



Protective effect of kumquat fruits and carrot seeds extracts against brain aging in rats

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ABSTRACT

Introduction: Protection of brain against accelerated aging helps avoiding the occurrence of neurodegenerative diseases. So, the current work was conducted to evaluate the rescuing role of kumquat fruits crude ethanol extract, carrot seeds ethanol and petroleum ether extracts against the brain aging induced by D-galactose in rats.

Methods: Forty male Sprague Dawley rats were divided equally into five groups. Group I was served as normal control, rats of group II were daily injected intraperitoneally (i.p.) with 150 mg/kg BW of D-galactose. Rats of group III, IV and V were daily injected i.p. with the same dose of D-galactose and administered orally with 250 mg/kg BW/day of kumquat fruits crude ethanol extract, carrot seeds ethanol extract and carrot seeds petroleum ether extract, respectively. After 6 weeks the rats were scarified, brain tissues were analyzed for malondialdehyde (MDA), catalase (CAT) as well as histological examination. Also, the plasma was analyzed for MDA, tumor necrosis factor- α (TNF- α), creatinine and urea levels, as well as CAT, butyrylcholinesterase (BChE), aspartate transaminase (AST) and alanine transaminase (ALT) activities.

Results: From the results, it was elucidated that the tested extracts suppressed both the reduction in CAT and the elevation in MDA either in brain or plasma and the increase in plasma TNF- α , BChE as well as liver and kidney parameters.

Conclusion: The tested extracts can be served as potent protective agents against the accelerated aging parameters which may be due to anti-oxidant and anti-inflammatory activities.

Implication for health policy/practice/research/medical education:

Kumquat fruits and carrot seeds crude ethanol extracts can be served as potent protective agents against accelerated aging.

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Introduction

Aging is a complex natural phenomenon not only associated with the age but also with the environmental factors which contribute to free radicals production (1). Brain aging may be one of the causes of several neurodegenerative diseases, such as Parkinson's disease, mild cognitive impairment as well as Alzheimer's disease (2). Brain aging is related to mitochondrial dysfunction which is combined with increased free radical production especially that the brain is more prone to oxidative stress and senescence due to the high content of lipid and high oxygen consumption (3). In this context, the use of natural antioxidants may be effective in the protection of

brain against the accelerated aging. D-galactose (D-gal) can mimic aging in animal models by inducing oxidative stress and inflammation (4). D-gal accumulation elevates oxidative stress due to elevation of reactive oxygen species (ROS). Elevation of ROS induces glycation of amino peptides and proteins. Also, this accumulation leads to downstream activation of nuclear factor kappa B (NF- κ B), followed by release of ROS and pro-inflammatory mediators (4).

Kumquat (In Chinese means golden orange) is one of the smallest citrus fruits (Figure 1), which is characterized by the acidic taste of flesh and soft edible peel, where the fruit can be eaten along with the peel either in the form

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of row fruit or as juice. It can also be used as pickles and marmalades (5). Beside the nutrients, in kumquat there are several phytochemicals in fruits including carotenoids, essential oils, ascorbic acid as well as flavonoids (6). Such phytochemicals, with various beneficial biological effects, provide kumquat a great importance as a folk medicine (7). Allam et al (8) demonstrated the hypocholesterolemic effect of kumquat. According to Dosoky and Setzer (9) kumquat exhibited antifungal, antibacterial as well as antioxidant activities. Also Nouri and Shafaghatlonbar (10) declared that the essential oil of kumquat was considered a potent antioxidant. Carrot seeds (Figure 1) produced when flowering process is induced; and the plant turns from the vegetative phase to the reproductive phase thus producing a rod or floral tassel at the end of it an umbels which carry the seeds (11). Although the carrot plant is planted primarily with the aim of obtaining roots, but the seeds are used as a flavoring agent in many food products and cosmetics in addition to its utilization as medicinal purposes. Carrot seeds oil not only has antioxidant effect but also possesses counteractive role for several disease such as cancer, diabetes and inflammation (12). Ethanol extract of carrot seeds possessed hepatoprotective impact against thioacetamide (13). Also, Shebaby et al (14) proved that oil fractions of carrot had hepatoprotective effect against carbon tetra chloride. Carotol, one of the compounds found in the carrot seeds oil attributed to its antifungal effect (15) and pleasurable aroma and taste (12). Kumarasamy et al (16) suggested that luteolin was responsible for the antibacterial activity of methanol extract of carrot seeds. The current study was designed to estimate the rescuing role of kumquat fruits crude ethanol extract, carrot seeds ethanol and petroleum ether extracts against the brain aging induced by D-galactose in rats.

Materials and Methods

Materials

Plant samples

Carrot seeds (Figure 1) (*Daucus carota* L., Family Apiaceae) and kumquats fruit (Figure 1) (*Citrus japonica*, Family Rutaceae) were purchased from local markets, Cairo, Egypt.

Animals

Forty male Sprague Dawley rats, 133-178 g were used. Animals were kept individually in stainless steel cages at

room temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity of about 55%; water and food were given *ad libitum*.

Diets

A balanced diet composed of 10% protein supplemented from casein, 10% corn oil, 23.5% sucrose, 47% maize starch, 5% fiber, 3.5% salt mixture (17) and 1% vitamin mixture (18) was prepared for feeding animals all over the experimental period.

Materials and Methods

Preparation of crude ethanol extract of kumquat fruits.

Kumquat fruits were washed by tap water and cutting to small slices, then dried using Solar furnaces. The dried kumquat fruits were powdered and extracted successively in a continuous extraction apparatus (soxhlet) until exhaustion with ethanol for preparation of crude ethanol extract. The solvent was completely removed by evaporation under reduced pressure at a temperature not exceeding 40°C . Crude extract of kumquats fruits was kept in deep-freeze till used.

Preparation of carrot seeds oil

The oil was extracted from crushed seeds with petroleum ether ($40\text{-}60^\circ\text{C}$) in a Soxhlet apparatus. The extract was evaporated in vacuum. The lipid extract was collected in a flask. The extracted lipid was weighed to determine the oil content and stored under nitrogen at 4°C for further analysis.

Preparation of ethanol extract of the carrot seeds

The defatted meal of carrot seeds was extracted with ethanol in a Soxhlet apparatus. The solvent was completely removed by evaporation under reduced pressure at a temperature not exceeding 40°C . The ethanol extract was stored in deep-freeze till used.

Total phenolic content

The Folin-Ciocalteu assay, adapted from Singleton and Rossi (19), was used for the determination of total phenolics present in the crude ethanol extract of kumquat fruits and ethanol extract of carrot seeds. Total phenolics were calculated with respect to gallic acid standard curve (concentration range: $0\text{-}12 \mu\text{g mL}^{-1}$). Results are expressed in mg of gallic acid g^{-1} of extract.



Figure 1. Kumquats fruit and carrot seeds.

Total flavonoid content

Total flavonoids in the crude ethanol extract of kumquat fruits and ethanol extract of carrot seeds were measured using a colorimetric assay adapted from Zhishen et al (20). Total flavonoids were calculated with respect to quercetin standard curve (concentration range: 50–200 $\mu\text{g mL}^{-1}$). Results are expressed in mg of quercetin g^{-1} extract.

Assessment of fatty acids of carrot seeds oil

Fatty acid methyl esters of carrot seeds oil were prepared according to AOAC (21) to be subjected to GLC analysis of fatty acids. Assessment of the methyl ester was carried out by injecting 2 μL into a Hewlett Packard HP-system 6890 gas chromatograph equipped with FID. HP-5 capillary column (30 m \times 0.32 mm i.d.; 0.25 μm film thickness) was used to separate the different methyl esters. The chromatographic analysis conditions were: initial temperature 70°C with a hold for 1 minute, then rose to 120°C at a rate of 40°C/min with 2 minutes hold then the temperature was finally raised to 220°C at a rate of 4°C/min with another 20 minutes hold. The injector and detector temperatures were 250°C and 280°C respectively. Identification of the fatty acid methyl esters were carried out by direct comparison of retention times of each of the separated compounds with standards of the fatty acid methyl esters analyzed under the same conditions. Quantization was based on peak area integration.

Design of the animal experiment

After one week of adaptation the animals were divided into five groups ($n = 8$, each). Group I was served as control group (normal group). Rats of group II were daily injected i.p. with 150 mg/kg BW of D-galactose (D-gal group). Rats of group III were daily injected i.p. with the same dose of D-gal and administered orally with 250 mg/kg/d of kumquat crude ethanol extract (kumquat ethanol extract group). Rats of group IV were daily injected i.p. with the same dose of D-gal and administered orally with 250 mg/kg/d of carrot seeds ethanol extract (carrot seeds ethanol extract group) while the animals of group V were daily injected i.p. with the same dose of D-gal and administered orally with 250 mg/kg/d of carrot seeds petroleum ether extract (carrot seeds petroleum ether extract group). The experiment lasted 6 weeks during which all rats were fed on balanced diet also body weight and food intake were recorded weekly. At the end of the study total food intake, body weight gain and feed efficiency ratio were calculated. At the end of the study, rats were anesthetized using ether and the blood was withdrawn from all rats after an overnight fast. Rats were dissected and brain was immediately separated from each rat and weighed then part of each brain was immersed in 10% formaldehyde solution for histological examination while the other part was immediately analyzed for malondialdehyde (MDA)

as indicator of lipid peroxidation according to Satoh (22) and catalase (CAT) according to Aebi (23). Also, plasma MDA and CAT were determined according to the same mentioned methods. Tumor necrosis factor- α (TNF- α) was assayed as inflammatory biomarker using Eliza technique. Plasma butyrylcholinesterase (BChE) activity was estimated according to the method of Vaisi-Raygani et al (24). In addition, plasma was analyzed for the activities of aspartate transaminase (AST) and alanine transaminase (ALT) according to Reitman and Frankel (25) as well as creatinine and urea according to the methods of Houot (26) and Fawcett and Scott (27), respectively.

Statistical analysis

Statistical analyses were done using SPSS version 22. The results were expressed as mean \pm standard error (SE) and analyzed statistically using one-way analysis of variance (ANOVA) followed by Duncan test. The statistical significance of difference was taken as $P \leq 0.05$.

Results

As shown in Table 1, carrot seeds ethanol extract recorded the highest contents of both total phenolic and total flavonoids (36.4 mg GAE/g extract and 10.6 mg/g extract, respectively).

Table 2 shows the fatty acid profile of carrot seeds oil. Palmitic acid (4.76%) was the major saturated fatty acid presence in the carrot seeds oil. Oleic acid (76.75%) was the major unsaturated fatty acid presence in carrot oil.

Table 1. Total phenolic (mg GAE/g extract) and total flavonoid (mg/g extract) contents of kumquat and carrot seeds ethanol extracts

Extract	Total phenolic content (mg GAE/g extract)	Total flavonoids (mg/g extract)
Kumquat crude ethanol extract	30 \pm 1.283	8.1 \pm 0.439
Carrot seeds ethanol extract	36.4 \pm 1.436	10.6 \pm 0.572

Table 2. Fatty acids contents of carrot seeds petroleum ether extract (oil) (as percentage of total fatty acids)

Fatty acids (%)	Carrot seeds oil
Caprylic acid (C8:0)	1.02
Capric acid (C10:0)	0.263
Lauric acid (C12:0)	1.66
Myristic acid (C14:0)	0.244
Palmitic acid (C16:0)	4.76
Oleic acid (C18:1) ω -9	76.75
Linoleic acid (C18:2) ω -6	7.13
Linolenic acid (C18:2) ω -3	0.185
Total saturated fatty acids	7.947
Total unsaturated fatty acids	84.063

Total saturated fatty acids were present by 7.947% in carrot oil, while total unsaturated fatty acids were present by 84.063%. The present results revealed that carrot oil is rich in mono-unsaturated fatty acids (MUSFs), especially oleic acid (ω -9).

The biochemical parameters of brain tissue and plasma of the studied groups are summarized in Table 3. It was evident that the injection with D-gal mediated oxidative stress which observed via the elevation of MDA and the reduction of CAT either in brain tissue or in the plasma. On the other side, the oral administration of kumquat fruits crude ethanol extract, carrot seeds ethanol extract or carrot seeds petroleum ether extract suppressed the elevation of MDA and the reduction of CAT either in brain tissue or in the plasma and thus attenuated the oxidative stress. Carrot seeds ethanol extract was the most promising in this concern. Rats which injected with D-gal and had no treatment exhibited the highest levels of BChE and TNF- α . The rats which were orally administrated with the studied extracts suppressed the raising of BChE and TNF- α . Also carrot seeds ethanol extract was the most promising in this concern. The liver functions (indicated by AST and ALT levels) as well as the kidney functions

(indicated by urea and creatinine levels) significantly increased in the rats of D-gal group. On the other hand, the studied extracts combated the elevations of both liver and kidney functions.

From the data tabulated in Table 4, it was evident that a significant reduction in the final body weight was caused by the injection of D-gal. On this basis, rats which were injected D-gal only, recorded the lowest body weight gain. This group also recorded the lowest total food intake, while the administration of kumquat crude ethanol extract, carrot seeds ethanol extract or carrot seeds petroleum ether extract improved the body weight gain.

Histopathological examination of brain tissue.

The histopathological examination of brain tissue of the rats of normal control group showed normal brain tissue, normal nerve cells and nerve fibers (H&E X400) (score lesions: 0) (Figure 2A). Brain tissue of D-gal control group showed vacuolated nerve cells (arrows) and demyelinated nerve fibers (H&E X400) (score lesions: +++) (Figure 2B). Brain tissue of D-gal control group showed diffuse gliosis; proliferated and aggregated glia cells (arrow in Figure 2C; score lesions: +++) (H&E X400). As it can

Table 3. Biochemical parameters of different experimental groups

	Normal control	D-Galactose control	kumquat crude ethanol extract	Carrot seeds ethanol extract	Carrot seeds petroleum ether extract
Brain tissue					
CAT (U/g tissue)	657.25 ^d ± 8.53	409.50 ^a ± 7.37	624.84 ^c ± 4.10	657.50 ^d ± 6.05	554.17 ^b ± 7.94
MDA (nmol/g tissue)	11.27 ^a ± 0.36	23.30 ^d ± 0.32	14.01 ^b ± 0.41	13.30 ^b ± 0.44	15.36 ^c ± 0.43
Plasma					
CAT (U/L)	462.99 ^d ± 3.96	328.14 ^a ± 9.34	387.78 ^b ± 6.86	450.09 ^{cd} ± 6.07	436.83 ^c ± 8.35
MDA (nmo/mL)	5.10 ^a ± 0.36	9.57 ^b ± 0.30	5.65 ^a ± 0.42	5.26 ^a ± 0.52	6.22 ^a ± 0.39
TNF- α (pg/mL)	14.29 ^a ± 0.30	29.87 ^d ± 0.83	25.50 ^c ± 0.69	19.37 ^b ± 0.57	24.62 ^c ± 0.57
BChE (U/L)	315.52 ^a ± 5.58	511.56 ^d ± 10.02	370.91 ^c ± 7.67	325.23 ^{ab} ± 9.71	342.11 ^b ± 7.48
AST (IU/L)	19.50 ^a ± 1.08	28.50 ^c ± 1.65	25.75 ^{bc} ± 0.98	23.50 ^b ± 1.13	23.87 ^b ± 1.57
ALT (IU/L)	10.37 ^a ± 0.70	23.00 ^c ± 1.35	14.62 ^b ± 1.16	10.62 ^a ± 0.90	16.37 ^b ± 1.24
Creatinine (mg/dL)	0.77 ^a ± 0.02	1.21 ^d ± 0.03	1.06 ^c ± 0.04	0.93 ^b ± 0.04	0.94 ^b ± 0.03
Urea (mg/dL)	30.35 ^a ± 1.06	40.65 ^c ± 1.63	35.22 ^b ± 1.65	29.35 ^a ± 0.88	28.02 ^a ± 1.03

Data are expressed as mean ± SE.

Values with different superscript letters in the same row are significantly different at $P < 0.05$ levels.

Table 4. Nutritional parameters of different experimental groups

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Total food intake (g)	Feed efficiency ratio	Relative brain weight
Normal	155.50 ^a ± 3.70	262.62 ^c ± 2.92	107.12 ^d ± 3.78	539.25 ^c ± 6.26	0.20 ^b ± 0.01	0.61 ^a ± 0.01
D-Galactose	155.12 ^a ± 5.70	235.62 ^a ± 6.45	80.50 ^a ± 2.48	456.50 ^a ± 6.20	0.18 ^a ± 0.01	0.63 ^{ab} ± 0.02
Kumquat crude ethanol extract	155.25 ^a ± 2.87	253.50 ^{bc} ± 2.99	98.25 ^c ± 2.78	536.12 ^c ± 4.45	0.18 ^{ab} ± 0.01	0.61 ^a ± 0.02
Carrot seeds ethanol extract	155.12 ^a ± 3.16	260.50 ^c ± 3.34	105.37 ^{cd} ± 2.61	537.37 ^c ± 3.25	0.20 ^b ± 0.01	0.61 ^a ± 0.01
Carrot seeds petroleum ether extract	155.00 ^a ± 2.14	245.25 ^{ab} ± 2.08	90.25 ^b ± 1.92	485.25 ^b ± 7.07	0.19 ^{ab} ± 0.01	0.67 ^b ± 0.01

Data are expressed as mean ± SE.

Values with different superscript letters in the same row are significantly different at $P < 0.05$ levels.

be seen in Figure 2D & 2E, the brain tissue of rats given kumquat crude ethanol extract showed improved brain tissue with congested blood vessels (arrow), (H&E X400) (score lesions: ++). Brain tissue of rats given carrot seeds ethanol extract (Figure 2F & 2G) showed normal nerve cells and nerve fibers (H&E X400) (score lesions: 0). Brain tissue of the rats given carrot seeds oil (Figure 2H) showed slightly congested blood vessel (arrow) (H&E X400) (score lesions: +). Also, brain tissue of the rats given carrot seeds oil (Figure 2I) showed intraneural edema with vacuolated nerve cells (arrow) (H&E X400) (score lesions: +).

Discussion

Aging is age related process however, it can be accelerated by various factors such as stress, environmental factors and nutritional deficiencies (28). Food and plant bioactive compounds can help slow down aging (29-31). In the present study aging was progressed in rats via the injection of D-gal which demonstrated to mediate the brain aging process in mice and rats in several studies (32-34). Fatemi et al (32) declared that oxidative stress was mediated by D-gal through not only the reduction in the antioxidant enzyme activities but also through catalyzing free radicals production. Thus, the brain aging can be developed by oxidative damage and inflammation. Indeed, the results of the present study pointed to the occurrence of oxidative stress via the injection of D-gal which demonstrated by the reduction in CAT as one of the antioxidant enzymes and elevation in MDA either in brain tissue or in plasma. Also, our results indicated the incidence of inflammation following injection of D-gal which could be observed via the elevation in TNF- α . It was confirmed via the results that the oral administration of kumquat fruits crude ethanol extract, carrot seeds ethanol extract or carrot seeds petroleum ether extract (at dose 250 mg/kg BW)

suppressed the reduction in CAT and elevation in MDA either in brain tissue or in plasma. Also, administration of the mentioned items reversed the raising of TNF- α as well as liver and kidney functions. These effects of the studied extracts may be attributed to their contents of the bioactive components. Lou et al (35) reported that poncirin, phloretin, rhoifolin, acacetin and apigenin-8-C-neohesperidoside among the flavonoids in kumquat to which various biological activities such as anti-oxidant and anti-inflammatory can be attributed. Also, the antioxidant and anti-inflammatory activities of kumquat may be contributed to its content of d-limonene (36). The brain anti-aging impact of kumquat may be attributed to its content of polyphenols such as kaempferol, luteolin, hesperidin and quercetin (7). Sarubbo et al (37) stated that polyphenol possess brain anti-aging effect. Also the improvement of the memory, learning, as well as the motor coordination by polyphenols may be originated from their ability to attenuate the oxidative stress and inflammation (via modification of NF- κ B levels and cytokines). Additionally, kumquat according to Hosseini et al (38) is a good source of vitamin C to which the protective effect of kumquat fruits against brain aging may be attributed. Monacelli et al (39) declared that oxidative stress, over production of the inflammatory agents, telomere attrition, as well as chromatin disorganization can be suppressed by vitamin C. Also, the two hallmarks of biological aging (immune-senescence and inflamm-aging) can be modulated by vitamin C.

Mani and Parle (40) concluded that the elevation in acetyl cholinesterase activity and the memory deficits can be reversed by the oral administration of carrot seeds extract. Mani et al (41) suggested the beneficial effect of carrot seeds in Alzheimer's disease. Our results pointed to the superiority of carrot seeds ethanol extracts in the

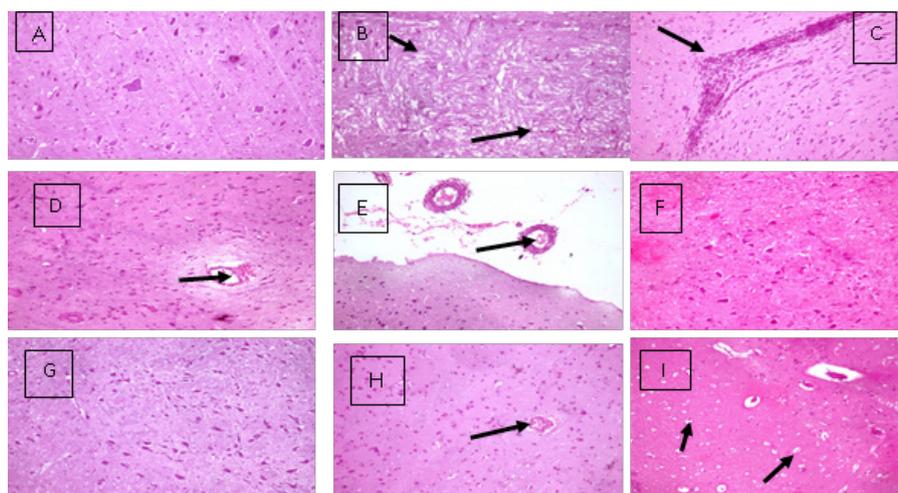


Figure 2. Histopathological examination of brain tissue of the rats in different groups.

(A): Brain tissue of normal control (H&E X400). (B & C): Brain tissue of D-galactose control (H&E X400). (D & E): Brain tissue of rats given kumquat crude ethanol extract. (F & G): Brain tissue of rats given ethanol extract of carrot seeds. (H & I): Brain tissue of rats given carrot seeds oil.

protection against the negative effect of D-gal might be due to the high content of phenolic compounds and flavonoids as found in the present study. Also, Ksouri et al (42) reported that the methanol extract of carrot seeds exhibited high radical-scavenging activity more than α -tocopherol. Rezaei-Maghadam et al (43) confirmed the antioxidant activity of ethanol carrot seeds extract and its ability to suppress the lipid peroxidation in the liver tissue. Flavones in carrot seeds including apigenin, kaempferol, luteolin, luteolin 3-O- β -D-glucopyranoside and luteolin 4-O- β -D-glucopyranoside contribute in the antioxidant activity of carrot seeds (44). Such flavonoids have the ability of free radicals scavenging and metal chelating in addition to the inhibition of the generative enzymes of free radicals including lipoxygenase, nitric oxide synthase, cyclooxygenase, xanthin oxidase as well as monoamine oxidase (45).

The anti-aging effect of carrot seeds petroleum ether extract (oil) may be attributed to the presence of oleic acid as monounsaturated fatty acid. Fatty acids stimulate gene expression and neuronal activity, boost synaptogenesis and neurogenesis, and prevent neuro-inflammation and apoptosis. By doing so, they promote brain development, ameliorate cognitive functions, serve as anti-depressants and anticonvulsants, bestow protection against traumatic insults, and enhance repairing processes (46). It was reported that intake of sufficient quantity of MUFAs prevented the age related deletion of mitochondrial DNA in the brain of aged animals (47). In this regards, palmitic acid (16:0), stearic acid (18:0), upstreamn-3 PUFAs, and AA(20:4n-6) triggered higher secretion of A β peptide compared to long chain downstream n-3 PUFAs and MUFAs (48). It also was reported that oleic acid supplementation in a mouse model of Alzheimer's disease reduced AD-type neuropathology (49).

Conclusion

Oral administration of kumquat fruits crude ethanol extract, carrot seeds ethanol extract or carrot seeds petroleum ether extract could reverse the negative effects of D-galactose in rats including the reduction in CAT and the elevation in MDA either in brain or in plasma and the elevation in plasma TNF- α , BChE as well as the liver and kidney functions. On this basis, it was concluded that the studied extracts have promising protective effect against the accelerated brain aging. Carrot seeds ethanol extract had the most promising effect in this concern. These beneficial effects may be due to the bioactive components with anti-oxidant and anti-inflammatory properties of the tested extracts.

Authors' contributions

DM designed all experimental works; prepared plants extracts, contributed in the analysis of tissue samples and wrote the final manuscript and the final reviewing of the

manuscript. KF participated in all animal interventions (animal experiment and blood analysis), made the statistical analysis of the results, prepared the final tables of the manuscript and contributed in writing the manuscript. IM contributed in designing the biological part and in writing the manuscript. The histological examination was done by SSA. The final paper was read and approved by all authors for publication.

Conflict of interests

The authors declare no conflicts of interest.

Ethical considerations

Ethical issues including plagiarism, misconduct, data fabrication, falsification, double publication or submission have been carefully checked by authors. The animal experiments were carried out according to the Ethics Committee, National Research Centre, Cairo, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

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