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The Efficacy of Egyptian Clementine oil identified by GC/MS analysis on Alzheimer's disease –induced rats

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Abstract

Essential oils have been used as remedies since ancient times for the treatment of numerous illnesses on account of their wide range of biological activities. Varying pharmacological responses in the nervous system leading to anxiolytic, antidepressant, sedative, and anticonvulsant effects. This research aims to extract oil from Egyptian Clementine by Screw Press to improve the properties of producing oil and to evaluate the effect of it on Alzheimer's disease (AD) in rats -induced by $AlCl_3$. The maximum yield of Clementine oil was about 11 %, healthy and low fatty acid at screw temperatures 40°C and motors speed 25 rpm. The fatty acid composition was analysed by GC/MS. In *In vivo* experiment ; AD-induced rats were orally administered 100mg/kg b.wt of AlCl3 for two months. Clementine oil was administered at a dose of 100 ul /kg b.wt. for 6 weeks compared to reference drug donepezil 10 mg/kg b.wt.. Acetylcholine esterase (AchE), Acetylcholine (Ach), ,noradrenaline (NA), adrenaline (AD),serotonin (5-HT),oxidative stress marker ;malondialdehyde (MDA), superoxide dismutase (SOD),catalase (CAT),total antioxidant capacity (TAC), tau protein and β-amyloid were estimated in brain tissue and serum of AD-induced rats and therapeutic group compared to control. Histological investigation of hippocampal of AD rats revealed severe neuropathologic damage exhibited by shrunken and necrosis of pyramidal neurons associated with the formation of neurofibrillary tangles, the proliferation of glia cells, and neuronophagia. Strong immune reactivity of hypertrophied astrocytes with deeply brown stained GFAP positive processes was noticed in the cerebral cortex and hippocampus of AD rats. The hippocampus of rats treated with reference drug revealed necrosis of some pyramidal neurons and neurofibrillary tangles. However, rats treated with oil showed marked restoration of the histological structure with only sporadic necrosis of pyramidal neurons. The cerebral cortex showed numerous neuropathologic alterations described by psychosis and necrosis of neurons as well as the formation of neurofibrillary tangles and focal gliosis. Further, oil treatment exhibited regression of the histopathological damage. While down-regulation of the immune GFAP immune reaction was seen in the cortex and hippocampus of rats treated with either reference drug or oil. Additionally, noticeable improvement was detected in all measured biochemical parameters upon treated AD-induced rats with Clementine oil. It could be concluded that remarkable improvement in all detected parameters resulting from treatment of AD rats with Clementine oil extracted by Screw press techniques due to its high antioxidant activity. Essential oils (Eos) has shown anticholinesterase activity, which might be advantageous for the development of drugs for AD treatment since cholinesterase has been recognized as one of its potential targets.

Keywords: Screw press; Alzheimer Disease (AD); neurotransmitters; oxidative stress; antioxidant; Clementine oil.

1. Introduction

Organic products and natural consumers are becoming increasingly popular in agricultural product marketing around the world. Essential oil products are extracted from various plant parts, such as flowers, leaves, roots, and fruits, which contain a variety of chemical-like hydrocarbons, alcohol, aldehydes, esters, ethers, ketones, oxides, phenols, and terpenes [1] .The clementine is a spontaneous citrus hybrid that evolved in the late nineteenth century in Misserghin, Algeria, in the garden of the orphanage of Brother Marie-Clément, C.S.Sp. Clementine mandarin fruits, which belong to the genus Citrus, may be an excellent alternative in this regard, as customers are increasingly demanding easy to eat fresh fruits with certain features such as appealing flavour, color, easy to peel rind, balanced sugar/acid ratio, firmness, and lack of seeds. Furthermore, citrus fruits are of great biomedical

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interest because a diet high in them appears to be associated with a lower risk of colorectal, esophageal, gastric, and stomach cancers [2]. Clementine is a mandarin-orange hybrid that is very similar to mandarin. Its fruits are shaped like mandarins but are sweeter. Citrus spp. fruits, in particular, have high antioxidant activity due to their high content of bioactive compounds that act as free radical scavengers and exert various antiradical activities. Vitamin C and flavonoids are the health-promoting compounds responsible for these beneficial effects. Because of its activity against free radicals, carcinogenesis, and cardiovascular diseases, as well as in stimulating the human immune system, vitamin C is regarded as one of the most important antioxidants [3]. Flavonoids act as free radical scavengers, inhibit cellular proliferation, can reduce high blood pressure or cholesterol levels, and have antibiotic, antiallergic, anti-diarrhea, and antiulcer properties [4,5]. Flavone glycosides, such as hesperidin and naringin, have antioxidant, anticarcinogenic, and blood lipid-lowering properties. Hesperidin, in particular, improves venous tone, improves microcirculation, and is used to treat chronic venous insufficiency, whereas naringin inhibits specific cytochrome P-450 enzymes, resulting in drug interactions [6]. Citrus fruits' chemical composition, like that of other fruits, is influenced by a variety of factors, including cultivar, genotypic differences, ripening stage, production area, and agricultural practices [7]. Clementines are high in antioxidants, which aid in the reduction of inflammation and the protection of cells from free radical damage. As a result, antioxidants may help to prevent type 2 diabetes, cardiovascular disease, and a variety of other conditions. In addition to vitamin C, these fruits contain a variety of citrus antioxidants such as hesperidin, naringin, and beta-carotene. Some animal and test-tube studies have revealed that the citrus antioxidant hesperidin has potent anti-inflammatory properties, but more human studies are required. Eventually, animal and test-tube studies have shown that flavonoids like hesperidin can improve mental health and may be effective in the treatment of Alzheimer's disease. More human research, however, is required. Citrus antioxidants such as hesperidin, naringin, and beta-carotene Some animal and test-tube studies have shown that the citrus antioxidant hesperidin has potent anti-inflammatory effects, but more human research is needed. Finally, animal and test-tube studies have shown that flavonoids like hesperidin can improve mental health and may be effective in treating of Alzheimer's disease. More human research, however, is required.

The effects of essential oils on the human nervous system vary, such as the relaxing effect of lavender oil, the vigilance effect of jasmine oil and rosemary oil, and the attention effect of rose oil and orchid oil on the brain and cognitive functions [8-10]. The action of an essential oil begins with the inhalation of the essential oil molecules through the nostril and attachment to odorant receptors, which activates G protein-coupled receptors. It will then cause synaptic transmission to the central nervous system to begin [11]. Finally, the signal input is transmitted to several brain regions that are in charge of olfactory perception, autonomic homeostasis, and other higher brain functions [12]. In the present study, we are focus on the effect of Clementine oil on the brain function neurotransmitters in Alzheimer's disease induced by AlCl3 in rats. Acetylcholine (Ach), acetylcholine esterase (AchE), noradrenalin (NA), adrenaline (AD) serotonin (5-HT), oxidative stress malondialdehyde (MDA),antioxidant enzymes ;superoxide dismutase (SOD),catalase (CAT),Total Antioxidant Capacity (TAC), total protein and β-amyloid were estimated in brain tissue and serum of treated animals .

2. MATERIALS AND METHODS

2.1 Chemicals

Donepezil, reagents, and kits were purchased from the Sigma Chemical Company (USA), whereas aluminum chloride ($AlCl_3$) was purchased from CDH, India.

2.2 Materials

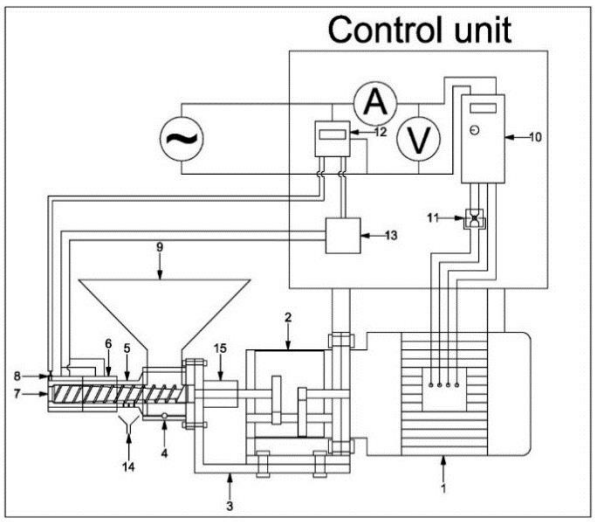
The seeds of Clementine were obtained from the local market in June 2020 in Cairo, Egypt.

Fig.1 Clementine seeds

2.3 Methods

2.3.1 Clementine Oil and Screw press Extraction Method

The screw press is a continuous process, it is used to extract oil from seeds according to the method was designed [13]. This will reduce power consumption and time to half. Screw press has control on temperatures and speed to try and give maximum oil yield.



1	AC Motor	2	Gear box	3	Base A
4	Base B	5	Housing	6	Heaters
7	Screw	8	Temperature sensor	9	Feeding hopper
10	Inverter	11	Direction switch	12	Thermostat
13	Relay	14	Oil collector	15	Coupling

Fig. 2 Design of the screw press produces tangerine oil.

2.3.2 Identification of non-polar compound (methylated hexane fraction) by GC/MS (Gas Chromatography/Mass Spectrometry) analysis

The bioactive fractions were subjected to GC/MS analysis to identify the non-polar compounds present in the non-polar fraction. Identification of the constituents was carried out by comparison of their retention times and fragmentation patterns of mass with those of published data [11]and/or with those of the Wiley 9 and NIST 08 mass spectra libraries [14] .

2.3.3 Biological experiment

2.3.3.1 Animals

The male Wistar albino rats (150 ± 10 g) were provided by the Animal House of the National Research Centre (NRC) and housed in a group of 10 rats per cage, maintained in a controlled environment condition at 26-29 °C. They were provided with a fixed light/dark cycle for one week (w) as an adaptation period to acclimatize under normal combination with free access to water and food. The study was approved by the Ethical Committee of the NRC, Egypt, provided that the animals will not suffer at any stage of the experiment under ethical approval no: 44123-2021.

2.3.3.2 Induction of $AlCl_3$ induced-Alzheimer's disease (AD)

$AlCl_3$ solutions were made freshly at the beginning of each experiment. For oral administration, $AlCl_3$was dissolved in drinking water and administered orally in a dose of 100 mg/kg, to rats daily for 8 weeks 0.5 mL/100 g b.wt. [15]. Donepezil drug was daily administrated post-induction of $AlCl_3$ for 8 weeks in a dose of 10 mg/kg b.wt. parallel with mandarin oil [16].

2.3.3.3 Acute Toxicity Study

Forty mice were used in the acute toxicity study four rats for each concentration. Serial concentrations of Clementine oil were used for the determination of acute toxicity, 100ul -1000 ul. No mortality and no toxicity signs were observed up to 1000ul /kg b.wt for 48 h.

2.3.3.4 Behavioural assessment

Assessment of Cognitive Abilities and Motor Coordination T-maze is locally constructed at N.R.C. Cognitive ability and impairment of spatial memory of rats was evaluated after chronic AlCl3 (two months) at the end of the treatment period [17]. Motor ability was assessed using the beam balance test as previously described [18].

2.3.3.5 Experimental design

Fifty rats were divided randomly into five groups of 10 rats each. Group 1: Normal of healthy control rats. Groups 2: Clementine oil-treated normal rats. Group 3: serving as AD rats, where rats were orally administered with $AlCl_3$. Group 4: was the AD-treated rats with Clementine oil 100 ul/kg b.wt.), daily for 6 weeks, respectively [1/10 LD_{50}]. Group 5: was the AD-treated rats with standard drug donepezil 10 mg/kg b.wt.), daily for 6 weeks.

2.3.3.6 Blood samples preparation

Overnight fasted animals were sacrificed under slight diethyl ether anaesthesia. The blood was collected by puncture of the sublingual vein in the clean and dry test tube. Blood was left 10 min to clot and centrifuged at 3000 rpm to obtain serum. The separated serum was used for biochemical analysis of total antioxidant capacity (TAC).

2.3.3.7 Brain tissue sampling and preparation

At the end of the experiment, the rats were fasted overnight, subjected to anaesthesia, and sacrificed. The whole brain of each rat was rapidly dissected, washed with isotonic saline, and dried on filter paper. The brain was weighed and homogenized in an ice-cold medium containing 50 m MTris/HCl and 300 mM sucrose at pH 7.4 to give a 10% (w/v) homogenate[15,19]. This homogenate was centrifuged at 1400 ×g for 10 minutes at 4°C. The supernatant was stored at −80°C and used for antioxidant and oxidative stress biomarkers, MDA, GSH, SOD, CAT, neurotransmitters markers, Tau protein, and amyloid β. The animals were disposed of in bags provided by

the Committee of Safety and Environmental Health, NRC.

2.3.3.8 Estimation of brain neurotransmitters

Serum AChE and Ach were measured by a quantitative enzyme-linked immunosorbent assay (ELISA) according to Engvall and Perlman [20]. The concentrations of brain NA, AD, DA, and serotonin (5-hydroxytryptamine; 5-HT) were determined using high-performance liquid chromatography with electrochemical detection (HPLC-ED) technique according to Giday et al. [21]. Tau, protein, and Amyloid-β concentrations were assayed using ELISA kits according to manufacturer instructions.

TAC was determined in serum by the colorimetric assay method of Koracevic et al. [22]. All animal groups were subjected to determine glutathione reduced (GSH) [23], malondialdehyde (MDA) [24]; enzymatic antioxidants; superoxide dismutase (SOD) [25], and catalase (CAT [26], in brain tissues by using standard diagnostic kits according to manufacturer instructions.

2.3.3.9 Histological examination of brain tissue

Specimens of the brain were harvested from all rats/groups and then fixed in 10% neutral buffered formalin. Paraffin sections of 5µm thickness were prepared and stained with Hematoxylin and Eosin (H&E) for histopathological examination by a light microscope [27] .An experienced pathologist blinded to treatments performed the histological analysis. Neuropathologic damage in the cerebral cortex and hippocampus were graded from (0-4) as follow :(0) indicated no changes; (1) indicated percentage area affected (<10%); (2) indicated percentage area affected (20-30%); (3) indicated percentage area affected (40-60%) and (4) indicated percentage area affected (>60%) [28].

2.3.3.10 Immunohistochemistry

Glial fibrillary acidic protein (GFAP)

The deparaffinized and rehydrated sections were incubated with GFAP, mouse monoclonal antibody (1:500) (Dako, N-series Ready- to use primary antibody). The immune-staining was amplified and completed by Horseradish Peroxidase complex (Dako, REALM EnVision TM / HRP, Mouse ENV). Sections were developed and visualized using 3, 3-diaminobenzidine (Dako, REALM DAB + Chromogen). The substrate system produced a crisp brown end product at the site of the target antigen. Sections were counterstained with hematoxylin, then dehydrated in alcohol, cleared in xylene and cover slipped for microscopical examination [29]. Quantification of GFAP was estimated by measuring the area % expression from 5 randomly chosen fields in each section and averaged using image analysis software

(Image J, version 1.46a, NIH, Bethesda, MD, USA).

2.4 Statistical analysis

All data sets were expressed as mean ± SD. The data were analyzed statistically for normal distribution using One Way Analysis of Variance (ANOVA) software and co–State for Windows, version 8. Values of different letters are statistically significant at $P< 0.05$.

3. RESULTS

GC/MS Identification of *Clementine* seeds oil extracted by (Screw press)

The probabilities of the structures of the detected compounds are listed in Table (1) were Hexadecanoic acid, trimethylsilyl ester 18.36%is the major constituent of seeds oils, While the dominant components of clementine seed oil were1-Hexadecanol 6.13%, (Cis)-2-nonadecene 5.27%, Phenol, 2,4-bis(1,1-dimethyl ethyl) 4.74%, except in case of Clementine peels, where D-limonene was the major component (56.70%%) Table 1. In general, the percent of hydrocarbons is higher than the percentage of oxygenated compounds in all the oil samples under investigation. In the present work, the GC-MS chromatogram of the Clementine peel oil extract displayed 37 peaks indicating the presence of 37 compounds. Flavonoids, especially poly ethoxy flavones, and flavones (hesperidin, narration, and naringin) which are identified according to (Hamdan et al., 2020). It has been found by (Bozkurt et al.017) that the total oil yield from sweet orange 0.80%, eureka lemon 0.90%, and mandarin 0.80% which were lower than our results. On the other hand, the results obtained by Bozkurt et al.(2017), clarified that the essential oil of *Citrus clementine* contained *d*-limonene (66-93%), α-pinene, sabinene, *β*-pinene, *β*-myrcene, linalool, *m*-cymene, and *4*-terpineol (. Zhang et al., 2017) reported that limonene (56.8–93.3%) and α -terpinene (0.1–36.4%) were the major components of volatile fraction in clementine juice and peels. While the dominant components clementine peel essential oils were à-Sinensal 7.27%, 4-Methyl-Benzimid Azolone 5.53, à-Farnesene 4.09%, Zingiberene 2.61%, 1, 5- Heptadien-4-one, 3,3,6-trimethyl 1.88%, Decanol 1.24, Gingerol 0.30% were identified by the GC/MS analyses(Table1).

Table (1). Main compounds of *Clementine* seeds oil extracted by (Screw press) identified by GC/M

S.N.	R_t[a]	Compound name	Area %[b]	Molecular formula
1	5.13	1,5- Heptadien-4-one, 3,3,6-trimethyl	1.88	$C_{10}H_{16}O$
2	6.43	(P)-Phenyl[1+1]cycloamide	0.39	$C_{45}H_{68}N_2O_2$
3	9.29	DL-Limonene	56.70	$C_{10}H_{16}$
4	13.92	1- Propyne	0.42	C_3H_4
5	14.04	Linalyl propionate	0.24	$C13H_{22}O_2$
6	14.12	Hexane,2,2,5,5-tetramethy	0.31	$C_{10}H_{22}$
7	14.36	, Decanal	1.24	$C_{10}H_{20}O$
8	16.62	4-Methyl-Benzimid Azolone	5.53	$C_8H_8N_2O$
9	17.00	5,7-Dodecadiyn-1,12-diol	0.35	$C_{12}H_{18}O_2$
10	19.19	4-Octadecynol	1.20	$C_{18}H_{34}O$
11	19.39	1-Decanol	0.40	$C_{10}H_{22}O$
12	19.80	4H-3,1-Benzoxazin-2-thione	3.56	C_8H_7NOS
13	19.98	Trans- Caryophyllene	0.58	$C_{15}H_{24}$
14	20.94	N-Acetyl-DL-penicillamine	0.31	$C_7H_{13}NO_3S$
15	21.46	Isolepidozene	0.24	$C_{15}H_{24}$
16	21.53	Benzene,1-(1,5-dimethyl-4-hexenyl)-4-methyl	1.10	$C_{15}H_{22}$
17	21.83	Zingiberene	2.61	$C_{15}H_{24}$
18	22.12	à-Farnesene	4.09	$C_{15}H_{24}$
19	22.40	Phenol, 2,4-bis- (1,1-dimethylethyl)	1.39	$C_{14}H_{22}O$
20	22.51	(+)-á-Funebrene	1.38	$C_{15}H_{24}$
21	23.61	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	0.25	$C_{21}H_{38}O_2$
22	23.95	1-Tridecanol	1.90	$C_{13}H_{28}O$
23	24.12	Hexadecane	0.40	$C_{16}H_{34}$
24	25.50	Butan-2-one, 4-(3-hydroxy-2-methoxy phenyl)	1.15	$C_{11}H_{14}O_3$
25	26.31	Pentadecane	0.40	$C_{15}H_{32}$
26	27.19	4-ethynylhept-6-en-1-ol	1.07	$C_9H_{14}O$
27	27.62	à-Sinensal	7.27	$C_{15}H_{22}O$
28	28.25	(Cis)-2-nonadecene	1.87	$C_{19}H_{38}$
29	28.81	E-sesqui-lavandulol	0.41	$C_{15}H_{26}O$
30	30.97	Hexadecanoic acid, methyl ester	1.36	$C_{17}H_{34}O_2$
31	32.16	Hexadecen-1-ol, trans-9	1.66	$C_{16}H_{32}O$
32	32.26	Oxalic acid, decyl propyl ester	0.37	$C_{15}H_{28}O_4$
33	34.11	13-Tetradece-11-yn-1- ol	1.27	$C_{14}H_{24}O$
34	34.22	13-Docosenoic acid, methyl ester, (Z)	0.30	$C_{23}H_{44}O_2$
35	37.67	Gingerol	0.30	$C_{17}H_{26}O_4$
36	43.54	3,5-Dimethyl butyrolactone	037	$C_6H_{10}O_2$
37	45.46	Cis- Pinane	0.39	$C_{10}H_{18}$

[a]Rt: retention time (min).
[b]The percentage composition was computed from the gas chromatography peak areas.

Behavioural tests

Numerous behavioural tests have been designed to assess the extent of brain aging. In our study, both T-Maze beam balance tests showed that AlCl3 caused significant deterioration in brain cognitive functions, as shown in (Table 2). However, treatment of rats with donepezil or mandarin oil extract resulted in an improvement in behavioural status as represented by an improved coordination and improved cognition, as shown in (Table3).

Table 2: Effect of oil of Clementine by using T- maze test in Alzheimer's disease induced rats

	Groups				
Parameters	Control	Normal + **Clementine** oil	Al Cl3 -AD	**Clementine** oil-AD	Donepezil drug
GSH (µg/mg protein) % of change % of improvement	3400.00±55.00a	3660±66.00a -7.65	1600.87±69.29b -52.92	2980.0±94.00d -12.35 40.56	2800.00±100.60d -17.65 35.27
MDA (µg/mg protein) % of change % of improvement	31.60±1.120e	29.87±2.10e -5.47	121.00±5.00f +282.91	49.60±3.00g +56.96 225.95	66.08±4. 76g +109.11 173.80
SOD (µmol/mg protein) % of change % of improvement	2100.00±100.88h	2324.00±98.70h +10.67	800±30. 62i -61.90	1978.25±93.00h -5.79 56.11	1677.00±70.00j -20.14 41.76
CAT (U/g tissue) % of change % of improvement	188.00±20.00k	200.00±10.09k +6.38	42.23±2.53l -77.54	166.88±10.80n -11.23 66.30	123.00±8.80m -34.57 40.96
TAC (U/g tissue) % of change % of improvement	2. 78±0.03o	2. 90±0.13o +4.32	0.53±0.05p -80.94	1.87±0.12q -32.73 48.20	1.23±0.14s -55.755 25.18

Data were expressed in seconds as mean ±SD (n=10). Groups with a similar letter are not significantly different; while, those with the different letters are significantly different at $P \leq 0.05$.

Table 3: Effect of Clementine oil by using the beam balance test in Alzheimer's disease induced rats

	Baseline	Induction two month	Treatment one (months)
Control	12.08±0.30a	11.66±0.23a	12.23±0.21a
Al Cl3 -AD % change	10.78±0.19b %-10.76	3.00±0.21c %-74.27	-
Clementine oil +AD % change % of improvement	-	-	11.08±0.30d (%)-9.40 66.10
Donepezil drug % change (% of improvement)	-	-	10.77±0.23d (%)-11.93 63.53

Data were expressed in seconds as mean ±SD (n=10). Groups with similar letters are not significantly different; while, those with different letters are significantly different at $P \leq 0.05$.

Table 4: Effect of Clementine oil on some antioxidant enzymes level in brain tissue of AD-induced rats

	Groups				
Parameters	Control	Normal + **Clementine** oil	Al Cl3 -AD	**Clementine** oil-AD	Donepezil drug
Ach (nmol/g tissue) % of change % of improvement	50.20±2.02a	54.70±4.00a +8.96	19.15±0.56b -61.85	39.80±3.00c -20.72 41.14	39.00±2.21c -22.31 39.54
AchE (µmol/g tissue) % of change % of improvement	3280.00±700.00d	3220.00±100.00d -1.83	9000.00±200.00e +174.39	4340.00±120.00g +32.32 142.10	5200.03±155.00f +58.53 115.85

Data were expressed in seconds as mean ±SD (n=10). Groups with similar letters are not significantly different; while, those with different letters are significantly different at $P \leq 0.05$.

Table (4): revealed insignificant change in normal rats treated with mandarin oil in oxidative stress and antioxidant markers as compared to control. AD–induced rats showed a significant reduction in GSH, SOD, CAT and TAC with percentages -52.92, -61.90,- for GSH, MDA, SOD, CAT, and TAC respectively compared to standard drug.

The present study clearly indicated significant reduction in Ach, while significant increase in AchE in brain tissue of AD rats with percentages change -77.54, and -80.94%respectively. While a significant increase in MDA level reached 282.91 was detected. Treatment of AD rats with mandarin oil revealed noticeable improvement in all detected parameters amounted to 40.56, 225.95, 56.11, 66.30, and 48.20 %, 61.85 and +174.39%, respectively. Noticeable amelioration in Ach and AchE levels upon treatment of AD rats with Clementine oil with percentages of improvements reached to 41.14 and 142.10%, respectively compared to reference drug (39.54 and 115.85%, respectively for Ach and AchE) (Table 5).

Table 5: Effect of Clementine oil on the levels of Ach and AchE in brain tissues of AD-induced rats

	Baseline	Induction two months	Treatment one (months)
Control	13.00±0.50a	14.00±1.10a	14.60±0.56a
Al Cl3 -AD% change	15.60±0.60b	49.00±3.22c	-
Clementine oil	-	-	23.00±0.12d (%) +57.53 178.08
Donepezil drug (% of improvement)	-	-	24.00±1.00d (%)+ 64.38 171.23

From table (6) we can deduced that, insignificant change in all neurotransmitters levels in normal rats treated with Clementine oil .However, significant reduction in all transmitters in brain tissues of AD rats with percentages reduction of -63.66,-61.78,-75.12 and -68.98%, for AD,NA, DA and 5-HT respectively .Treatment of AD rats with Clementine oil showed higher improvement percentages amounted to 31.52, 45.78, 46.47 and 44.81% ,respectively than standard drug.

Table (6): Effect of Clementine oil on the levels of AD, NA, DA, and 5-HT in brain tissues of AD –induced rats

	Groups				
Parameters	Control	Normal + **Clementine** oil	Al Cl3 -AD	**Clementine** oil-AD	Donepezil drug
Tau protein (ng/l) % of change % of improvement	332.00± 11.45a	323.76±10.12a -2.48	1234.00±16.405b +271.69	524.90±11.66e -58.10 213.58	588.98±14.00e -77.40 194.28
Amyloid β (g/l) % of change % of improvement	665.00±19.10h	644.40±30.00h -3.10	1667.23±13.00i +150.71	889.10±3.12j +33.69 117.01	904.89±20.12j +36.07 114.63

Data were expressed as means ± SD (n=10). Groups with similar letters are not significantly different; while, those with different letters are significantly different at $P \leq 0.05$. Ach: Acetylcholine; AchE: Acetylcholine esterase.

Table (7) declared insignificant change in tau protein (and amyloid-β in brain tissues of normal rats treated with Clementine oil compared to control rats. Significant increase in the levels of Tau protein and amyloid-β in AD –induced rats with percentages increase 271.69 and 150.71%, respectively. Higher percentages of improvement was recorded for Clementine oil than standard drug upon treatment AD rats with oil.

Table (7): Change in tau protein and amyloid-β in brain tissues of normal rats

	Groups				
Parameters	Control	Normal + **Clementine** oil	Al Cl3 -AD	**Clementine** oil+ AD	Donepezil drug
AD (ng/gm tissue) % of change % of improvement	289.00±13.00 a	300.00±12.00 a +3.81	105.00±12.22 b -63.66	196.08±11.55d -32.15 31.52	160.00±21.21d -44.64 19.03
NA (ng/g tissue) % of change % of improvement	225.00±11.30 d	229.00±12.20 d +1.78	86.00±7.00 e -61.78	189.00±8. 09f -16.00 45.78	167.00±8.25 f -25.78 36.00
DA(ng/g tissue) % of change % of improvement	88.43± 4.90Gad	85.59± 5.24g -3.21	22.00±1.23h -75.12	63.10±2.88i -28.64 46.47	58.90±3.20i -33.39 41.73
5-HT(ng/g tissue) % of change % of improvement	102.87±7.35j	98.99±6.90j -3.77	31.90±3.02k -68.98	78.00±5.22l -24.18 44.81	62.00±4.00m -39.73 29.26

Data were expressed as means ± SD (n=8). Groups with similar letters are not significantly different; while those with different Letters are significantly different at $P \leq 0.05$. AD: adrenaline, NA: Noradrenaline, DA: Dopamine, 5-HT: Serotonin.

Table (8) demonstrated lower reduction in lesion score in Clementine oil treated -AD rats than reference drug compared to AD- induced rats.

Table (9) showed significant increase in the percentage of GFAP area in cerebral cortex in AD induced rats, while significant recovery upon treatment of neurotoxic rats with oil. The same

observation was recorded in the area of GFAP in the hippocampus (Table 10).

Table (8): Histopathological lesions score in cerebral cortex and hippocampus of all experimental groups.

Histopathological lesion	Normal	control oil	+ve control	Treat reference	Treat oil
Cerebral cortex Necrosis of neurons	0	0	4	2	2
Neuronophagia	0	0	3	1	1
Neurofibrillary tangles	0	0	3	1	0
Gliosis	0	0	3	1	0
Hippocampus Necrosis of pyramidal neurons	0	0	4	1	1
Neurofibrillary tangles	0	0	3	1	0
Proliferation of glia cells	0	0	2	0	0

Grade 0: no change grade 1: low change, grade 2: middle change and grade 3: high change

Table (9): %Area of GFAP staining in the cerebral cortex.

group	%Area of positive GFAP staining
normal control	3.56 ± 0.11^{a}
Normal-treated oil	4.16 ± 0.21^{a}
AD-induced rats	17.04 ± 1.01^{b}
AD- treated reference drug	4 ± 0.32^{c}
AD- treated oil	5.85 ± 0.21^{d}

Each group represented by 6 different areas i.e. Mean± SD of 6 rats in each group.

Table (10): %Area of GFAP staining in the hippocampus:

group	%Area of positive GFAP staining
normal control	2.92 ± 0.05^{a}
Normal-treated oil	3.60 ± 0.16^{a}
AD-induced rats	13.80 ± 1.65^{b}
AD- treated reference drug	6.98 ± 0.88^{c}
AD- treated oil	5.03 ± 0.52^{c}

Each group represented by 6 different areas i.e. Mean± SD of 6 rats in each group

Histopathology

-Cerebral cortex

Microscopically, the cerebral cortex of normal control rats, as well as rats treated with oil only, exhibited the normal histological architecture of brain tissue with no histopathological alterations figures 3a& b).On contrary, the cerebral cortex of AD rats (control +ve) showed numerous neuropathologic alterations described by pyknosis and necrosis of neurons as well as the formation of neurofibrillary tangles (Figure 3c) and focal gliosis (Figure 3d). On the other hand, the cerebral cortex of rats treated with reference revealed necrosis of some neurons and neuronophagia (Figure 3e). Furthermore, sections from rats treated with oil exhibited regression of the histopathological damage examined cases described necrosis of sporadic neurons (Figure 3f).

Examination of the hippocampus of normal control rats, as well as rats treated with oil only, revealed the normal histological architecture of the hippocampus, normal pyramidal neurons with large vesicular nuclei (figures 4a & b). On contrary, hippocampal sections from AD rats exhibited severe neuropathologic damage exhibited by shrunken and necrosis of pyramidal neurons(Figure 4c & d)associated with the formation of neurofibrillary tangles, the proliferation of glial cells (Figure 4c), and neuronophagia (Figure 4d). However, the hippocampus of rats treated with reference drug revealed necrosis of some pyramidal neurons and neurofibrillary tangles (Figure 4e). Furthermore, sections from rats treated with oil

marked the restoration of the histological structure, examined sections exhibited only sporadic necrosis of pyramidal neurons (Figure 4f). (Figure 4f).Table 7summarizes the histopathological lesion scores in the cerebral cortex and hippocampus of different experimental groups

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-Immunohistochemistry

Glial fibrillary acidic protein (GFAP)

Immunohistochemical expression of GFAP proteins in the cerebral cortex and hippocampus were demonstrated in Figures5 (a & b) and 6(a& b) a figure 6and tables (8 & 9). In brief microscopic examination of cerebral cortex and hippocampus of normal control rats as well as rats treated with oil only exhibitednormal small-sized astrocytes with lightly stained GFAP positive short processes (Figures 3(a & b)& 4 (a & b)). On contrary, strong immunoreactivity of hypertrophied astrocytes with deeply brown stained GFAP positive processes was noticed in the cerebral cortex and hippocampus of AD rats (Figures5c&6c). Down regulation of the immune GFAP immune reaction was seen in the cortex and hippocampus of rats treated with either reference drug or with the oil (Figures 5 (d & e) and 6 (d & e).

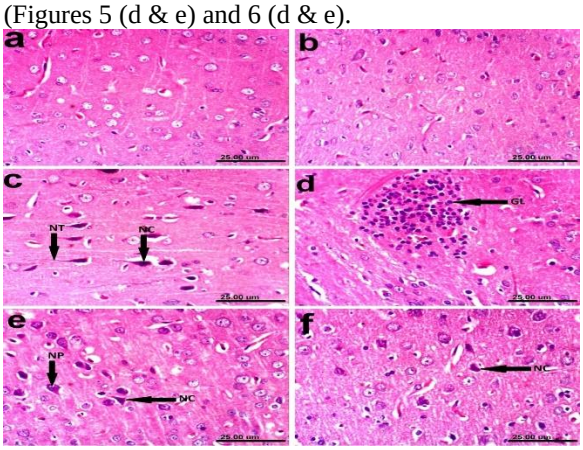

Figure (3): Photomicrographs of H&E stained sections of the cerebral cortex of rats: a) control normal showing the normal histologically. b) Oil control, exhibited no histopathological alterations. c) AD (+ve control) showing necrosis of neurons (NC), and formation of neurofibrillary tangles (NT).d) AD showing focal gliosis (GL). e) Treated with reference, showing necrosis of some neurons (NC) and neuronophagia (NP). f) Treated with oil, showing necrosis of sporadic neurons (NC). (Scale bar 25µm).

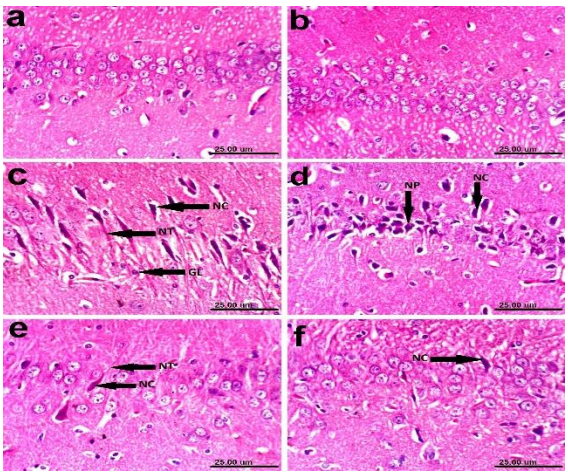

Figure (4): Photomicrographs of H&E stained sections of CA1 region of the hippocampus of rats: a) control normal showing the normal histologically. b) Oil-control, exhibited no histopathological alterations. c) AD (+ve control) showing necrosis of pyramidal neurons (NC), formation of neurofibrillary tangles (NT), and proliferation of glia cells (GL). d) AD showing necrosis of pyramidal neurons (NC) and neuronophagia (NP). e) Treated with reference, showing necrosis of some neurons (NC) and formation of neurofibrillary tangles (NT). f) Treated with oil, showing sporadic necrosis of pyramidal neurons (NC). (Scale bar 25µm).

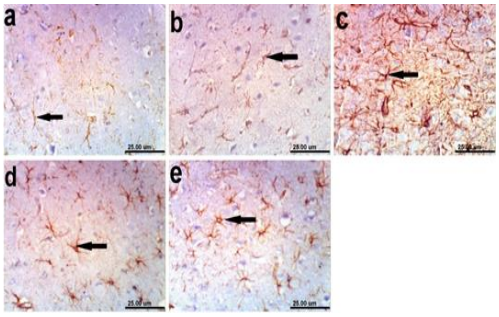

Figure (5): Photomicrograph of GFAP-immuno-reactive cells in the cerebral cortex of rats: a) and b) control and oil control respectively showing normal small-sized astrocytes with lightly stainedGFAP positive short processes (arrows). c) AD, showing strong immunoreactivity of hypertrophied astrocytes with deeply stained GFAP positive brown processes (arrow). d) and e) reference treated and oil-treated respectively, exhibited mild immunoreactive astrocytes with lightly stained processes (scalebar, 25 µm).

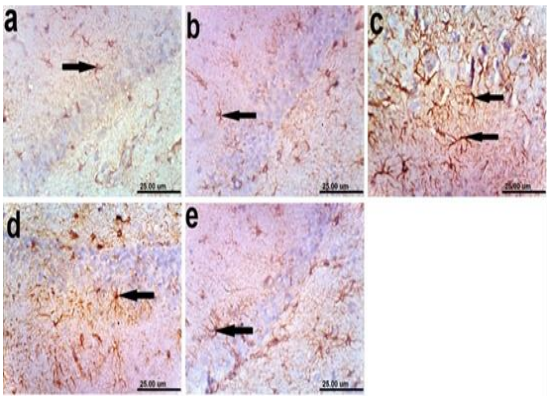

Figure (6): Photomicrograph of GFAP-immuno-reactive cells in the hippocampus of rats: a) and b) control and oil control respectively showing normal small-sized astrocytes with lightly stained GFAP positive short processes (arrows). c) AD, showing strong immunoreactivity of hypertrophied astrocytes with deeply stained GFAP positive brown processes (arrow). d) and e) reference treated and oil-treated respectively, exhibited mild immune reactive astrocytes with lightly stained processes (scale bar, 25 μm).

4.Discussion

The current results of both T-Maze beam balance tests showed that $AlCl_3$ caused significant deterioration in brain cognitive functions, as shown in Table (1). However, treatment of rats with donepezil or mandarin oil extract resulted in an improvement in behavioral status as represented by improved motor coordination and improved cognition, as shown in Table (2)

These findings are consistent with previous findings that $AlCl_3$-neurointoxicated rats took longer to catch food in the T-maze than control rats, indicating deteriorated neuro-cognitive function [19, 30]. The disruption in antioxidant defense mechanism and ROS excessive generation are considered as the main causes of mitochondrial dysfunction induced intracellular damage. So, the use of mandarin oil as antioxidants is regarded as a useful therapy for ROS-induced brain damage [31] .

The current results revealed a significant elevation in the oxidative damage biomarkers in brain tissue following AlCl3 induction. This result is in agreement with Johnson et al. [32] ,Aly et al. [33] ,Aly et al. [34] , declared that Al–linked neurotoxicity could be causing an increase in lipid peroxidation Furthermore, Nehru and Anand [35] reported a significant increase in thiobarbituric acid reactive substances in rats brains following aluminium induction, which was related to Fe^{3+}carrying protein transferring bounding, thus lowering Fe^{2+} binding and increasing free intracellular $Fe^{2+,}$ which causes membrane lipids, protein peroxidation, and later membrane destruction, despite loss of membrane fluidity , alterations in membrane potential, an increase in membrane permeability, and disturbances in receptor function [36] Furthermore, the current study stated that the increase in MDA in AD-induced rats was associated with inhibition of antioxidant enzymes involved in the removal of ROS, such as SOD, CAT, and GSH in brain tissue, implying Al's pro-oxidant action. Instead, Sumathi et al. [36] reported that aluminium exposure promotes neuronal lipid destruction, which is associated with changes in the enzymatic antioxidant defense system. Furthermore, the presented results revealed a significant decrease in GSH level in brain tissue of rats induced with $AlCl_3$, which could be attributed to the high level of H_2O_2 induced cytotoxicity in brain endothelial cells due to glutathione reductase inhibition [33]. The noticeable drop in brain TAC in AlCl3-induced AD rats may be attributed to the fact that long-term exposure to $AlCl_3$ causes an increase in lipid peroxidation, as well as the depletion and exhaustion of several antioxidant enzymes [36]. Furthermore, EL-Baz and Aly [37] attributed the decrease in TAC in AD-induced rats to a decrease in axonal mitochondria transformation, impairment of the golgi, and reduction of synaptic vesicles, which results in the release of oxidative products such as hydroperoxide and carbonyls as well as peroxy nitrites, as well as a decrease in antioxidant enzymes and glutathione within the neurons. The high level of Fe promoting ROS in AD induced rats is due to the high brain content of polyunsaturated fatty acids, which can easily interact with elaborated radicals and afford oxidative destruction. Administration of AlCl3 caused histological aberrations, elevated activity of ACh E while reduction in NA, AD, DA, 5-HT in AD-induced rats was detected. AD is involved the deficiency of the neurotransmitters AChE and DA that are linked to malfunctions in the catechol aminergic and cholinergic systems. Memory and cognitive function are affected by the acetylcholine-containing neurons. In the current study, the Alzheimer' group recorded low DA, NA and 5-HT than those of controls. In previous study, the level of NA are depressed in the cortex and cerebellum of animals have administered low copper or high aluminum [38] .β-amyloid (Aβ) plaques were mostly made of aggregated Aβ proteins and neurofibrillary tangles (NFTs) formed by hyper phosphorylated tau protein. Aβ induces neurotoxicity through induction of apoptosis and inflammation, disruption of calcium homeostasis, oxidative stress, and activation of complement [39]. It was indicated that oligomeric Aβ is more toxic than other forms, reducing spine density and suppressing long-term

potentiation [40]. Tau is crucial in the toxicity caused by Aβ. Reducing the levels in AD model animals consistently resulted in significant improvements in spatial learning performance, as well as reduced premature mortality, hypoactivity, and excite toxicity [41]. Besides that, tau is linked to mitochondrial dysfunction in Alzheimer's disease [42]. As a result, the primary strategy in AD therapy is thought to be the prevention or reduction of toxic Aβ and tau species production.

The amelioration in all investigated antioxidant, oxidative stress markers, brain neurotransmitters, tau protein and β myeloid may be explained on the basis of all citrus oils exhibited antioxidant activity as DPPH free radical scavenger and reducing power in dose dependent manner. Mandarin oil showed the strongest activity compared to clementine and wilking essential oils. The oils may be recommended as safe plant based antimicrobials as well as antioxidants for enhancement of shelf life of food commodities. Phenolic and terpenoid compounds present in the chemical composition of EOs are closely associated with their antioxidant function, mainly due to their redox properties exerted by various possible mechanisms: free radical-scavenging activity and hydrogen Donors, etc [43] . EOs have drawn attention from scientists, practitioners, and therapists for their biological activities such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anticancer, and antinociceptive properties [44]. Human and animal studies have shown that several EOs produce diverse pharmacological responses in the nervous system resulting in anxiolytic, analgesic, antidepressant, anticonvulsant and sedative effects. Therefore, it has been suggested that EOs could be effective for mitigating the symptoms of various mental illnesses including depression, anxiety and dementia. Numerous studies suggest that only some of the major compounds of the EOs contribute significantly to their anxiolytic and antidepressant effects including linalool, limonene, and pinene Hence, EOs with high content of these compounds are expected to have anxiolytic and antidepressant properties [45] .

Variety of EOs has been shown to have free radical scavenging and antioxidant properties that confer neuroprotective effects which can improve cognitive function and reduce brain damage. Cognitive function in this context, refers to various mental abilities including memory, reasoning, planning, decision-making, attention span, speech, language, and judgment [46]. The deterioration of cholinergic neurons has been shown to lead to cognitive deficits, especially in the case of degenerative diseases. Varying EOs have shown anticholinesterase activity, which might be advantageous for the development of drugs for AD treatment since cholinesterase's have been recognized as one of its potential targets [47] .

EOs can affect hypothalamic–pituitary–adrenal (HPA) axis by decreasing glucocorticoid levels producing a calming effect. On the other hand, the pro inflammatory response may be suppressed by down regulating NF-kB as in the case of cinnamon EO, resulting in an anxiolytic effect [48]. Moreover, calming effects are also produced by increasing serotonin levels while decreasing glucocorticoids like in the case of ylang-ylang EO [47] .Furthermore, it has been demonstrated experimentally that other EOs like rosemary [49], stimulate the adrenergic system resulting in psycho stimulant and cognitive-enhancer effects. Additionally, bergamot [50], lemongrass [51] and lavender [52] EOs can exert its anxiolytic effects by activating the GABA adrenergic system.

In vivo study revealed that limonene significantly increased -aminobutyric acid (GABA) and other neurotransmitter changes [1].GABA is acting though GABAA receptor, which is potentiating chloride current resembling alcohol, barbiturates, and benzodiazepines. Moreover, other neurotransmitters were reported to change after limonene administration such as dopamine (DA), serotonin (5-HT), and glutamate (Glu). These neurotransmitters play an important role related with the waking system of the brain so the direct effect of the oil to neurotransmitterfunction may be the underlying mechanism related with its sedative and vigilance effects [1] .

5. Conclusion

Significant improvement in all detected parameters as result of treatment of AD rats with Clementine oil due to the high antioxidant activity. EOs has shown anticholinesterase activity, which might be advantageous for the development of drugs for AD treatment since cholinesterase have been recognized as one of its potential targets.

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Conflicts of interest

"There are no conflicts to declare".

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