Original Article

The Effect of Extra Virgin Olive Oil on The Behavior and Biochemical Analysis of Brain Tissues of Rats with Alzheimer's Disease

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Abstract

Background: Alzheimer's disease (AD) is the most common form of dementia in older people and is a progressive disease of the brain that leads to shrinkage of the brain tissue and irreversible loss of neurons.

Results: The aim of this study was to evaluate the effects of olive oil on brain aging in rat and to verify whether these antioxidant and anti-inflammatory activities were involved. A rat was fed with extra-virgin olive oil (Coratina (C) and Koroneiki (K) olive oils (0.3 ml/kg of body weight/ day). Behavioral tests were employed to assess object recognition test and Morris Water Maze apparatus in treated animals. Parameters of oxidative status and inflammation were measured in different brain areas at the same time and evaluated for correlation with behavioral changes. The present study was designed to evaluate the neuroprotective properties of olive oil in an aluminum chloride (AlCl3)-induced model of AD in Wistar rats. Wistar rats were administered with dietary oils for 60 days before induction of Alzheimer's disease (AD) using AlCl3. Spatial memory was assessed using Brain antioxidant parameters such as lactate dehydrogenase (LDH) activity malondialdehyde MDA levels (lipid peroxidation product) and nitrite levels were determined. In addition, tau protein and amyloid precursor protein (APP) - Amyloid (β1-42) expression mRNA expression and the levels of acetylcholinesterase in serum biochemical.

Conclusion: This work points out that natural extra-virgin olive oil can improve some age-related dysfunctions by differentially affecting different brain areas. Such a modulation can be obtained with an olive oil intake that is normal in the Mediterranean area, provided that the oil has a sufficiently high polyphenols content.

Keywords: Alzheimer's disease, Amyloid β, tau protein.

INTRODUCTION

The olive tree (Olea europaea L.) is one of the most important crops in the Mediterranean countries (Foscolou et al., 2018). The roots of olive tree cultivation go back to legends and traditions. It probably began about 5,000-6,000 years ago within a wide strip of land on the Mediterranean coast and in adjacent regions comprising Asia Minor and part of India, Africa and Europe (Fernandez Diez, 1971, Muzzalupo and Perri, 2008andPhoenicians Loukas and Krimbas, 1983). The olive fruit is an important product because it produces nutritious and edible oils with potential medicinal functions (Ribarova et al., 2003), oleaeuropaea products have been used as an aphrodisiac, emollients, laxatives, nutraceuticals, sedatives, and tonics. Specific conditions that are traditionally treated include colic, alopecia, paralysis, rheumatic pain, sciatica and high blood pressure (Gikni et al., 2005, Ryan and Robards, 1998 and Gooch, 2005).

Extra virgin or VOOs contain more monounsaturated fatty acids than other olive oils (Fernandez Diez, 1971; Psomiadou et al., 2000 Bouaziz et al., 2010 and Muzzalupo et al., 2011). Another health benefit of olive oil is that it appears to replace omega-6 fatty acids, with no effect on omega-3 fatty acids. Olive oil helps to construct a healthier balance between omega-3 and omega-6 fats (Caravita et al., 2007, Servili et al., 2004, De Nino et al., 2000).

Redundant abecedarian olive oil painting (EVOO) conforming of phenolic secoiridoid, (-) oleocanthal (OC) is one of the most biologically active phenols with provedanti-inflammatory and antibacterial conditioning Pei etal., 2016 and Curiel, 2018. It

also possesses proven neuroprotective conditioning by perfecting concurrence of the Alzheimer's hallmark α, β amyloid and by guarding against H2O2 convinced oxidative stress (Qosa etal., 2015a; Batarse etal., 2017 and Giusti etal., 2018). EVOO to ameliorate cognitive performance and decelerate the progression of memory impairment (Scarmeas etal., 2006; Scarmeas etal., 2009 and Valls- Pedret etal., 2015).

Oleocanthal is responsible for the affable bitter taste of pure olive oil painting and hasanti-inflammatory and antioxidant parcels analogous to thenon-steroidalanti-inflammatory medicine ibuprofen Beauchamp etal., 2005. Oleocanthal and phenolic composites of EVOO retain important neuroprotective conditioning against Alzheimer's complaint (announcement) (Pitozzi etal., 2010; Farr etal., 2012; Abuznait etal., 2013; Grossi etal., 2013 and Luccarini etal., 2014). Oleocanthal has been shown to act on major intercessors of Alzheimer's complaint. Pathogenesis, amyloid- β (Aβ) and hyperactive phosphorylated tau proteins (Pitt etal., 2009; Monti etal., 2011 and Abuznait etal., 2013), which contribute significantly to neurodegeneration and memory loss (Gu etal., 2010 and Selkoe, 2001). Oleocanthal stopped the accumulation of hyperphosphorylated tau (τ proteins) by locking (τ protein) into a naturally unfolding state (Monti etal., 2011), and altered the oligomerization state of answerable Aβ42 oligomers that defended neurons from their synaptic pathological effect (Pitt etal., 2009).

Alzheimer's disease (AD) is the most common form of dementias affecting people of usually over 65 years old and cause an increase in the global health challenge with 40–50 million people currently living with dementia (Wu et al., 2017, HardyandSelkoe, 2002andLombardo et al., 2018).

The aim of this study was to evaluate the effects of olive oil on brain aging in rat and to verify whether these antioxidant and anti-inflammatory activities were involved. This work points out that natural extra-virgin olive oil can improve some age-related dysfunctions by differentially affecting different brain areas. Such a modulation can be obtained with an olive oil intake that is normal in the Mediterranean area, provided that the oil has a sufficiently high polyphenols content.

Materials and methods

Materials

Source of olive fruits:Two varieties of olive fruits (Olea europaea L.) Koroneiki and Coratina cultivars were handpicked during the 2019–2020 season in Khatatba, Sadat city, Minufiya Governorate, Egypt, and transported in the same day to the laboratory. Only healthy fruits, without any kind of infection or physical damage were processed.

Animals: All animal treatments were carried out with the agreement of (Ethical approval code BSU-IACUC. 021-154). Adult male Albino Wistar rats weighing 180–220 g was purchased from the animal house colony of National Research Centre. They were maintained at a controlled temperature of 24 ±1◦C with a 12–12 h light-dark cycle, also were given unlimited access to water and normal food.

Reagents, solvents and standards: All solvents in this study were purified and distilled before use. Folin-Ciocalteau reagent was obtained from Gerbsaure Chemical Co. Ltd., Germany. Sigma–Aldrich (St. Louis, Missouri, USA) delivered the Aluminum tri chloride (AlCl3).

Methods

Chemical composition of olive fruits: Moisture and oil contents were determined according to A.O.A.C (2016).

Oil extraction: After harvest, fresh olives (1.5-2.0 kg) were washed and deleafed, crushed with mill and pressed using hydraulic laboratory (Carver) press. Oil produced from each extraction was 200-250 ml/kg, filtered then transferred into dark glass bottles and stored in the dark at 4˚C until analysis.

Quality parameters: Acidity, peroxide value and UV absorption characteristics, K232nm (conjugated dienes) and K270nm (conjugated trienes) and ΔK [ΔK = k270 – (k266-4) + (k274+4)/2] were carried out following the analytical methods described by A. O. A. C. (2016).

Oil stability: Oxidative stability was evaluated by the Rancimat method (Gutierrez and Dobarganes, (1988). Stability was expressed as the oxidation induction time (h), measured with the Rancimat 679 apparatus (Metrohm Co., Herisou, Switzerland), using 5.00 g oil heated to 100˚C ± 2˚C with an air flow of 20 l/hr-1.

Tocopherol analysis: The total tocopherol content in oils was determined according to the method of Wong et al., (1988).

Total phenolic content: was determined according to Gutfinger, 1981.

Total flavonoid content: Total flavonoid content was determined according to Zhishenet al 1999).

Experimental design: Rats were randomly divided into four groups (n =6): Group 1; Cont. group received daily saline injections intraperitoneal (IP) for 60 days. Group 2; AlCl3 group receivedAlCl3 (Sigma, USA), dissolved in saline, daily IP at a dose of

17 mg/kg per injection for 60 days (Krasovskiiet al.,1979). Group 3; K group received K (0.3 ml/kg) concurrently with AlCl3 (17 mg/kg). Group 4; C group received C (0.3mg/kg) concurrently with AlCl3 (17 mg/kg). Following the treatments, the animals were slaughtered and beheaded after being evaluated behaviorally. Brain tissues were separated, washed, and stored at 80 degrees Celsius until biochemical tests were done. Following that, additional brain samples were preserved in 10% formalin for histological evaluation. The experimental parameters were illustrated in the flowchart below.

Behavioral assessment: evaluation of memory and learning measured by Morri's water maze (MWM). The Morris water maze test, developed by Richard G. Morris in 1981, is one of the most widely used and well-established behavioral tests for assessing rat spatial learning and memory (Morris, 1981). Object recognition test equipment was built in the manner described by (Ennaceur and Delacour, 1988).

Brain tissues biochemical analysis: Malondialdehyde (MDA) content by Ruiz-Larrea et al., 1994, Lactate dehydrogenase (LDH) activity and Acetylcholinesterase (AchE) activity were determined using Ellman's technique (1961).

Quantitative real-time PCR: For total tau mRNA and amyloid precursor protein (APP) gene expression.

RNA extraction

Brain tissues from all groups were homogenized in ice before total RNA was extracted using Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA), and quantity and quality were determined using a Beckman dual spectrophotometer (USA).

Real time PCR:

Super script IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific and Waltham, MA USA) was utilized for reverse transcription of extracted RNA followed by PCR. The relative quantitation (RQ) of each target gene is quantified according to the calculation of 2-ΔΔCt method.

Table 1. Primer sequences used for the RT-PCR.

Gene symbol	Forward	Reverse
Tau	AGTGGATCTGAGCAAGGTG	AGGTGCCGTGGAGATGTG
APP	GGATGCGGAGTTCG GACATG	GTTCTGCATCTGCTCAAAG
GAPDH	ATGACTCTACCCACGGCAAG	GATCTCGCTCCTGGAAGATG

Histopathological examination

The brains from all groups were cut and fixed in 10% neutral buffer formalin for 48 hours. For assessment the neuronal loss, the normal neurons in the cerebral cortex and hippocampus CA2 region were quantified in five areas of 1µm2 each per group according to the method of (Khalil et al., 2019), with some modifications.

Immunohistochemical analysis

Immnuo histochemical staining was performed to demonstrate neuro inflammation marker according to Saleh et al., 2020.

Statistical analysis

Values are presented as means ±standard error of the means (SE). One-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons were conducted for comparisons between different groups. However, the spatial memory and learning test measured by MWM and the comparison of total exploration time in T1 and T2 in the ORT which was carried out using 2-way ANOVA followed by Tukey's multiple comparisons test and when comparing the exploration times of the F and N objects in T2, Student's t-test was used. GraphPad Prism software, version 7 (USA) was utilized to perform these statistical tests whereas, the difference was considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

1. Moisture and oil contents (%) of olive fruit varieties.

The Moisture and oil contents (%) of the two olive fruit varieties are given in Table (2). Moisture content is a major factor for olive fruits as it generally contributes to more than 50% of the fruit weight. Coratina cultivar shows the significantly higher oil (47.89%) and less moisture (54.96%) contents than Koroneikivariety (45.31% and 59.34%, respectively). These results are in agreement with those obtained (Boskou, 1996b; El-Mahdy and Rashwan, 1997; Salvador et al., 2001; Najafian et al., 2009, Atta et al., 2010; Dabbou et al., 2010b and Al-Okaby, 2015).

Table 2. Moisture and oil contents (%) of KoroneikiandCoratina varieties olive fruits.

Samples	Moisture content %	Oil content %
Koroneiki	59.34 ± 6.0^{a}	45.31 ± 4.5^{b}
Coratina	54.96 ± 5.4^{b}	47.89 ± 4.8^{a}

Values represent the mean ± standard deviation of three independent biological replicates. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

2. Physical and chemical properties of oils.

a. Percent of free acidity.

The quality indicators of the analyzed samples are shown in Table No. (3). The data shows the free acidity values of the two oils were less than 1.0, the sample of Koroneiki cultivar was 0.21 ± 0.02 g of oleic acid per 100 g of oil and of the Coratina cultivar which was 0.34 ± 0.02 g of oleic acid per 100 g of oil and falls within the acceptable values for extra virgin olive oil (EVOOs) and virgin olive oil (VOOs) as the free acidity standard for extra- VOO and VOO are 0.8 and 2.0 grams per 100 grams maximum (EEC, 1991; IOC, 2006 and Dabbou et al., 2010b).

b. Peroxide value.

The data Table (3) depicts the in all cultivars the PVs were not higher than (3.10 ± 0.30 meq O2 kg-1) in Koroneiki. In the recant results the maximum peroxide value for extra virgin olive oil (20 meq O2 kg-1) IOC (2006). These results concur with those obtained for Coratina (1.56 ± 0.15 meq O2 kg-1) cultivar by (Clodoveo et al., 2007 and Benincasa et al., 2011).

c. Specific extinction coefficient at 232 nm, 270 nm and ΔK

K232 parameter is especially reflective of the conjugated dienes. Data in Table (3) depicts the minimum and outside values for the absorbance at 232 nm recorded independently for Coratina (0.018±0.001) and Koronaiki (0.022±0.002) oil painting. The absorbance at 270 nm, K232 parameter is especially reflective of the conjugated dienes. reflective of the conjugation of trienes and of the presence of carbonylic composites, gives the minimal value for Coratina oil painting (0.017±0.001) and the maximum value for Koroneiki oil painting (0.019±0.001). The values at 232 and 270 nm for the two anatomized samples match the limits for redundant abecedarian olive oil painting. Also, all ΔK values fall within the limits for redundant abecedarian olive oil painting (IOC, 2006).

d. Refractive index at (25 C°).

No significant differences ($p \leq 0.05$) were observed in refractive index for both extracted Koroneiki and Coratina oils. Refractive index of extracted Koroneiki and Coratina olive oils were inside the ranges reported by (E.O.S, 2005 and IOC, 2013).

e. Oxidative stability at 100˚C by (Rancimat).

Table (3) shows that the antioxidant activity of the extracted oils is resistant to oxidation due to their high content of bioactive com-pounds and low content of unsaturated fatty acids with high content of one double bond (oleic acid) in the fatty acids. It could be noticed that the oxidative stability recorded a positive correlation between antioxidant activity and total polyphenols; it means that the variations between antioxidant activities of the studied oils may be related to the phenolic contents. High content of phenols possesses much great antioxidant activity to scavenge the free radicals. These observations are agreed with those of (Issaoui et al., 2009).

f. Color index

Two types of natural colors, chlorophyll and carotenoids are responsible for the color of olive oil painting. The former composites, chlorophyll, contribute to the greenness of vegetable canvases, while the carotenoids are responsible for their yellowing. Chlorophyll is encountered as phytovitin. Pheophytin a attention in olive oil painting ranges from3.3 to 40 ppm (Psomiadou and Tsimidou, 1998 and Natella et al., 1999).

Table (3) show that the color index red and blue value for Coratina oil (1.00 and 1.00 mg kg-1) and Koroneiki oil (2.30 and 1.00 mg kg-1) respectively, they act as antioxidants and anti-inflammatory agents and hold great promise for cognitive health for older adults. These observations are agreed with those of (Psomiadou and Tsimidou, 1998 andNatella et al., 1999McGeer et al., 2000; Pratico and Trojanowski, 2000; Pappolla et al., 2002; Pratic`o 2002; Tarkowski et al., 2003; Teunissen et al., 2003; Engelhart et al., 2004; Keller et al., 2005; Ravaglia et al., 2005 and Wyss-Coray, 2006).

g. Total Tocopherol and total Flavonoids

Data in Table (3) shows that total tocopherol value for Coratina oil (189.00 mg kg-1) and total tocopherol value for Koroneiki oil (215.00 mg kg-1).

Table 3. Properties of olive oil extracted from Koroneiki and Coratina varieties.

Properties		Koroneiki olive oil	Coratinaolive oil
Refractive index at (25 C°)		1.4679 ± 0.14	1.4679 ± 0.14
Color index	**Yellow**	35.00 ± 0.30	35.00 ± 0.30
	Red	2.30 ± 0.20	1.00 ± 0.10
	Blue	1.00 ± 0.10	1.00 ± 0.10
Acid value (% as oleic acid)		0.21 ± 0.02	0.34 ± 0.02
Peroxide value (meq. O_2/kg oil)		3.10 ± 0.30	1.56 ± 0.15
Uv Absorbance at 232 nm		0.022± 0.002	0.018 ± 0.001
Uv Absorbance at 270nm		0.019 ± 0.001	0.017 ± 0.001
Δ K		0.00	0.00
Total polyphenols (mg kg^{-1})		428.00 ± 42.93	530.00 ± 55.30
Total flavonoids (mg kg^{-1})		113.50 ± 11.40	110.00 ± 11.10
Total tocopherol (mg kg^{-1})		215.00 ± 21.45	189.00 ± 19.15
Oxidative stability at 100˚C by (Rancimat)		51.20 ± 5.13	48.50 ± 4.85

** Values represent the mean ± standard deviation of three independent biological replicates.*
Where: ΔK= variation of specific extinction.

These findings appear to agree with the results obtained by other authors (Benincasa et al., 2011 and Ergönüla and Köseoğlu, 2014).

Flavonoids are low molecular weight compounds with a C6–C3–C6 structure, while this classification includes various compounds. Inside the molecular structure of flavonoids, the aromatic ring A is derived from the acetate/malonate pathway, while the B ring is produced from phenylalanine via the shikimate pathway. The Flavonoids may have beneficial effects for many diseases, involving cancer, cardiovascular diseases, and neurodegenerative disorders. These benefits are attributed to its antioxidant activity and its effect on cellular oxidative status.

Table (3) show that the olive oil contains about 113.50 -110.00 mg / kg total flavonoids in both Koroneiki and Coratina olive oils, respectively. The amount of flavonoids was marginally higher in Koroneiki oils than that in Coratina oils.

3. Behavioral variations

a. Memory and learning assessment by the Morris water maze (MWM).

a. 1. The mean escape latency time (MELT)

On the first day of training, there were no significant differences between groups in the MELT. However, as compared to the control rats, AlCl3 significantly increased the MELT of the rats on the second and third days of the trial. On the second day of the experiment, treating the AlCl3-injected rats with C resulted in a significant decrease in MELT compared to the AlCl3-treated group (Table 5).

a. 2. The time spent on the target.

Intraperitoneal AlCl3 injection significantly reduced the mean time spent by rats in the target quadrant by 48%, indicating impaired learning and memory processes (Fig. 3). These findings are in a good agreement with data published by (Farr et al., 2012; De Nicoló et al., 2013 and Nampoothiri et al., 2015).

Table 5. Effect of K and C on the mean escape latency time (sec) in Morris's water maze in AlCl3-induced Alzheimer's disease (AD) in rats (Acquisition Phase).

Groups	Day 1	Day2	Day 3
Normal	30.37 ±2.24	29.05 ±2.33	25.25 ±2.18
$Alcl_3$	56.75 ± 4.32[a]	52.6 ±2.38[a]	47.9 ± 4.51[a]
K+ $AlCl_3$	39.44 ± 3.12[b]	36.65 ± 3.68[b]	32.7 ± 2.57[b]
C+ $AlCl_3$	31.92 ± 2.58[b]	30.00 ± 2.31[b]	27.35 ±2.41[b]

* Data was expressed as mean ±SEM, n =6rats/group, a Significantly different from the normal control on the corresponding day at P <0.05 and b Significantly different from the AD group on the corresponding day at P <0.05.

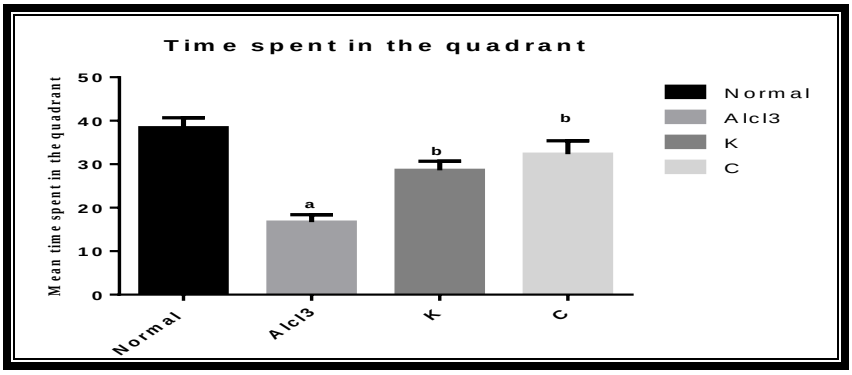

Fig. 3. Effect on time spent in the quadrant in Alzheimer's disease (AD)-induced rats.

* Data was expressed as mean ±SEM, n=6 rats/group, a Significantly different from the normal control and b Significantly different from the AD group at P <0.05.

b. Effect on recognition memory by Object recognition test.

Rats were administered an i.p dosage of AlCl3 at a rate of 17 mg/kg daily for 60 days, their locomotor activity and recognition memory were lower than in the control group. While rats were administered K and C (0.3ml/kg) administered orally to rats for 60 days in combination with AlCl3 might normalize the previously stated parameters (Figure 4 a, b and c). These findings are in a good agreement with data published by (Farr et al., 2012; De Nicoló et al., 2013 and Nampoothiri et al., 2015).

Alzheimer's disease caused by AlCl3 revealed that T1 in the ORT had no significant effect on overall exploration time in T1 and T2. In contrast, rats with Alzheimer's disease treated for 60 days with K and C (0.3ml/kg) exhibit a substantial difference in total exploration time in T1 and T2. During T2, AlCl3-induced Alzheimer's disease rats showed no significant change in their N exploration time compared to their F exploration time. Alzheimer's disease rats treated with AlCl3 examined N and F items in the same way. Alzheimer's disease rats given K and C (0.3ml/kg) performed similarly to donepezil and examined the N item substantially more than F.

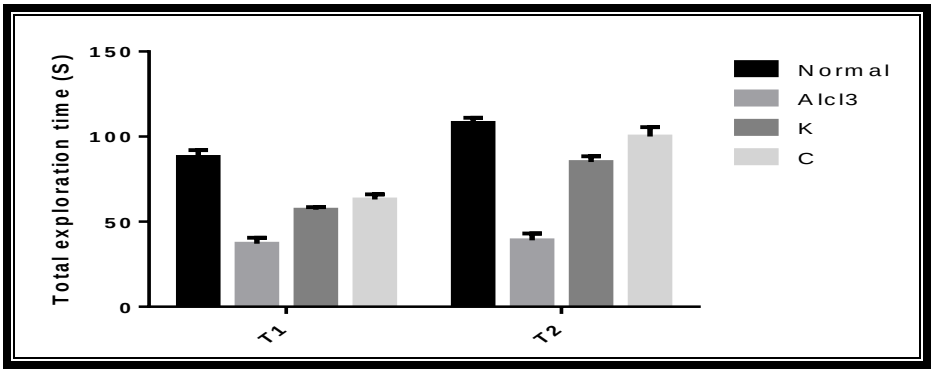

Fig. 4a. Effect of K and C (0.3ml/kg) on AlCl3 induced Alzheimer's disease (AD) in rats using the object recognition test.

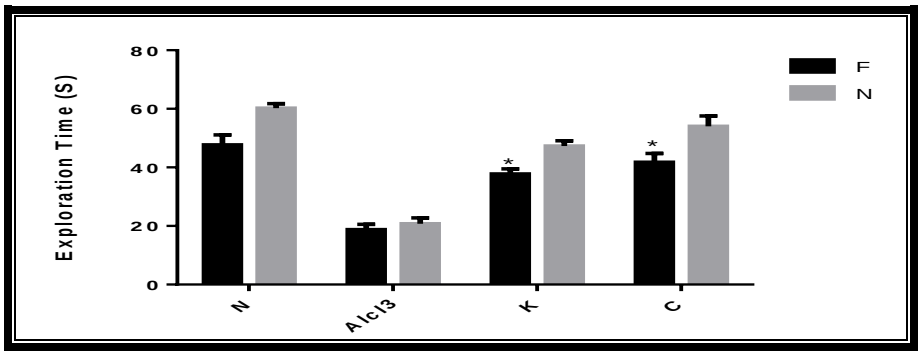

Fig. 4b. Effect of K and C (0.3ml/kg) on AlCl3 induced Alzheimer's disease (AD) in rats using the object recognition test.

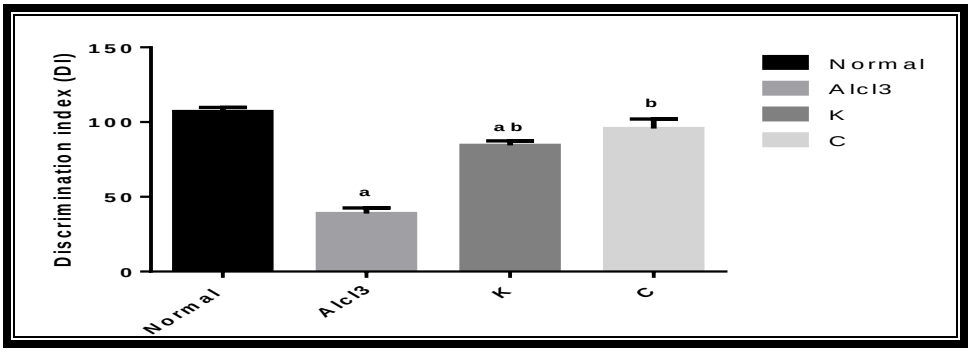

Fig. 4c. Effect of K and C (0.3ml/kg) on AlCl3 induced Alzheimer's disease (AD) in rats using the object recognition test.

* Significant difference versus correspondent N group at P < 0.05, a significant difference from the normal group at P < 0.05 and b Significant difference from control (AlCl3) group at P < 0.05

4. Brain biochemical levels.

a. Effect on oxidative stress activity in Aluminum Chloride (induced rats).

The impact of oral administration of K and C on oxidative stress was assessed by measuring MDA levels (lipid peroxidation product) in brain tissue from the control and treatment groups, as shown in Figure (5) and Table (6) MDA levels in AlCl3-induced rats were substantially higher (p>0.05) than in control rats, but treatment with K and C significantly lowered (p>0.05) MDA levels in AlCl3-induced rats. The findings might imply that antioxidant-rich treatments have a protective effect against AlCl3-induced oxidative stress in rats. Extra virgin olive oil demonstrated their protective effect by causing a decrease in brain MDA levels. The results of this study are almost agreed with the results of (Nampoothiriet al., 2015 and Kumar et al., 2019).

Table 6. Effect on brain oxidative stress (MDA) in Aluminum chloride induced Alzheimer's disease (AD) in experimental rats.

Groups	MDA (nmol/mg protein)
Normal	0.55±0.04
Aluminum chloride	2.77±0.15[a]
K	1.3±0.04 [ab]
C	*1.3±0.07 [ab]*

* Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test. -a significant difference from normal group at P< 0.05.-b significant difference from control (Alcl3) group at P<0.05.

Table 7. Effect on brain oxidative stress LDH in Aluminum chloride induced Alzheimer's disease (AD) in experimental rats.

Groups	LDH (ng/mg protein)
Normal	1.6 ± 0.08
Aluminum chloride	4.8 ± 0.15[a]
K	3 ± 0.1[ab]
C	2.9 ± 0.11[ab]

* Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test.-a significant difference from normal group at P< 0.05.-b significant difference from control (Alcl3) group at P<0.05.

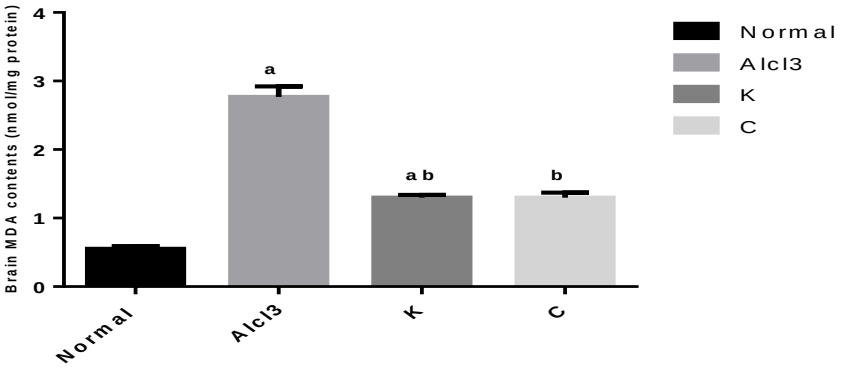

Fig. 5. Effect on brain oxidative stress (MDA) in Aluminum chloride induced Alzheimer's disease (AD) in experimental rats.

* Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test. -a significant difference from normal group at P< 0.05.-b significant difference from control (Alcl3) group at P<0.05

b. Effect on neuronal injuries in Aluminum Chloride (AlCl3) induced rats.

Figure (6) and Table (7) depicts the effect of oral administration of K and C on neuronal injuries by evaluating the lactate dehydrogenase (LDH) activity of brain tissue in the control and treatment groups. The results demonstrated that LDH activity was significantly higher (p>0.05) in the AlCl3-induced rats compared to the control rats. When rats treated with K and C at 0.3 ml/kg were compared to AlCl3 induced animals, a significant difference (p>0.05) in LDH activity was detected. These findings indicated that K and C therapy improved neuronal damage related with blood flow regulation in the brain, extra virgin olive oil (EVOO) reduce the levels of lactate dehydrogenase (LDH). These findings are in a good agreement with data published by (Lin, 2007).

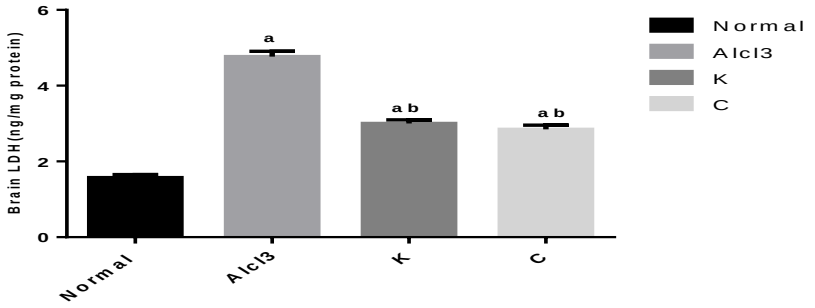

Fig. 6. Effect on brain oxidative stress LDH in Aluminum chloride induced Alzheimer's disease (AD) in experimental rats.

*Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test. -a significant difference from normal group at P< 0.05.-b significant difference from control (Alcl3) group at P<0.05.

c. Effect on pro-inflammatory cytokinesin Aluminum Chloride (induced rats).

Figure (7) and Table (8) depicts the effect of oral K and C administration on iNOS as pro-inflammatory cytokines in the control and treatment groups. iNOS levels were higher in animals given AlCl3-induced AD than in control rats. Rats treated with K and C on the other hand, considerably reversed (p>0.05) the AlCl3 impact.

Table 8. Effect on brain pro-inflammatory cytokines (iNOS) inAluminum chloride induced Alzheimer's disease (AD inexperimental rats.

Groups	iNOS (ng/mg protein)
Normal	2.03 ±0.11
Aluminum chloride	5.9 ±0.15[a]
K	3.5 ±0.09 [ab]
C	3.2 ±0.2[ab]

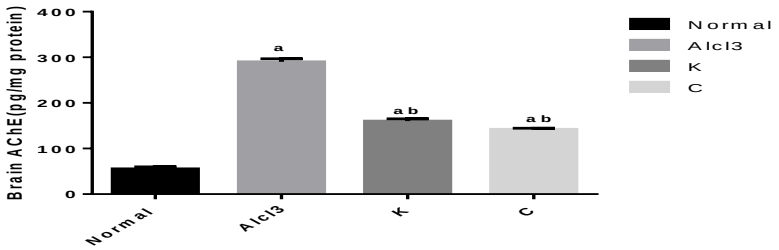

Fig. 7. Effect on brain pro-inflammatory cytokines (iNOS) inAluminum chloride induced Alzheimer's disease (AD) in experimental rats.

*Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test. -a significant difference from normal group at P< 0.05.-b significant difference from control (Alcl3) group at P<0.05.

d. Effect on cholinergic function in Aluminum Chloride induced rats.

Talesa, 2001 demonstrated adjustments in acetyl cholinesterase (AChE) activity and changes in polymorphisms in the brain as well as in cerebrospinal fluid (CSF) and blood.Co-localization of the enzyme in the aging panel provided evidence for its aberrant features. It has also been shown that acetyl cholinesterase forms a stable complex with components of the aging plate through its peripheral anionic site. The neurotoxicity of amyloid components increases due to the presence of acetylcholinesterase. The occurrence of an altered glycosylation of some acetyl cholinesterase forms in Alzheimer's disease is closely related to the presence of amyloid formations.

The present data recorded in Table (9) and Figure (8) shows the effect of oral administration of K and C on cholinergic function by assessing Acetylcholinesterase (AChE) activity in brain tissue in the control and treatment groups. AChE level elevation with a significant difference ($p<0.05$) between rats from the AlCl3 induced group and the control group. Following that, the K and C treatments effectively inhibited the AChE ($p<0.05$). This can be explained by the therapy of K and C, which improves cholinergic function in the brain, which is important for learning, memory, and motor control. Increased AChE activity promotes Aβ aggregation. At present, cholinesterase inhibition is considered to be used for improving the cholinergic deficits. The administration of EVOO and its fractions could attenuate the decreased level of AChE in (K and C) treated rats. Intake of olive oil caused a decrease in AchE activity in the frontal cortex. The results of this study are almost agreed with the results of Bonesi et al., 2010; Singh et al., 2013; Amel et al., 2016 and Kumar et al., 2019.

Table 9. Effect on brain pro-inflammatory cytokines AChE in Aluminum chloride induced Alzheimer's disease (AD in experimental rats.

Groups	AChE (pg/mg protein)
Normal	55.4 ± 4.4
Aluminum chloride	291 ± 6.7[a]
K	160 ± 5.3[ab]
C	142 ± 2.5[ab]

Results are expressed as Mean ± SE (n=6), Statistical analysis was carried out by one-way ANOVA followed byTukey's multiple comparison test.

*-a significant difference from normal group at P< 0.05.

-b significant difference from control (Alcl3) group at P<0.05.

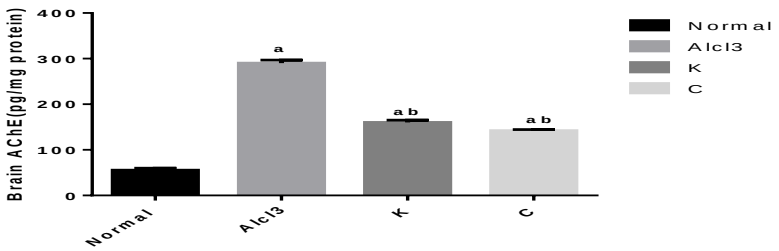

Fig. 8. Effect on brain pro-inflammatory cytokines AChE in Aluminum chloride induced Alzheimer's disease (AD in

experimental rats.

e. Effects on relative quantification (RQ) of tau mRNA gene expression.

EVOO has a protective effect against Aβ diseases by reducing the levels of soluble toxic Aβ oligomers and the amounts of various forms of insoluble Aβ in the brains of rat (Pitt et al., 2009).

The present data recorded in Table (10) and Figure (9) shows the effect of oral administration of K and C on tau mRNA expression in brain tissue in the control and treatment groups. Quantitative real time-PCR revealed that the level of tau mRNA expression in the AlCl3 group were significantly higher than those in the control group (P< 0.05). Oral administration of K and C treatments effectively decreases. Results showed that both EVOO (K and C) consumption treatment significantly stopped the accumulation of hyperphosphorylated tau (τ proteins). These findings are in a good agreement with data published by (Beauchamp et al., 2005; Monti et al., 2011; Abuznait et al., 2013 and Lopez et al., 2014).

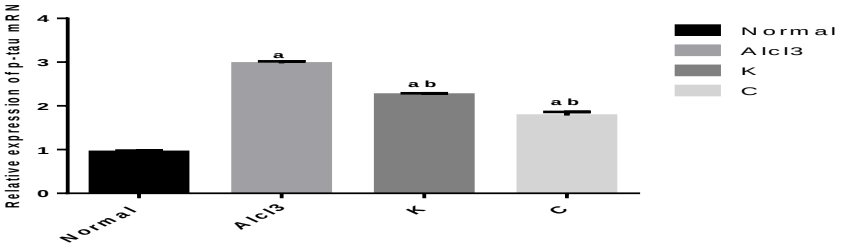

Fig. (9). Effect of K and C on brain tau mRNAexpression inAlzheimer's disease (AD)-induced rats.

* Data was expressed as mean ±SEM, n=6 rats/group. a Significantly different from the normal controlat P <0.05.

b Significantly different from the AlCl3 group at P <0.05.

Table 10. Effect on relative quantification (RQ) of tau mRNA gene expression.

Groups	relative quantification tau mRNA gene expression
Normal	0.95 ± 0.032
Aluminum chloride	2.97 ± 0.05
K	2.26 ± 0.028
C	1.78 ± 0.08

* Data were expressed as means ± SD (n=6) and were tested by one-way ANOVA followed up by Tukey post hoc test. p value < 0.05 is considered to indicate statistical significance. a: significance relative to Control groupb: significance relative to AlCl3 group

f. Effect on relative quantification (RQ) of amyloid precursorprotein (APP) mRNA gene expression.

Effect of oral administration of K and C on relativequantification (RQ) of amyloid precursor protein (APP) mRNA gene expression in brain of aluminum chloride (AlCl3)-treated rats. Continuous AlCl3 administration to rats demonstrated a significant rise in the brain APP gene expression when compared to control rats (p <0.05).

Table 11. Effect on relative quantification (RQ) of amyloid precursorprotein (APP) gene expression.

Groups	Relative quantification (APP) gene expression
Normal	0.42 ±0.034
Aluminum chloride	3.22 ± 0.031
K	2.33 ± 0.03
C	1.9 ± 0.07

* Data were expressed as means ± SD (n=6) and were tested by one-way ANOVA followed up by Tukey post hoc test. p value < 0.05 is considered to indicate statistical significance. -a: significance relative to Control group. -b: significance relative to AlCl3 group

However, concomitant administration of K and C with AlCl3 significantly decreased brain APP gene expression when compared to the AlCl3-treated rats (p < 0.05). The brain APP gene expressions of rats treated with K and C are still significantly raised when compared to control group (p < 0.05), The present data recorded in Table (10 and 11) and Figure (10). Results showed that both EVOO consumption (K and C) treatment significantly reduced Aβ42. These findings are in a good agreement with data published by (Beauchamp et al., 2005; Monti et al., 2011; Abuznait et al., 2013 and Lopez et al., 2014).

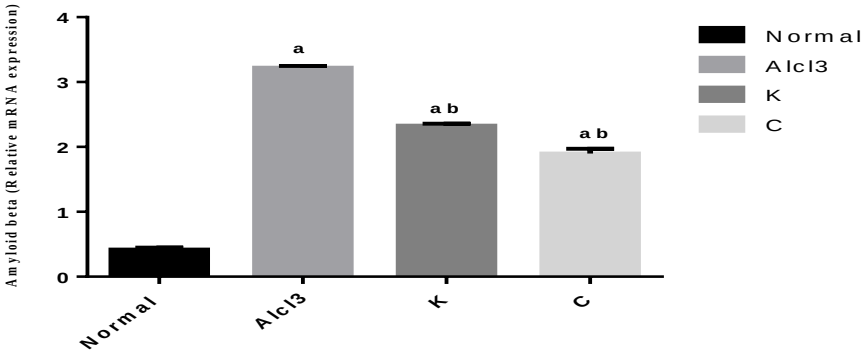

Fig. (10). Effect of KandC on brain Amyloid (β1-42) expressionin Alzheimer's disease (AD)-induced rats.

* Data was expressed as mean ±SEM, n=6 rats/group. a Significantly different from the normal controlat P <0.05. b Significantly different from the AlCl3 group at P <0.05.

5. Histopathology

The results of pathologic scoring of neuronal cell count recorded in the cerebral cortex and hippocampus are illustrated in Table 12. Microscopic examination of brain sections from normal group showed normal histological structure with normal cerebral cortical and hippocampal neurons (Fig 11a & 12a, respectively). Whereas, marked reduction of normal neurons associated with widespread neuronal degeneration and activation of microglia and astrocytes were demonstrated in the cerebral cortex of Aluminum chloride group (Fig. 11b). One of the characteristic lesions demonstrated in the cerebral cortical neurons was presence of flame-shaped neurofibrillary tangle (Fig. 11c).

Table (12): Illustrates the results of viable neuronal cell count recorded in the cerebral cortex and hippocampus of different treated groups.

Group	Viable neuronal cells in the cerebral cortex (count/1µm^2) (mean±SE)	Viable neuronal cells in the hippocampus (count/1µm^2)
Normal	23.1^a±2.29	33.20^a±1.48
ALCl$_3$	9.7^c±1.22	18.90^c±1.27
K	10.00^c±1.49	24.60$^{b,\ c}$±2.62
C	16.50b±1.32	23.60b, c±3.04

More severe pathological alterations were demonstrated in the hippocampus of this group, which revealed neuronal loss with pronounced decrease of normal neurons (Table 12) and activation of glial cells particularly astrocytes (Fig. 12b). Significant amelioration was recorded in C group, with little improvement was recorded in the cerebral cortex and hippocampus of K group (Table 12), in which the normal cerebral cortical and hippocampal neurons were decreased and gliosis was marked (Fig 11d& 12c). In the same way, decreased neuronal degeneration and activation of astrocytes and microglia were demonstrated in the cerebral cortex and hippocampus of C group (Fig 11e& 12d).

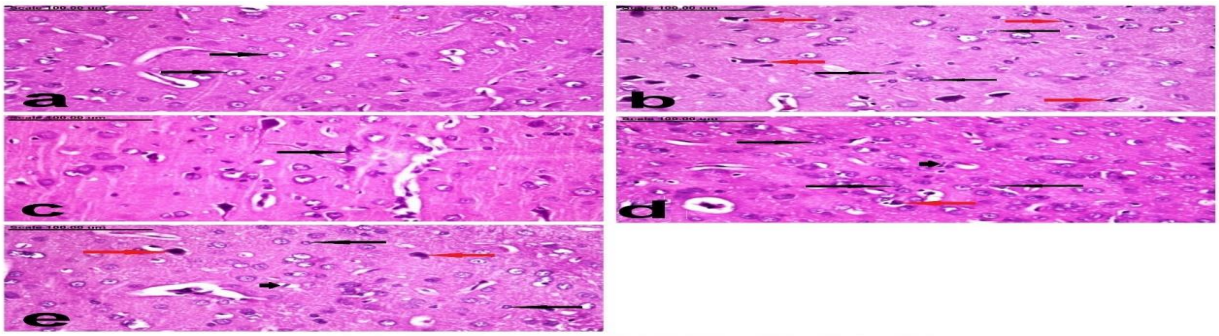

Figure 11: Cerebral cortex of (a) normal group showing normal cerebral cortical neurons with large round nuclei and prominent nucleoli (black arrows), (b , c) Aluminum chloride group showing reduction of normal neurons associated with widespread neuronal degeneration (red arrows) and activation of microglia and astrocytes (black arrows) (b) and flame-shaped neurofibrillary tangle (black arrow) (c), (d)K group showing degenerated neuron (red arrow) and activation of astrocytes (black arrows) and microglia (arrow head), (e) C group showing decreased neuronal degeneration (red arrows) and activation of astrocytes (black arrows) and microglia (arrow head), (Stain: H&E, Scale bar=100µm).

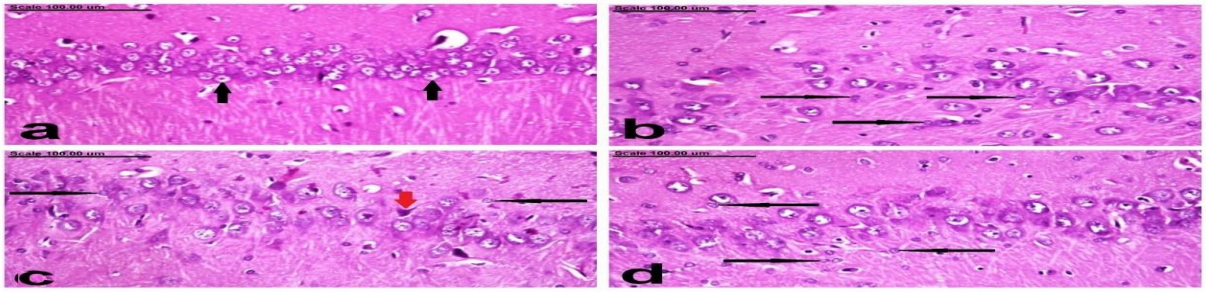

Figure 12: Hippocampus of (a) normal group showing normal neurons (black arrows), (b) Aluminum chloride group showing pronounced decrease of normal neurons and marked activation of astrocytes (black arrows), (c) K group showing degenerated neuron (red arrow) and activation of astrocytes (black arrows), (d)C group showing activation of astrocytes (black arrows), (Stain: H&E, Scale bar=100µm).

6. Immunohistochemistry

The results of caspase-3 and TNF-α expression in the brain of normal and treated groups are illustrated in Table 13.

Table 13: Illustrates the results of caspase-3 and TNF-α expression in the cerebral cortex and hippocampus of normal and treated groups.

Group	**Caspase-3 expression (% of positive cells/HPF) (mean±SE)**		**TNF-αexpression (% of positive cells/HPF) (mean±SE)**	
	Cerebral cortex	Hippocampus	Cerebral cortex	Hippocampus
Normal	0.10^{d}±0.10	0.10^{d}±0.10	0.00^{e}±0.00	0.00^{e}±0.00
AL Cl_3	2.90^{a}±0.10	2.80^{a}±0.13	2.90^{a}±0.10	2.80^{a}±0.13
K	1.80^{b}±0.20	1.90^{b}±0.27	1.80^{b}±0.24	1.80^{b}±0.20
C	1.40^{b}±0.16	$1.60^{b, c}$±0.16	$1.60^{b, c}$±0.16	1.30^{c}±0.15

a. Caspase-3 expression

Immunohistochemical examination of the brains of normal group revealed no caspase-3 expression in the cerebral cortex and hippocampus (Fig. 13a &1 3b, respectively). On the contrary, increased caspase-3 expression, with increased % of positively stained cells, was recorded in the cerebral cortex (Fig. 13c) and hippocampus (Fig. 13d) of Aluminum chloride group. K group where caspase-3 immune stained cells with strong brown staining were decreased in the cerebral cortex and hippocampus (Fig. 13e& 13f, respectively). While the % of cells expressing caspase-3, with strong brown staining, was significantly decreased in the cerebral cortex and hippocampus of C group, (Fig. 13g& 13h, for cerebral cortex and hippocampus respectively).

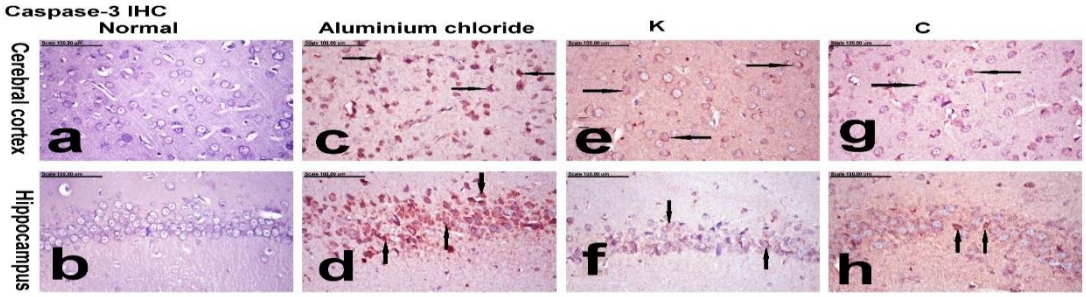

Figure13:photomicrograph displaying the cerebral cortex and hippocampus, immunohistochemically stained with anti-caspase-3 antibody, of the following: (a, b) normal group showing no caspase-3 expression in the cerebral cortex (a) and hippocampus (b), (c , d) Aluminum chloride groupshowing increased caspase-3 positively stained cells with strong brown cytoplasmic and / or nuclear staining in the cerebral cortex (c) and hippocampus (d), K groupshowing scattered caspase-3 positively stained cells with strongk cytoplasmic staining in the cerebral cortex (e) and hippocampus (f). Cgroup showingdecreased caspase-3 positive cells in the cerebral cortex (g) and hippocampus (h),(Caspase-3 immunohistochemical staining; Scale bar=100µm).

b. TNF-α expression

No expression of TNF-αwas demonstrated in the cerebral cortex and hippocampus (Fig. 14a & 14b, respectively) of

normal group, but the cells expressing TNF-α were significantly increased in the brain of Aluminum chloride group (Table 13). The immune reactive cells revealed intense brown cytoplasmic staining (Fig 14c & 14d).

In contrast, the % of cells expressing TNF-α, with strong brown staining, was significantly decreased in the cerebral cortex and hippocampus of K and C groups, with significant difference between them (Table 13), in which few TNF-α- positively stained cells with strong brown cytoplasmic staining were demonstrated in the cerebral cortex and hippocampus of K group (Fig. 14e &14f, respectively). In the same way, in group C few TNF-α positive cells with strong brown staining were demonstrated in the cerebral cortex (Fig. 14g) and hippocampus (Fig. 14h).

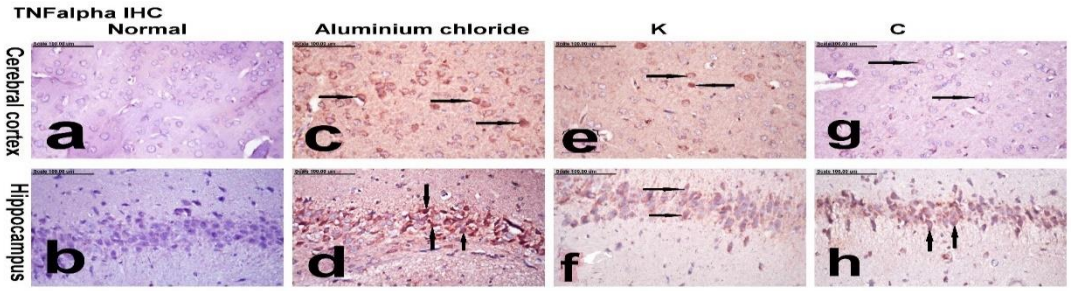

Figure 14: photomicrograph displaying the cerebral cortex and hippocampus, immunohistochemically stained with anti-TNF-α antibody, of the following: (a, b) normal group showing no TNF-α immune stained cells in the cerebral cortex (a) and hippocampus (b), (c , d) Aluminum chloride group showing numerous TNF-α-positively stained cells with intense brown cytoplasmic staining in the cerebral cortex (c) and hippocampus (d), (e , f) K group showing few TNF-α- positively stained cells with strong brown cytoplasmic staining in the cerebral cortex , (g, h)C group showing TNF-α- weakly stained cells in the cerebral cortex (g) and moderately stained cells in the hippocampus (h).

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