X). (O) Enlarged view of selected area of Fig. N exhibit irregular features with blood vessels (BV), neurons (N), and oligodendrocytes (O) (400 X). (P) Cerebral cortex region of brain after HEDTA treatment exhibit improved histological features (400 X). (Q) Cerebral cortex region of brain after propolis treatment exhibit better recovery in tissues (400 X). (R) Cerebral cortex region of brain after combined treatment of HEDTA and propolis exhibit better formed histological features (400 X).

due to hypoactivity of lipoprotein lipase in blood vessels, which breaks up triglycerides. The higher level of serum cholesterol and low concentration of albumin might be due to hepatic dysfunctions [57]. Cholesterol content in brain was found to be higher in $Al(NO_3)_3$-intoxicated animals [54]. Urea and uric acid were elevated in $Al(NO_3)_3$-exposed rats, which clearly revealed nephrotoxicity. These findings were corroborated by Mahieu et al. [50]. Treatment with a combination of HEDTA and propolis could reverse all these variables toward control due to improvements in organ functioning toward control.

AChE activity was significant decreased in forebrain, midbrain, and hindbrain after $Al(NO_3)_3$ exposure that might be due to the interference with either synthesis of AChE or inhibited choline uptake by synaptosomes. Al^{+++} may also bind with active sites of AChE and thus decrease the activity of AChE in all three parts of brain. When this enzyme is inhibited, ACh is not hydrolyzed and accumulates in cholinergic sites, causing alteration in the normal nervous system function. Significant decrease in the acetylcholinesterase activity in brain of rats after $Al(NO_3)_3$ exposure had already been reported [47]. Concomitant administration of HEDTA and propolis extract showed neuroprotection by enhancing AChE in all parts of the brain. The present data also showed that aluminum inhibited ATPase enzyme, which is involved in maintaining Na^+, and K^+ ion gradients across the cell membrane. Since this enzyme is susceptible to the cell membrane's lipid environment, inhibition of this enzyme might largely be a consequence of aluminum-induced LPO.

Thus, it can be concluded that combined treatment of HEDTA and propolis express excellent therapeutic value over monotherapy in preventing complications associated with aluminum-induced toxicity.

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