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Modulatory Effects of *Casimiroa Edulis* on Aluminium Nanoparticles - Associated Neurotoxicity in A Rat Model of Induced Alzheimer's Disease

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Abstract

This study evaluated the biological effectiveness of aluminum oxide nanoparticles (Al2O3NPs), on brain rats by analyzing brain function neurotransmitters, oxidative stress and antioxidant biomarkers. Rats were administered Al2O3 nanoparticles (NPs)of 20 nm diameter at dose 50 mg/kg body weight and examined for induction of Alzheimer's disease (AD) in rats model through determination of acetylcholine esterase (ACHE), noradrenaline (NA), adrenaline (A), serotonin , dopamine , apoptotic markers; caspase -3, oxidative stress, lipid peroxide (MDA), nitric oxide (NO) antioxidant markers , glutathione reduced (GSH), total antioxidant capacity (TAC) and calmoduline (CaM) . Exposure to Al2O3 NPs induced AD in rats showed significant reduction in NA, A with percentages decrease 42.18 and 34.09%, respectively , while significant increase in ACHE activity (64.83%), as compared to normal control rats. However AD induced rats showed significant reduction in serotonin and dopamine with percentages decrease 38.17and 54.02%, respectively as compared to normal control rats. However, Al NPS intoxicated rats showed significant increase in caspase -3 with percentages increase 99.10%. However, Al NPS induced rats showed significant decrease in TAC and GSH levels with percentages 59.70 and 61.00%, respectively. While, significant increase in CaM(+206.09%), lipid peroxide (108.74%) and NO (74.43%) levels as compared to normal control rats was detected. Histopathological investigation of NPs induced rats' demonstrated marked amelioration in all parameters under investigation upon treated intoxicated rats with *Casimiroa edulis* total extract as compared to dopenzil standard drug. These results are conformed by histopathological examination which revealed improvement in brain neuron architectures with various degrees. It could be concluded that *Casimiroa edulis* total extract may be useful in ameliorating Al NPs induced AD in rats' model which may be used as an intelligent candidate nutraceutical.

1. Introduction

Particles of nano scale are one of the rapidly developing area in research of materials and are recently being used in a great range, as in manufactories of military , biomedical applications, owing to their characteristics of unique size. The major distinctive things of the nanoparticle, called quantum size and surface-induced effects, as a leading cause of its tiny size. Numerous dental applications included nanoparticles have been progressed and are obtainable [1]. Titanium dioxide, silicon dioxide, aluminum oxide, and zinc oxide are widespread in dental products. Nanoparticle sizes are identical to that of lysosomes, nuclei and mitochondria as well as /they may be capable of to pass cell barriers, as a result of that unexpected reverse tissue impact. Hence, an critical point in research of NP about, where and how NPs are inserted into components of cells [1].In the sciences of medicine, it is serious not only to know the size modifying effect, but also to assess the nanoparticles penetration mechanisms, their activity, their metabolic pathways and the degree of accumulation inside the cells of different organs of living organisms.

One of the progressive fetal neurodegenerative diseases is Alzheimer's disease (AD) which included chronic inflammation of CNS resulting in markedly oxidative damage [2]. Progressive cognitive deterioration is considered the principle characteristic associated disease [3]. Combined with continuous obvious reduction in activities and behavioral alterations. Noticeable of cerebral cortex atrophy and cortical neurons loss are the important features of AD. Then neuropathological determination associated AD, in the brain of human, showed amyloid plaques [3], tangles of neurofibrillary [2], as well as degeneration of neurons with deteriorations of memory short-term. Although, a global experiments using animal model confirmed abnormalities in the cognitive, behavioral, biochemical, and histopathological examination associated AD patients do not found [3]. Nevertheless, different partial reproductions of AD reports have been attained with several approaches of pharmacology such as cholinergic and amyloid peptide related -dysfunction in animal models [2]. Aluminium

(Al) silicate granules have been observed in the brain patients with AD [2], and the aluminium chloride ($AlCl_3$) symptoms induced brain toxicity in mice similar those of AD [3]. Al, a known neurotoxicant [4], has been declared under normal physiological conditions to alter the blood–brain barrier (BBB), so, it can easy to pass the central nervous system (CNS) and accumulates in the different brain regions [4]. It has been demonstrated to be implicated in the etiology of various neurodegenerative diseases [5]. Further, Al being a powerful cholinotoxin [4], promotes neuronal apoptosis related to acetylcholinesterase (AChE) high levels in the brain [5]. Al is ascertained to promote Fe^{2+}peroxidative effect suggested its role in oxidative damage[2].Al administration induced oxidative stress produced membrane lipids, proteins and defense of antioxidant enzymes. *In vivo* studies report on the apoptosis like changes [2]. Further, Al produces amyloid-b-protein accumulation; hyper phosphorylated tau-protein aggregation which includes neurofibrillary tangles (NFTs) beside, it promotes cholinergic neurotransmission alterations [3].

Several evidences documented the use of plant-derived antioxidants for the neurodegenerative disorders control [6]. Neuroprotective effect (apoptosis prevention) may occur by antioxidants which may be also have neurodegenerative efficacy, by decreasing or inverting cellular destruction through decelerating loss of neuronal cells progression. Several phytochemicals are ascertained to have neuroprotection effect however their across towards BBB needs to be documented. There are several plants that have various biological properties and are recently used in folk medication. *Sapote blanche or Casimiroa edulis* Llave et Lex (Rutacae)is one of such plants, which is promising for its sedative efficacy as induce of sleep[8]. The tree is cultivated in Egypt for its edible fruits. Previously, extensive works have been run on the separation and chemical constituents identification of the bark, seeds and root of *Casimiroa edulis* [**7**]. The separated chemical constituents comprised casimiroedine and dimethyl histamine [9], *N*a, *Na*-dimethyl histamine [10], zapoterin, imidazole [11] and 2-quinolinone, and 4-quinolones [12]. The aqueous extract of *Casimiroa edulis* leaves has anti-inflammatory and diuretic effects [12]. However, the alcoholic extract of the leaves has anticonvulsant, antimutagenic, and sedative activities. The compounds isolated from leaves include isoimpinellin, casimiroin, skimmianine, 1-methyl-2-phenyl-4-quinol, edulein, and scopoletin methyl ether [13]. Hence, the aim of the present study is to determine aluminum oxide NPs effects (20 nm diameter) on rats model upon treated rats with 50mg /kg body weight to induce Alzheimer´s disease (AD) and the therapeutic ameliorative role of *Casimiroa edulis* total extract.

2. Material and Methods

2.1. Chemicals

Donepezil and all chemicals were purchased from Sigma Co (USA) and aluminum chloride from BDH Laboratory Supplies, Poole (UK).

2.2. In vitro toxicity study

In vitro study revealed that Al-nanoparticles were tested against rate embryo hippocampus neuronal culture. Sample concentrations ranges between 100 10 0.78 ug/ml using MTT assay [11].

2.2.1. Plant material

The leaves of *Casimiroa edulis* LlaveetLex (Rotaceae) were collected from Dakahlia governorate, Egypt, in 2001. The plant identification was verified by the late Prof. N. El-Hadidi, Professor of Botany, Botany Department, Faculty of Science, Cairo University, and a voucher specimen has been deposited in the herbarium of the Desert Research Center, Cairo, Egypt. The plant

2.2.2. Preparation of plant extract

The air-dried powdered of *C.edulis* leaves (1 kg) were extracted in a soxhlet apparatus with 95% ethanol [7],at room temperature and the residues were repercolated four times, collected and then concentrated under reduced pressure at a temperature not exceeding 35 ^{o}C to yield a dry extract of 89 g.

2.3. In vivo study

2.3.1. Experimental animals and design

Forty adult male Sprague-Dawley rats weighing 100–120 g were obtained from the Animal House Colony of the National Research Centre, Giza and acclimated in a specific pathogen-free area at 25 ± 1 ºC and controlled constantly humidity (55 %) with a 12 h light/dark cycle. The rats were housed with ad libitum access to standard laboratory diet consisting of 10 % casein, 4 % salt mixture 4 %,1 % vitamin mixture, 10 % corn oil and 5 % cellulose, completed to 100 % with corn starch. Animals were cared and sacrificed according to the guidelines for animal experiments by the Ethical Committee of NRC. Study was approved by the Ethical committee of the National Research Centre (NRC) Egypt. The animals were classified into four groups of 10 rats each. Group one: Normal control rats. Group two: Serving as Al nanoparticles -intoxicated rats were injected daily i.p. with

aluminum nanoparticles (Al_2O_3NPs) suspended in distilled water for one month in a dose 50 mg /Kg b. w.[15],for induction of AD. Group three: Al nanoparticles -intoxicated rats treated orally daily with 500 mg /kg body weight of total extract of *Casimiroa edulis* for one month .Group four: Al intoxicated rats treated orally daily with standard Donepezil drug daily for one month in a dose 10 mg /Kg b.w.[16].

2.3.2. Brain tissue sampling and preparation

At the end of the experiment, the rats were fasted overnight, subjected to anesthesia with diethyl ether and sacrificed. The whole brain of each rat was rapidly dissected, washed with isotonic saline and dried on filter paper. Each brain was divided sagittall into two portions. The first portion was weighed and homogenized in ice cold medium containing 50 mMTris/HCl and 300mM sucrose at pH 7.4 to give a 10 % (w/v) homogenate. This homogenate was centrifuged at 1400 × g for 10 min at 4 °C. The supernatant was stored at –80 °C and used for biochemical analyses that included oxidative stress biomarkers (NO and MDA),non-enzymatic antioxidant (GSH), apoptotic marker (caspase 3), cholinergic markers; acetylcholine esterase (ACHE), and neurotransmitters ; dopamine, adrenaline (AD) , noradrenaline (NA) and serotonin (5-HT) . The second portion of the brain was fixed in 10 % formalin for histological investigation. The ethical conditions were applied such that the animal suffered no pain at any stage of the experiment and the study was approved by the Ethics Committee of the National Research Centre. Animals were disposed of in bags provided by the Committee of Safety and Environmental Health, National Research Centre.

2.3.3. Biochemical analyses

2.3.3.1. Oxidative stress biomarkers

Nitric oxide was done using the method of Berkels et al. [17]. A lipid peroxidation product represented by malondialdehyde (MDA) was evaluated by the method of Satoh [18], using thiobarbituric acid (TBA) and measuring the reaction product spectrophotometrically at 534 nm.

2.3.3.2. Antioxidant enzymes activity

Brain glutathione (GSH) was measured colorimetrically according to the method of Beutler et al.[19].

2.3.3.3. Brain CaM , caspase-3 and ACHE enzyme activity

The activity of caspase-3 enzyme in the brain tissue homogenate is directly proportional to the color reaction according to the method of Thompson [20]. Brain ACHE was determined colorimetrically according to the methodof Den Blaauwenet al.[21]. Brain calmodulin(CaM): The activity an activator of cAMP phosphodiesterase was assayed in brain tissue homogenate by spectrophotometric assay method according to Garget al.[22].

2.3.3.4. Brain Neurotransmitters estimation (NA, AD, DA and 5-HT)

Frozen brain samples were homogenized in 30 ml butanol for catecholamine extraction as described by Brownlee and Springgs [23]. This extraction was then transferred to a 125 ml bottle and 2 ml of 0.01 N HCI and 5 g sodium chloride were added. The bottle was then shaken at room temperature with a speed of 50 strokes per minute for one hour. The mixture was then centrifuged at 2000 rpm for 5 min. After extraction, fluorimetric technique was used for estimation of neurotransmitters (NA, AD, DA and 5-HT) levels at excitation and emission wave length on spectrophotometer as described by Ciarlone [24].

2.3.3.5. Measurement of serum TAC levels

Serum TAC levels were determined using an automated measurement method, which is based on the bleaching of the characteristic color of a more stable 2, 2-azino-bis (3-ethylbenz-thiazoline- sulfonic acid, (ABTS) radical cation by antioxidants(Beckman Coulter - Fullerton, CA, USA) [25]. The ABTS radical cation is decolorized by antioxidants according to their concentrations and antioxidant capacities. The results are expressed in mmol Trolox equivalents/L.

2.3.3.6. Comet assay

0.5 g of crushed samples were transferred to 1 ml ice-cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was mixed with 600 µl of low-melting agarose (0.8% in PBS). 100 µl of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining with ethidium bromide 20µg/ml at 4°C. The observation was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (With excitation filter 420-490nm [issue 510nm]). The comets tails lengths were measured from the middle ofthe nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization

of DNA damage, observations are made of EtBr-stained DNA using a 40xobjective on a fluorescent microscope.

Although any image analysis system may be suitable for the quantitation of SCGE data, we use a comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample [26].

2.3.4. Histopathological examination

The brain tissue was fixed in 10 % formalin for one week, washed in running tap water for 24 h and dehydrated in ascending series of ethanol (50–90 %), followed by absolute alcohol. The samples were cleared in xylene and immersed in a mixture of xylene and paraffin at 60 ºC. The tissue was then transferred to pure paraffin wax of the melting point 58 ºC and then mounted in blocks and left at 4 ºC. The paraffin blocks were sectioned on a microtome at thickness of 5 µm and mounted on clean glass slides and left in the oven at 40 ºC to dryness. The slides were deparafinized in xylene and then immersed in descending series of ethanol (90–50 %). The ordinary haematoxylin and eosin (H & E) stain was used to stain the slides [27].

2.3.5. Statistical analyses

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

3. Results and discussion

In vitro study revealed that Al-nanoparticles were tested against rat embryo hippocampus neuronal culture. Sample concentrations ranges between 10, 15, 50 and 100 ug/ml uing MTT assay. The results revealed 100% death of neuronal cells up to 12.5 ug/ml (Table 1).

Table (1): In vitro Al oxide nanoparticles toxicity effect on brain neuronal rats

Sample	LC_{50}	LC_{90}	Remarks
1	100%up to 12.5 ug/ml	----------	----------
DMSO	----------	----------	1%at 100ppm
Negative control	----------	----------	0%

LC50: Lethal concentration of the sample which causes the death of 50% of cells in 48h.
LC90: Lethal concentration of the sample which causes the death of 90% of cells in 48 h.

Table (2) recorded insignificant change in NA, AD and ACHE levels in normal rats treated with total extract of *Casimiroa edulis* as compared to untreated control rats.

Table 2: Effect of total extract of *Casimiroa edulis* on adrenergic and neurotransmitters level of Al nanoparticles induced rats

Groups \ Biomarkers	NA(ng/gm)	A (ng/gm)	ACHE(ng/ml)
Normal control	187.20±11.23^{b}	289.15±9.30^{e}	96.67±6.23^{a}
Normal Control+ total extract %change	189.90±21.10^{b} +1.44	290.15±10.34^{e} +0.34	90.89±5.14^{a} -5.98
Al-neurotoxic rats %change	108.23±10.25^{a} -42.18	190.56±13.56^{g} -34.09	159.34±12.10^{h} +64.83
Total extract treated Al –intoxicated rats %change %of improvement	161.20±9.11^{b} -13.88 28.29	245.21±15.45^{g} -15.19 18.90	125.13±9.21^{e} +29.44 35.39
Standard drug treated Al –intoxicated rats %change %of improvement	167.29±15.73^{b} -10.63 31.55	249.10±7.25^{e} -13.85 20.25	105.88±10.12^{a} +9.53 55.30

Statistical analysis is carried out using SPSS and co-state computer program, where unshared letters are significant at $p \leq 0.05$.

However AD induced rats showed significant reduction in NA, A with percentages decrease 42.18 and 34.09% , respectively, while significant increase in ACHE activity (64.83%) , as compared to normal control rats . Treatment of neurotoxic rats with total extract declared insignificant change in NA level and ACHE activity as compared to normal control rats , while significant reduction in A level with percentage of improvement 18.90% . In addition, treatment of rats with standard drug showed insignificant change in NA, A and ACHE as compared to normal control rats.

Table (3) declared insignificant change in serotonin and dopamine levels in normal rats treated with total extracts of *Casimiroa edulis* as compared to control rats. However neurotoxic rats showed significant reduction in serotonin and dopamine with percentages decrease 38.17 and54.02%, respectively as compared to normal control rats. Treatment of stimulated neurotoxic rat with total extract of *Casimiroa edulis* declared significant reduction in serotonin and dopamine levels with percentages improvement 21.08 and 23.69%, respectively. While, the percentage of improvement reached to 26.17 and 23.76 %, in sertonine and dopamine levels respectively upon treated intoxicated rats with standard drug.

Table (3): Effect of *Casimiroa edulis* total extract on serotonine and dopamine levels in Al nanoparticles induced rats.

Biomarkers / Groups	5-HT(ng/gm)	DA(ng/gm)
Normal control	90.54±5.07^{a}	66.33±6.13^{ef}
Normal Control+ Total extract % change	92.23±6.33^{a} +1.83	69.20±7.11^{e} +4.33
Al –neurotoxic rats % change	55.98±8.30^{b} -38.17	30.50±3.22^{h} -54.02
Total extract Treated-neurotoxic rats % change % of improvement	75.07±4.11^{g} -17.09 21.08	46.22±4.22^{b} -30.40 23.69
Standard Drug % change %of improvement	79.67±3.44^{f} -12.00 26.17	50.90±4.15^{b} -23.26 30.76

Statistical analysis is carried out using SPSS and co-state computer program , where unshared letters are significant at p≤0.05

Table (4) showed insignificant change in caspase -3 activities in normal rats treated with total extract of *Casimiroa edulis* as compared to untreated control rats. However Al_2O_3 nanoparticle - induced rats showed significant increase in caspase -3 activities with percentage increase 99.10% comparing to normal control rats. Treatment of intoxicated rats with total extract of *Casimiroa edulis* as well as standard drug demonstrated significant deccrease in caspase –3 as compared to intoxicated rats with percentages of improvement 67.83and 72.95%, respectively.

Table (4) : Effect of *Casimiroa edulis* total extract on caspase -3 level in Al nanoparticles induced rats.

Parameters / Groups	Caspase-3 (pg/ml)
Normal control	9.98±1.55^{a}
Control rats + Total extract %change	9.70±1.00^{a} -2.80
Al – neurotoxic rats %change	19.87±2.00^{b} +99.10
Total extract treated neurotoxic rats %change %improvement	13.10±1.34^{c} +31.26 67.83
Standard Drug–treated neurotoxic rats %change %improvement	12.59±1.56^{c} +26.15 72.95

Statistical analysis is carried out using SPSS and co-state computer program, where unshared letters are significant at $P \leq 0.05$.

Table (5) revealed insignificant change in CaM, TAC, GSH, lipid peroxide (MDA) and NO levels in normal rats treated with total extracts of *Casimiroa edulis* as compared to untreated control rats. However, neurotoxic stimulated rats showed significant decrease in CaM, TAC and GSH levels with percentages 50.32% , 59.70 and 61.00%, respectively , while significant increase in lipid peroxide (108.74%) and NO (73.43%) levels as compared to normal control . Treatment of Al nanoparticles induced rats with total extract of *Casimiroa edulis* declared significant increase in CaM, TAC and GSH levels with percentages of improvement reached to 23.72, 23.88 and 39.88%, respectively. While lipid peroxide and NO levels demonstrated improvement with percentages 62.25 and 48.91%, respectively. Concerning standard drug, it demonstrated insignificant change in TAC, while it showed significant increase in CaM and GSH with percent of improvement 16.98 and 40.96% %, respectively .In addition significant decrease in lipid peroxide and NO levels upon treated rats with standard drug with amelioration percent 71.12 and 60.96%, respectively.

Table (6) represented insignificant change in all comet parameters in normal rats treated with total extract of *Casimiroa edulis* comparing to untreated rats. AD rats showed significant increase in the all detected DNA comet assay parameters. Marked amelioration was detected in the parameters of DNA comet assay upon treated neurotoxic rats with total extract of *Casimiroa edulis* with more or less similar effect as standard drug.

Histopathological examination of normal control treated rats with ethanolic extract of *Casimiroa edulis* comparing to untreated one (Photomicrographs 1and 2).While , Al –intoxicated rats showed pyknosis and necrosis of neurons, focal cerebral hemorrhage ,pyknosis and necrosis of pyramidal cells (Photomicrographs 3 -5), comparing to control rats (Photomicrographs 1).While , treatment of intoxicated rats with ethanol extract of *Casimiroa edulis* demonstrated no histopathological changes (Photomicrograph 6),however necrosis of some sporadic pyramidal cells was detected (Photomicrograph 7). Further, donepezil standard treated intoxicated rats showing no histopathological changes (Photomicrographs 8, 9). Table (7) indicated histopathological lesion score in different groups, where (-) no change (+) mild change (++) moderate change (+++) severe change.

The current results revealed significant elevation in the oxidative damage biomarkers in brain tissue following AlNPs .This result is in agreement with Johnson et al. [28] and Aly et al.[29] , who declared that the Al –linked neurotoxicity may be a leading to rise in lipid peroxidation . Further, Nehru and Anand [5] added a marked elevation in thiobarbituric acid reactive substances in rats brain post aluminium induction which related to Fe^{3+}carrying protein transferring bounding, hence lowering Fe^{2+} binding and rising free intracellular Fe^{2+}that produces membrane lipids, proteins peroxidation and later membranes destruction, though loosing of membrane fluidity , alteration in potential of membrane, an elevation in permeability of membrane and disturbances in the function of receptors [30]. In addition, the present study declared that the rise in MDA in AD induced rats was associated with inhibition of antioxidant enzymes that is implicated in the removal of ROS, such as SOD, CAT and GSH in brain tissue, suggesting the pro-oxidant action of Al. Instead, Sumathi et al. [30], declared that aluminium, exposition promote destruction in neuronal lipid associated with modifications in the enzymatic antioxidant defense system.

Our results also exhibited significant increase in NO level in neurotoxic rats , which is in accordance with EL-Baz et al.[31], who found elevated NO level in Al induced brain neurotoxicity . As well Yang et al.[32], speculated that Al causes rise in NO level incorporated with significant elevation in neuronal nitric oxide synthase(Nnos) and inducible nitric oxide synthase (INnos).The exact mechanism for the induced activities of Nnos and iNOS ,may be through stimulating effect of Al on tumor necrosis factor -α (TNF-α) and interleukin -1 beta (IL-1β). These cytokines have been ascertained to promote NOS mRNA and consequently NOS expression activity [31]

Moreover , the presented results showed significant decrease in GSH level in brain tissue of rats induced with Al NPs which may be attributed to high level of H_2O_2 induced cytotoxicity in brain endothelial cells as a results of inhibition of glutathione reductase [29]. The significant reduction in brain TAC in Al NPs induced AD rats , may be attributed to the long term exposure to Al NPs leads to increase of lipid peroxidation with depletion and exhaustion of several antioxidant enzymes [29]. In addition , EL-Baz and Aly [33] explained the reduction in TAC in AD induced rats to the decrease in axonal mitochondria transformation, impairment of golgi and reduction of synaptic vesicles which results in the release of oxidative products like hydroperoxide and carbonyls as well peroxy nitrites, while decrease antioxidant enzymes and glutathione within the neurons. The high level of Fe promoting ROS due to high brain content of polyunsaturated fatty acids which can be easily interact with elaborated radicals and afford oxidative destruction in AD induced rats.

Further, our study clearly indicated significant reduction in CaM in Al NPs induced rats.

In concomitant with the the present findings, McLachlan et al. [34] showed, CaM levels in the Al-induced rats were markedly decreased (66%). The author added that CaM displayed decrement ability as an activator of 3', 5' cyclic nucleotide phosphodiesterase. The decreased levels of these principle proteins may influence homeostasis of calcium and the control of brain calcium-mediated activities. It was supposed that neurodegenerative diseases is a leading cause an imbalance of calcium levels in cells which precedes malfunctions in the signaling pathway and neuronal impairment [35].

It was hypothesized that the formation of amyloid plaques is well connected with CaM binding proteins leading to the AD symptoms and development,so CaM is markedly decreased in the individuals' brain of AD [35]. While , the significant increase in caspas -3 enzyme as well as comet assay biomarkers are in a good agreement with Guix et al. [42], who reported that, the high levels of brain nitric oxide (BNO) is responsible for oxidation and DNA damage in neurodegenerative diseases such as AD, stroke and Parkinson's diseases due to the formation of highly reactive peroxynitrite. Previously, Dorheim et al. [43], reported that the increase in the levels of BNO in AD patients may result from activation of NO synthesis, suggesting that BNO may play a role in neuronal cell degeneration in the AD disease.

Table(5): Effect of *Casimiroa edulis* total extract on CaM, TAC, GSH ,MDA and NO in Al-nanoparticles induced rats

Parameters / Group	CaM (ng/ml)	TAC (umol/l)	GSH(mg/g. tissue)	MDA (mmol/g. tissue)	NO (Umol/g tissue)
Normal control	3.12±0.22a	0.67±0.11a	225.95±2.15f	11.55±1.21a	82.00±5.20a
Total extract +Normal control	2.99±0.13a	0.70±0.12	249.88±9.10cd	8.59±0.13b	83.32±7.43a
%change	4.16	4.48	+10.59	-25.62	1.61
Al –neurotoxic	1.55±1.00b	0.27±0.10e	86.43±6.66g	24.11±2.11b	142.21±9.12b
%change	-50.32	-59.70	-61.00	+108.74	+73.43
Total extract treated neurotoxic rats	2.29±0.92c	0.43±0.16b	176.55±11.21a	16.92±1.23c	102.10±4.10e
%change	-26.60	-35.82	-21.86	+46.49	+24.512
%improvement	23.72	23.88	39.88	62.25	48.91
Standard Drug	2.08±0.90d	0.70±0.04	179.00±12.00a	15.90±1.22c	92.22±5.23d
%change	-33.96	+4.47	-20.77	+37.66	+12.46
%improvement	16.98	64.18	40.96	71.12	60.96

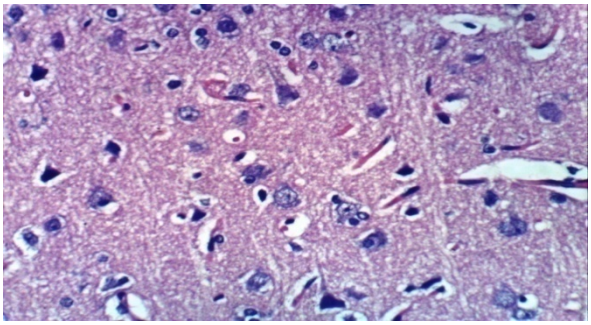
Statistical analysis is carried out using SPSS and co-state computer program , where unshared letters are significant at $p \leq 0.05$.

Table (6) : Effect of *Casimiroa edulis* total extract on comet assay parameters in Al-nanoparticle - induced rats .

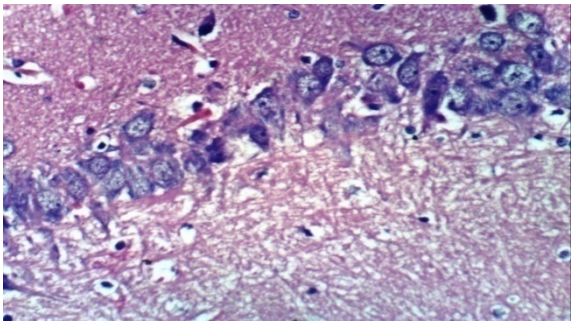
Groups	Parameters	Tailed %	Untailed %	Tail length µm	Tail DNA %	Tail Moment Unit
1	Normal control	5.00±0.22a	95.00±5.12a	1.02±0.07a	1.17±0.02a	1.19±0.12a
2	Al-neurotoxicity	17.00±1.22b +240.00	83.00±3.29b 12.63	3.67±0.62b +259.80	3.88±0.80b +231.62	14.24±1.92b +1096.63
3	Total extract- treated neurotoxic rats %change % improvement	11.00±1.00c 120.00 120	89.00±0.22c 6.32 6.32	3.22±0.20c +215.68 44.11	3.45±0.20c +194.87 36.75	11.11±1.30c +833.61 263.03
4	Standard drug treated neurotoxic rats % change % of improvement	10.00±0.92c 100 140	90.00±7.33c 5.3 7.36	3.05±0.40c 199.02 60.78	3.20±0.12c +173.50 58.11	9.76±0.90c 720.16 376.47

Statistical analysis is carried out using SPSS and co-state computer program , where unshared letters are significant at $p \leq 0.05$.
Values are % of the total counts in each assay.
Each parameter was done in doublets .
Tail Moment (unit) =Tail length x % total DNA.

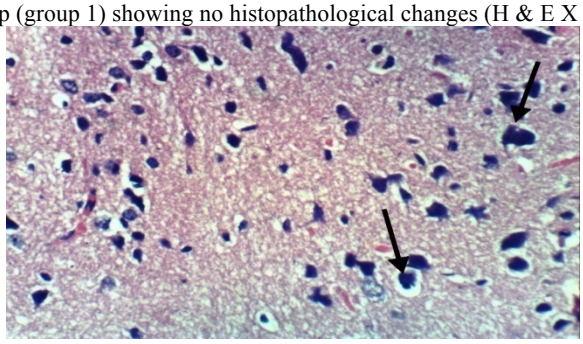
Aluminium induction caused a remarkable DNA damage, examined by agarose gel electrophoresis (Fig.1 , Slide 2) , which demonstrated an increase in DNA fragmentation and the number of observed comets resulting from Al- induced genotoxicity in cells comparing with control cell's DNA. The results also, clearly indicated significant increase in caspase enzyme activity in brain tissue of Al - intoxicated rats. The current results run in parallel with Sumathi et al.[30] and EL-Baz et al.[31, 44], who declared Al NPs toxicity proceeded an increment in DNA destruction as illustrated by the observed elevation in DNA fragmentation. Al is shown to stimulate the reactivity of different species as oxygen [29], which in turn cause deterioration of macromolecules such as DNA, protein and lipid. DNA damage is considered as one of the biomarkers and identical features of apoptosis [30]. Hence ,the toxicity resulting from Al-NPs can command rapidly apoptosis (Fig.1, Slide2), which displayed markedly disruption of cells, DNA fragmentation and increased comets have also been previously reported as a consequence of aluminium neurotoxicity [45]. Moreover, the present finding is in agreement with Sumathi et al.[30], who showed , aluminium toxicity can lead to faster apoptosis as seen in the photomicrographs (3-5) which revealed pyknosis and necrosis of neurons cells of cerebral cortex. This oxidative damage of aluminium could be attributed to its chemical nature of being a trivalent cation having high affinity for negatively charged groups such as phosphates and phosphorylated proteins in nucleic acids. Therefore, it may bind to DNA and RNA, inhibiting the activity of enzymes in addition to enhance the peroxidative damage of lipids and decreased the antioxidant status of the rat brain [46].



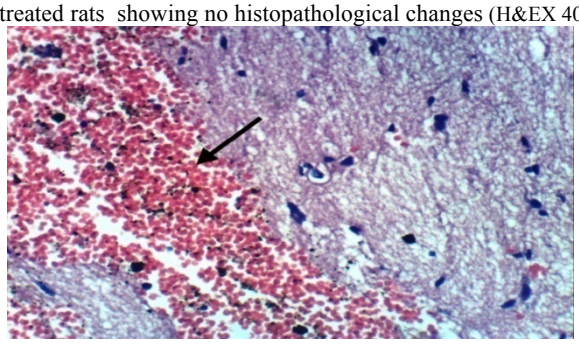
Photomicrograph (1): Cerebral cortex of rat from control, untreated group (group 1) showing no histopathological changes (H & E X 400)



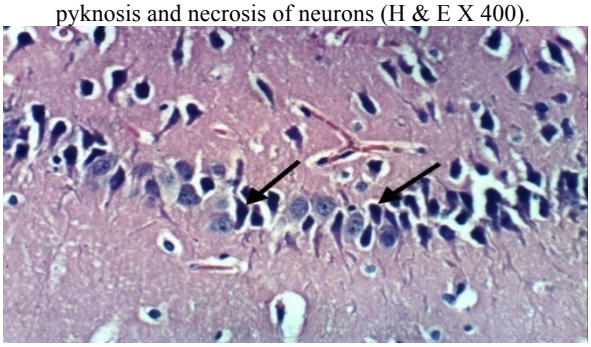
Photomicrograph (2): Hippocampus of rat from control, untreated rats showing no histopathological changes (H&EX 400)



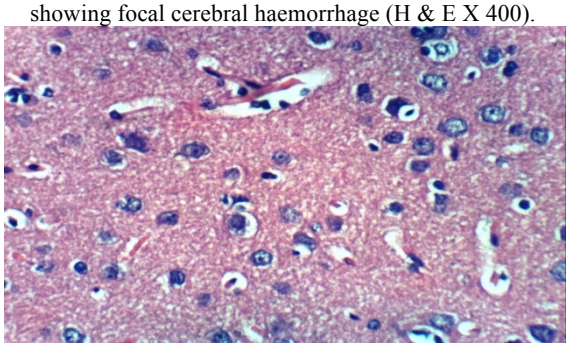
Photomicrograph (3): Cerebral cortex of intoxicated rat showing pyknosis and necrosis of neurons (H & E X 400).



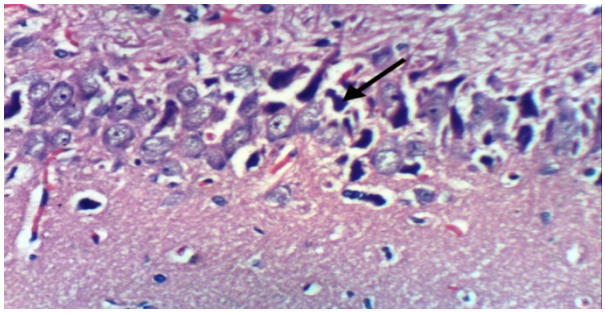
Photomicrograph (4): Cerebral cortex of intoxicated rat showing focal cerebral haemorrhage (H & E X 400).



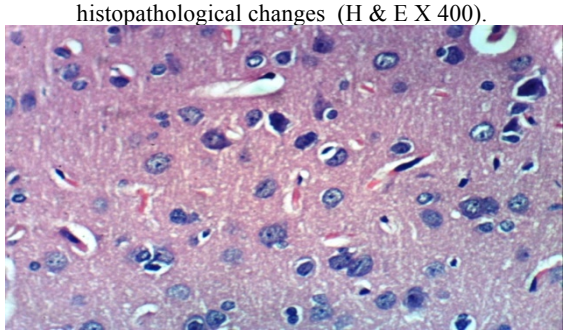
Photomicrograph (5): Hippocampus of intoxicated rat showing pyknosis and necrosis of pyramidal cells (H&EX 400)



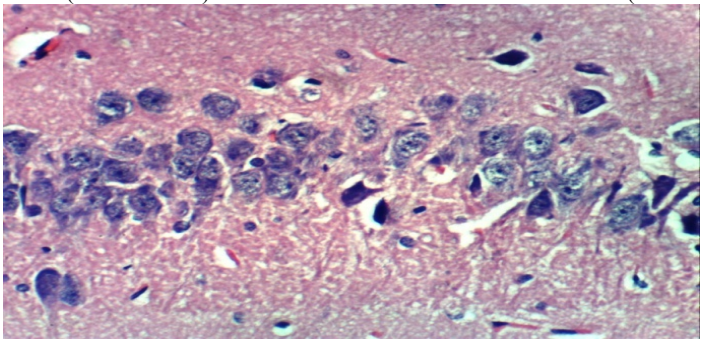
Photomicrograph (6): Cerebral cortex of intoxicated rat treated with ethanolic extract of *Casimiroa edulis* showing no histopathological changes (H & E X 400).



Photomicrograph (7): Hippocampus of intoxicated rats treated with ethanolic extract of *Casimiroa edulis* showing necrosis of sporadic pyramidal cells (H & E X 400).



Photomicrograph (8): Cerebral cortex of intoxicated rats treated with Donepezil showing no histopathological changes (H & E X 400).



Photomicrograph (9): Hippocampus of intoxicated rats treated with Donepezil showing no histopathological changes (H&EX400)

Table (7): histopathological lesions score of different groups

Histopathological lesion	Group 1	Group 2	Group 3	Group 4
Necrosis of neurons	-	+++	+	+
Neuronophagia	-	+	-	-
Cerebral haemorrhage	-	++	-	-
Necrosis of pyramidal cells of hippocampus	-	++	+	-

(-) no change (+) mild change (++) moderate change (+++) severe change

Table (7): demonstrated histopathological lesion score in Al –intoxicated rats as well as treated one, where (-) no change , (+) mild change (++) moderate change and (+++) severe change

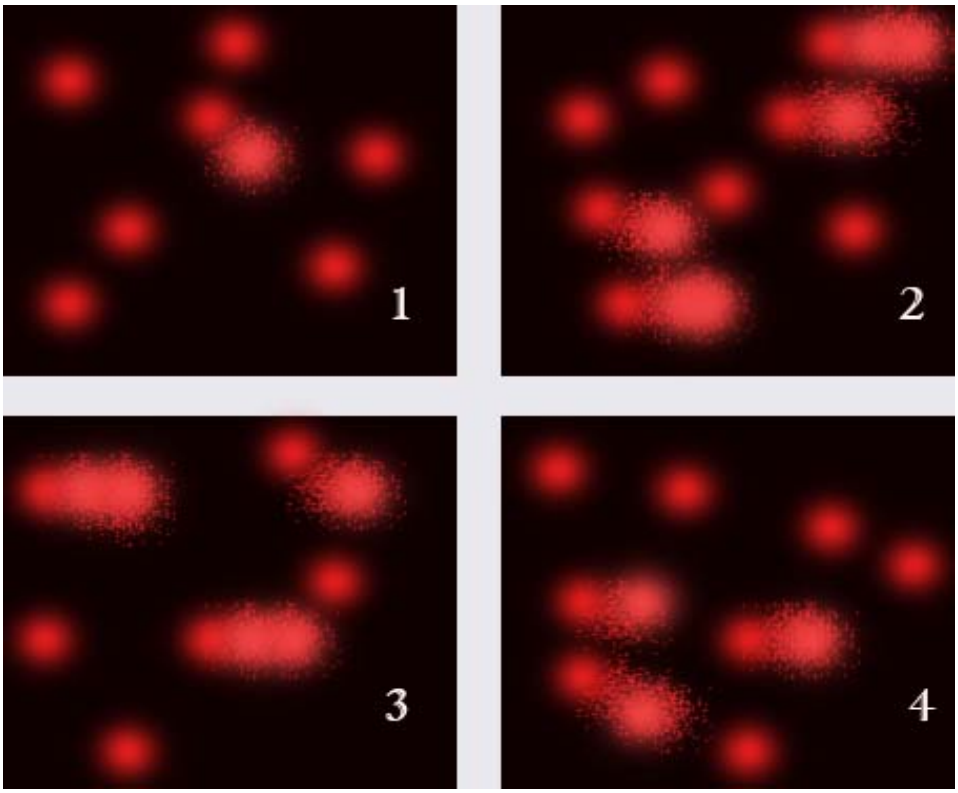


Figure. (1): Aluminium oxide nanoparticles -induced DNA damage in the brain tissue rats. Slide 1: represented normal control rats .Slide 2: represented Al- induced rats with comet tail of damage cells. Slide 3: represented AD- induced rats treated with total extract of *C. edulis* showed ameliorated comet tail of damage cells. Slide 4: represented Al-induced rats treated with standard drug indicated ameliorated comet tail of damage cells

On the other hand,treatment of AD induced rats with *C. edulis* showed marked amelioration in all detected biomarkers, Comet assay (Fig. 1, slids 3,4) as well as in histopathological examination of cerebral cortex and hippocampus (photomicrographs 6,7).The rats treated with the alcohol extract of *C. edulis* exhibited no markers of acute toxicity using up to 5000 mg/kg [47]. In addition, the alcoholic extract of the leaves displayed several activities as anticonvulsant, antimutagenic and sedative due to the presence of different isolated biologically active compound such as isoimpinellin, casimiroin, skimmianine, 1-methyl-2-phenyl-4-quinol, edulein, and scopoletinmethyl ether [13]. However the ethanol extract exhibited the strongest antioxidant activity due to the presence of flavones compounds [7]. Beside, *C. edulis* ripened fruits alcoholic and alkaloidal extracts demonstrated antihypertensive property[48].These presented properties are suggested to be attributed to the existence of the glucoside casimirosine, principally in seeds , bark and leaves. In addition, leaves and seeds decoction is determined in therapy of anxiety, insomnia and hypertension in Mexico[49, 50].Further, histamine derivatives like N, N-dimethyl histamine compounds were identified as cardiovascular activity [51].Also, leaves extract of *C. edulis* showed sedative effects combined with anxiolytic and anti-depressant effects in mice and rats[51,52]. *C. edulis* aqueous extracts of seed have been demonstrated to possess vaso-relaxing activity [53]. Other compounds were also identified such as coumarins, f1avonoids and limonoids, in *C. edulis* [12, 49 and 54], which are known to have diuretic or anti-inflammatory effects [55]. Hence, the ameliorative effect of *C.*

edulis in AD induced rats may be related to several biological active compounds which exhibited antioxidant and anti-inflammatory effects .

In summary, the present data indicated that Al NPs induced neurotoxicity which might be related to oxidative stress.These experimental results suggested that ethanolic extract of *Casimiroa edulis* have neuroprotective, antiapoptotic and antiamnesic effects against Al NPs -induced cerebral damages and cognitive decline. Overall, the results showed that *Casimiroa edulis* total extract can induce beneficial therapeutic effects against Al-induced neurochemical changes in the central nervous system (CNS) as well as potential protective effect against age-associated cognitive deterioration by suppressing the process mediated free radical formation; an action that could be related to the antioxidant characteristic of the extract.

Conclusion

Casimiroa edulis extract administrated to Al NPs intoxicated rats has the ability to down regulate free radicals elevation, improve brain functions, ameliorate brain neurotransmitters as well as normalize brain cells architectures. The tested sample administered post– Al NPs treatment recorded fluctuating percent of improvement in the majority of the selected biomarkers. So, *Casimiroa edulis* extract may be used as a new safe therapy that may delay brain injury progression and its complications.

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