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Protective effect of dietary supplements against streptozotocin-induced Alzheimer's disease in mice

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a neurodegenerative problem that is increased progressively due to the increment of aging worldwide. Phytochemicals play an important role in the protection from neurodegeneration. The present study aimed to evaluate the protective effect of two dietary supplements (DS) rich in betalains, anthocyanins, and omega-3 fatty acids against AD.

Methods: Two dietary supplements (DS I and DS II) were prepared; the first one was a mixture of anthocyanin-rich extract of purple carrot and flaxseed oil (DS I), while the second was a mixture of betalains-rich extract of beetroot and flaxseed oil (DS II). The protective effects of both DS were evaluated in an AD model. AD was induced in mice by intracerebroventricular (ICV) injection of streptozotocin (STZ) (3 mg/kg). Biochemical changes in brain tissue and plasma were determined. Behavioural of mice was evaluated through Y–maze test, Morris water maze, and novel object recognition test. Changes in brain tissues were assessed through histopathological examination. *In vitro* antioxidant activities of DS I and DS II were evaluated. Also, the contents of total phenolics, anthocyanins, betalains, and fatty acids profile were assessed.

Results: Both DS investigated in the present study showed significant improvement ($P<0.05$) in acetylcholinesterase, antioxidant enzymes, tumor necrosis factor-α (TNF-α) and malondialdehyde (MDA)in brain tissue and butyrylcholinesterase in plasma in association with amelioration in the behavioural tests and histopathological changes of the brain tissue.

Conclusion: Both DS showed protective effects against STZ induced AD in mice due to the presence of anthocyanins, betalains, and omega-3 fatty acids.

Implication for health policy/practice/research/medical education:
Dietary supplements containing anthocyanins, betalains, and ω-fatty acids from purple carrot, red beetroot, and flaxseed can be served as potent protective agents against AD due to their antioxidant and anti-inflammatory activities.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the deposition of β-amyloid plaque and neurofibrillary tangles, which lead to deterioration in cognitive function and memory impairment (1). The prevalence of AD is growing worldwide due to the increase in the aging world population (2). Inflammation, oxidative stress, and reactive oxygen and nitrogen species play an important role in the progression of AD (3,4). Fruits and vegetables are rich sources of nutrients and contain phytochemicals/bioactive compounds. The presence of phytochemicals in fruits and vegetables is an added value of their benefits. Phytochemicals are recognized for their nutraceutical effects and health benefits (5). There are a lot of vegetables and fruits growing in Egypt containing biologically active compounds such as phenolic compounds, polyphenols, and omega-3 fatty acids, which possess health-promoting activities (6-9). Red beetroot (*Beta vulgaris* L., family Chenopodiaceae) is a vegetable with a worldwide distribution; Egypt produced 11 045 639

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tons of beetroot (10). Beetroot is an important source of health promoting phytochemicals (11). It is one of the richest sources of betalains, which is a water-soluble nitrogenous pigment used for imparting red or yellow color (12). Besides, betalains in beetroot contains ascorbic acid (11,13), carotenoids (14), saponins, polyphenols, flavonoids, and high levels of nitrate (644–1800 mg/kg) (15). Carrot (*Daucus carota* L., family Apiaceae) is the most important root vegetable plant grown worldwide (16, 17). Purple carrot is a rich source of anthocyanin (17,18). The anthocyanins of carrot effectively reduce the risk of different types of chronic diseases such as cancer, diabetes, cardiovascular diseases, and dyslipidemia (19,20). Prophylactic strategies and nutritional interventions are promising approaches to retard neurocognitive dismiss and decrease the hazard of AD (21). Flaxseed (*Linum usitatissimum* L.) is one of the richest sources of ω-3 fatty acids and α-linolenic acid (C 18:3), which possess multiple biological activities such as anti-inflammatory, anti-diabetic, anti-cancer, and cardio-protective effects (22). Consumption of ω-3 fatty acids is associated with reducing the risk of cognitive impairment, especially AD (23). So, the present study aimed to evaluate the protective effects of two dietary supplements (DS) rich in betalains, anthocyanins, and omega-3 fatty acids against AD induced in mice by intracerebroventricular (ICV) injection of streptozotocin (STZ). In-vitro antioxidant activities of both DS were also evaluated, and the total phenolic contents of these DS were assessed. Anthocyanins, betalains, and fatty acids profile of purple carrot extract, beetroot extract, and flaxseed oil was determined, respectively.

Materials and Methods

Materials

Plant materials and chemicals

Purple carrot (*Daucus carota* L.) and red beetroot (*Beta vulgaris* L.) were purchased from local markets, Giza, Egypt. Flaxseed (*Linum usitatissimum* L.) was purchased from Agriculture Research Center, Giza, Egypt. Chemicals and pure reagents were purchased from Sigma Chemical Companies (Sigma-Aldrich, St. Louis, MO, USA).

Animals

Swiss male albino mice 25-30 g of body weight (age: 2-3 months) were used in the study. Mice were obtained from the animal house colony of the National Research Centre (Cairo, Egypt). The mice were housed in a temperature-controlled room (23–34°C), allowing a 12-hour light and dark cycle, food and water were provided *ad libitum*.

Diets

A balanced diet composed of 10% casein, 10% corn oil, 23.5% sucrose, 47% maize starch, 5% cellulose, 3.5% salt mixture, and 1% vitamin mixture was prepared for feeding the animals all over the experimental period.

Methods

Preparation of anthocyanin-rich extract, betalain-rich extract and flaxseed oil

The purple carrot and beetroot were washed, cut into thin slices, and dried using a sun-drying device; then the dried slices were ground into fine powder. The extraction process was carried out using the solid-liquid extraction method. Ethanol/water/citric acid mixture (70:30:0.5 v/v/w) was used in extraction of anthocyanin-rich extract of purple carrot, while ethanol/water/citric acid mixture (50:30:0.5 v/v/w) was used in the betalain-rich extract of beetroot. Purple carrot and beetroot were macerated in the extraction solvent (1/3 w/v) for 1 hour in a shaker (SCILOGEX- SK-0330-Pro). The extract was then centrifuged at 3000 × g for 15 minutes, and the residue was re-extracted by the same solvent till each plant being colorless. The solvent was completely removed from the collected extract by evaporation under reduced pressure at a temperature not exceeding 40°C. Anthocyanin-rich extract of purple carrot and betalain-rich extract of beetroot were kept in deep freeze till used. Flaxseed was crushed and pressed with laboratory type of screw press machine with speed 15 rpm and 35°C Carver hydraulic press under 10.000 Ib/in (pic) pressure for 1 hour at room temperature according to the method of Üstun et al (24). The produced oil was kept in deep freeze until used.

Determination of betalain in red beetroot extract

The concentrations of the betalain pigments, betanins (betacyanins), and betaxanthins were measured spectrophotometrically at wavelengths 538 nm and 480 nm, respectively, using a spectrophotometer following the method of Stintzing et al (25). The absorbance reading was used to calculate the betalain concentration for each sample. The betalain content (BC) was calculated as BC (mg/L) =[(A×DF×MW×1000)/ (e×l)], where A is the absorption, DF the dilution factor, and l the path length (1 cm) of the cuvette. For quantification of betacyanins and betaxanthins, the molecular weights (MW) and molar extinction coefficients (e) (MW=550 g/mol; e=60 000 L/mol cm in H_2O) and (MW=308 g/mol; e=48 000 L/mol cm in H_2O) were applied.

Determination of total anthocyanin in purple carrot extract

Total anthocyanin as cyanidin-3-O-glucoside was measured according to the method of Sims and Gamon (26).

Total anthocyanin as cyanidin-3-O-glucoside (mg/L) = (Abs × M.W. × D.F. × 1000)/ (ε ×1)

Where MW (molecular weight of cyanidin-3-O-glucoside) is 449.2 g/mole, D.F.= dilution factor, 1= path length in cm, ε (molar absorbance coefficient for cyanidin-3-O-glucoside = 26900 molar extinction coefficient in 1L ×

mol/L × cm^{-1}), 1000 factor conversion from g to mg (27).

Determination of fatty acids profile of flaxseed oil

Fatty acid methyl esters of the studied oil was prepared according to methods of Association of Official Analytical Chemists (AOAC) (28) to be subjected to gas-liquid chromatography analysis of fatty acids. Assessment of the methyl ester was carried out by injecting 2 ul into a Hewlett Packard HP-system 6890 gas chromatograph equipped with FID. HP-5 capillary column (30 m × 0.32 mm i.d.; 0.25 um film thickness) was used to separate the different methyl esters. The chromatographic analysis conditions were as follows: 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, and the temperature was finally raised to 220°C at a rate of 4°C/min with another 20 minute 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.

Preparation of dietary supplements

Two DS were prepared. The dietary supplement I (DS I) was a mixture of anthocyanin-rich extract of purple carrot and flaxseed oil in ratio 1:1, while dietary supplement II (DS II) was a mixture of betalain-rich extract of beetroot and flaxseed oil in ratio 1:1.

Preparation of dietary supplements emulsion for mice oral dose

DS I and DS II were prepared in the form of oil-in-water emulsions using Tween 80 as surfactant. All emulsions were stored (4°C) for one week during the experiment.

Determination of total phenolics content in the prepared dietary supplements

Total phenolics were determined colorimetrically in the prepared DS using Folin-Ciocalteu reagent (29). Absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE) in mg/g dietary supplement. The results were expressed as mean ± SD.

Determination of antioxidant activity of the prepared dietary supplements

Antioxidant activity of the prepared DS was assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) method (30) and the reducing power method (31). The percent DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) or percent inhibition= [A0 - A1 / A0] × 100.

Where A0 was the absorbance of control reaction and A1 was the absorbance in presence of test or standard sample. The reducing power of DS was determined according to the method of Oyaizu (31). Various concentrations of DS and BHT as standard (1, 2, 3, 4, and 5 mg/mL) in 1 mL of methyl alcohol were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 minutes, and then 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 minutes at 1000 × g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 minutes, 0.1%), and the absorbance was measured at 700 nm. The assay was carried out in triplicate, and the results were expressed as mean ± standard error (SE). An increase in the absorbance of the sample with concentrations indicates high reducing potential of the samples.

Induction of Alzheimer's disease in mice

Mice were randomly isolated into four groups (ten/group). These groups were defined as follows: Group one was served as normal control given daily oral dose of vehicle for a month. Group two were given daily oral dose of the vehicle (a mixture of water Tween 80, which was used in the preparation of both DS) for a month and served as AD control group. Groups three and four were given oral dose of DS I or DSII daily (100 mg/kg mice body weight). After one week from starting the experiment all mice, except the normal control group, were received ICV infusion of STZ (3 mg/kg ICV) once (32). After three weeks from STZ injection, mice behavioural assessment were assessed, then blood samples were collected from all mice. Plasma samples were separated for determination of butyrylcholinesterase (BTC) (33). Mice brain was removed, rinsed with ice-cold saline, and immediately homogenized using phosphate buffer (pH 7.4). The homogenates were centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant used for the determination of catalase activity (34), malondialdehyde (MDA) (33), acetylcholine esterase (SUNLOG, Cat No. SL002Ra, ELISA kit), glutathione peroxidase (GPx) (SUNLOG, Cat No.SL1033Ra, ELISA kit), superoxide dismutase (SOD) (SUNLOG, Cat No.SL1341Ra, ELISA kit) and tumor necrosis factor-α (TNF-α) (SUNLOG, Cat No.SL0722Ra, ELISA kit).

Behavioural assessment

Y-maze

The Y-maze test estimates short-term memory (36). The Y-maze is a metallic maze consisting of three arms in the form of a Y-like shape. The dimensions of each arm were 40 cm long, 30 cm high, and 15 cm wide, and the arm was fixed at 120° from a central platform. This test depends on the preference of normal rat to explore a new arm rather than the well-known arm. The test was performed on

two consecutive days. Rats were trained on the first day by placing each rat in the central platform to move freely throughout the Y-maze for 8 minutes. On the test day, the succession of the entered arms throughout an 8-minute session was recorded. Between each test interval, the maze was cleaned with 70% ethanol to avoid erroneous observations arising from olfactory cues.

The successive entries into the three arms recorded the actual alternation known as overlapping triplet sets. The total number of entered arms measures possible alternations. The ratio between the actual alternations to the possible alternations multiplied by 100 was used to calculate the percentage of spontaneous alternation behaviour (SAP) (actual alternations/total alternations) × 100 (37). The SAP value was directly proportional to the spatial memory. The percentage of spontaneous alternation was considered a measure of spatial memory (38).

Morris water maze

The Morris water maze test assesses the learning capacity and visuo-spatial memory of animals (39). The apparatus employed in the present study consisted of a large circular pool made of stainless steel (150 cm in diameter and 60 cm in height), which was half-filled with water maintained at room temperature. The pool was divided arbitrarily into four equal quadrants with the help of two threads perpendicular to each other that were affixed to the rim of the pool. A submerged platform (10 cm width, 28 cm in height), painted in black, was placed inside the target quadrant of this pool, 2 cm below the water surface. The platform position was kept constant throughout the test. In order to render the platform invisible, the water was made opaque by adding a purple-colored non-toxic dye. Normal animals usually learn quickly to swim directly toward the platform, thus reaching it in a shorter time. The procedure was performed on five successive days (40). Each mouse was subjected to two consecutive trials on the first four days of the test, with a gap of at least 15 minutes between the trials. The maximum time allowed for each trial was 120 seconds. If the mouse was able to find the hidden platform within the designated 120 seconds, it was allowed to remain there for 20 additional seconds before being removed. However, if the mouse failed to find the hidden platform during the allocated time, it was gently guided onto the platform and allowed to remain there for 20 seconds. The mean escape latency time is defined as the time taken by each mouse to find the hidden platform. It was recorded for each mouse during each of the trials performed over the four testing days and was noted as an index of acquisition or learning (41). On the fifth day, the mice were subjected to a probe-trial session in which the platform was removed from the pool and each mouse was allowed to explore the pool for 60 seconds. The time spent by each mouse in the target quadrant, where the hidden platform was previously located, was considered as an index of retrieval or memory (41).

Object recognition test

The object recognition test is used to test long-term memory and evaluate cognition (42). It is based on the concept of preference for novelty, which is the innate tendency of animals, which exhibit affinity for exploring to an oval object rather than a familiar one (43). The test employed for this purpose consisted of three phases conducted on three consecutive days. In the first phase (habituation phase), the mouse was placed in a wooden box of 30 × 30 × 30 cm dimensions and was left to adapt to the surroundings for 10 minutes (44). The second day was designated for the familiarization or training phase, whereby two wooden objects (to ensure that a non-toxic material was used), identical in shape, size, and color, were placed in opposite corners inside the box, 2 cm from the walls (45). Then, each mouse was presented with the two identical objects and was left to explore them for 10 minutes (44). On the third day, designated for the test phase, one of the two identical objects was removed and replaced with a novel object that was different in shape, color, and size from the objects mice were familiar with. Each mouse was left to explore the two objects for 5 minutes (46). Objects and arena were thoroughly cleaned with 70% ethanol between experiments with individual mice to ensure that their behavior was not guided by odor cues. The recognition time spent on exploring the novel object was recorded (42).

Histopathological examination

For histopathological examination, the brain was collected from all mice groups and fixed in 10% buffered formalin. After 24 hours, tissues were rinsed three times in 70% ethanol, dehydrated using a graded ethanol series and then embedded in paraffin wax. Paraffin sections were cut into 5 μm thick slices and stained with hematoxylin and eosin for light microscope examination. The sections were viewed and photographed using a digital microscope (Olympus BX50, Japan) (47). All tissues were screened for the presence of granulovacuolar degeneration, β-amyloid plaques, and neurofibrillary tangles, which are indicators of AD.

Statistical analysis

The results of animal experiments were expressed as the mean ± SE and analyzed statistically using the one-way analysis of variance (ANOVA) followed by Duncan's test. In all cases, $P < 0.05$ was used as the criterion of statistical significance.

Results

Phytochemicals and antioxidant activity

In the present study, the anthocyanin-rich extract of

purple carrot contained 51.7 ± 1.25 mg anthocyanin as Cy3G/g extract, while the betalains-rich extract of beetroot contained betacyanin and betaxanthin as 7.2 ± 0.287 mg/g and 2.6 ± 0.205 mg/g, respectively. Total phenolic contents in DS I and DS II were present by 42.9 ± 0.805 and 49.1 ± 1.187 mg GAE/g dietary supplement.

The antiradical activity of DS I and DS II was determined using the DPPH radical (Figure 1a). The antiradical activity of both DS increased in association with an increment in the concentration of the dietary supplement, and it was ranged from 42 to 62 for DS I and 45 to 75 for DS II. The IC_{50} of both DS against DPPH was 150 µg/mL. DS II was more effective in scavenging DPPH radical than DS I. Both DS showed the highest antiradical activity at the concentration of 250 µg/mL. The reducing power activity (Figure 1b) of the studied DS showed an increment in accordance with the elevation in the concentration of the dietary supplement used. The DS I was most promising than DS II.

Fatty acids methyl esters

Table 1 represents the fatty acids profile of flaxseed oil. Fatty acids methyl esters of flaxseed oil revealed the presence of a high percentage of unsaturated fatty acids 81.4% and a low percentage of saturated fatty acids 8.4%. α-Linolenic acid (C18:3) was the major unsaturated fatty acid present in the flaxseed oil (50.8%). Linoleic acid and oleic acid were present in the flaxseed oil by 17.8% and 12.8%, respectively. The ratio of omega-3 to omega-6 fatty acids was 2.8 in the flaxseed oil.

Table 1. Fatty acids' profile of flaxseed oil (as percentage of total fatty acids)

Fatty acids	Flaxseed oil
Palmitic C16: 0	6.3
Stearic C18: 0	2.1
Oleic, C18: 1	12.8
Linoleic, C18: 2	17.8
α-Linolenic, C18: 3	50.8
Total identified saturated fatty acids	8.4
Total identified unsaturated fatty acids	81.4
Ratio of omega-3/omega-6	2.8

Behavioural studies

AD was induced by ICV injection of STZ, which led to cognitive decline as appeared in the water maze test, Y maze test, and novel object recognition.

The Y–maze test

Y-maze test evaluates short-term memory. ICV injection of STZ led to a significant decrease in the percentage alternation noted in the AD group by 9.1% from those observed in the normal group (Figure 2). In addition, treatment with both DS resulted in a significant elevation in percentage alternation when compared to the AD group. However, the change was greater than that observed in the normal group in DSII group.

Morris water maze

The Morris water maze is used to explore the spatial reference learning and memory of mice. An average of the two trials held on each day for each group was taken and recorded (Figure 3). On the first day, mice in all groups

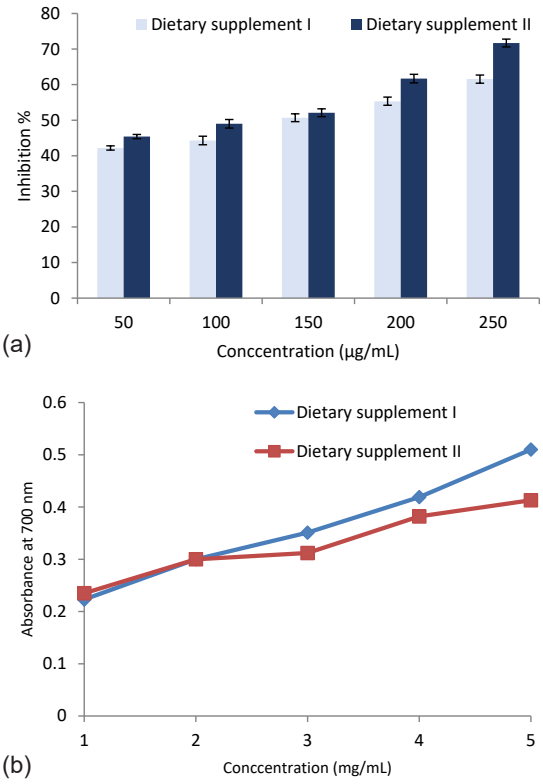

Figure 1. Free radical scavenging (a) and reducing power activities (b) of the studied dietary supplements.

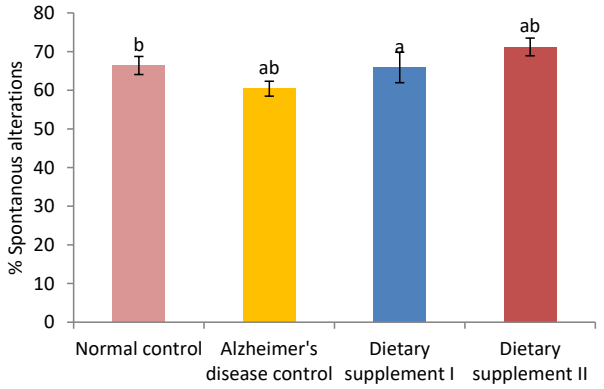

Figure 2. Y-maze test of the different experimental groups. Similar letters mean non-significant difference within groups at $P<0.05$.

took the full 60 seconds designated for the test to reach the platform. On the third and fourth days, the normal group and mice given oral administration by DS I or II started to show significant improvement, as compared to the AD group.

Effect of treatment with STZ and dietary supplements on the time spent in the target quadrant during the probe test

The mean time spent in the target quadrant for mice treated with DS I and DS II increased by 4 seconds compared to that recorded for the AD group. However, it was still significantly shorter (by 1 second) than that pertaining to the normal group (Figure 3).

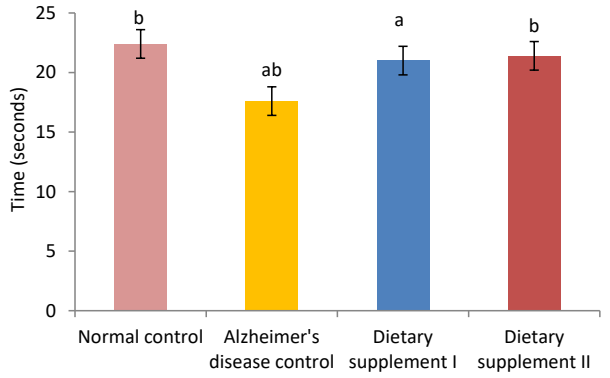

Figure 3. Morris water maze of the different experimental groups. Similar letters mean non-significant difference within groups at $P < 0.05$.

Novel object recognition

Novel object recognition explores non-spatial memory (long-term memory and cognition). The time the AD group (as well as DS I and DS II groups) spent exploring the new object was significantly lower by 21.2%, 18.9%, and 10.6%, respectively, from the time spent by the normal group exploring the novel object (Figure 4).

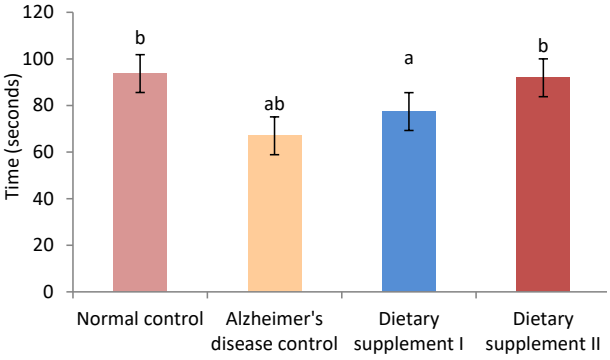

Figure 4. Time spent with new object recognition of different experimental groups. Similar letters mean non-significant difference within groups at $P < 0.05$.

Biochemical changes of mice

Plasma butyrylcholinesterase activity was increased significantly in AD control group compared with the normal group. Oral administration of mice by DS I or II significantly reduced the elevation in the BTC activity (Table 2). Acetylcholinesterase activity (AChE) increased significantly in AD control group compared with all mice groups indicating the neurodegeneration of the brain tissues. The reduction in AChE observed in mice groups given an oral dose of DS I or II was significant indicating their potencies as acetylcholinesterase inhibitors (Table 2). Brain MDA, as lipid peroxidation indicator, was elevated significantly in AD mice group compared with all groups (Table 2). This elevation was reduced significantly in mice administered by DS I or DS II. Brain antioxidant enzymes (SOD, GPx, and catalase) showed a significant reduction in AD group compared with the normal group (Table 2). DS I or II elevated antioxidant enzymes in brain tissue significantly, and DS II was promising in this concern. As appeared in table 2, TNF-α as an inflammatory marker showed significant elevation in the brain tissue of AD group compared with the normal group. Inflammation was reduced in accordance with administration of mice with DS I or DS II as observed by a significant reduction in TNF-α in brain tissue.

Table 2. Biochemical changes in plasma and brain tissue of the different experimental groups

Parameters	Groups			
	Normal	Alzheimer's disease	Dietary supplement I	Dietary supplement II
Plasma				
Butyrylcholinesterase (U/L)	$125.76^{a}\pm2.20$	$225.30^{c}\pm5.70$	$153.92^{b}\pm5.52$	$128.17^{a}\pm5.45$
Brain tissue				
Catalase (U/g)	$0.63^{b}\pm0.01$	$0.55^{a}\pm0.03$	$0.57^{ab}\pm0.02$	$0.59^{ab}\pm0.01$
AChE (ng/g)	$0.61^{a}\pm0.03$	$0.99^{b}\pm0.06$	$0.74^{a}\pm0.05$	$0.66^{a}\pm0.03$
SOD (U/mg)	$48.86^{c}\pm1.278$	$35.43^{a}\pm1.064$	$45.14^{b}\pm0.737$	$46.43^{bc}\pm1.409$
GPx (U/mg)	$50.00^{c}\pm1.07$	$36.57^{a}\pm1.36$	$44.86^{b}\pm1.89$	$47.14^{bc}\pm1.22$
MDA (nmol/g tissue)	$8.40^{a}\pm0.50$	$14.57^{c}\pm1.10$	$10.63^{b}\pm0.53$	$10.43^{ab}\pm0.50$
TNF-α (ng/g tissue)	$20.43^{a}\pm1.11$	$31.71^{c}\pm0.99$	$25.00^{b}\pm1.17$	$23.57^{ab}\pm1.06$

Abbreviations: SOD, Superoxide dismutase; GPx, Glutathione peroxidase; MDA, Malondialdehyde; TNF, Tumor necrosis factor; AChE, Acetylcholinesterase.

Histopathological results

Brain tissue from the normal control group showed normal white matter (all nerve fibers and blood vessels) (Figure 5a). Also, it showed normal grey matter consisting of outer molecular layer (granule cells axons, Purkinje cells dendrites, stellate cells, and basket cells), middle Purkinje layer having Purkinje cells bodies and inner granular layer having cell bodies, Golgi cells and Purkinje cell axons (Figure 5b). Granulovacuolar degeneration, β-amyloid plaques, and neurofibrillary tangles were clearly visible by microscopy in the brain of mice afflicted by AD, especially in the hippocampus. Plaques were dense, mostly with insoluble deposits of β-amyloid peptide and cellular material outside and around neurons (Figure 5c). Tangles (neurofibrillary tangles) were aggregates of the microtubule-associated protein tau, which became hyperphosphorylated and accumulated inside the cells themselves (Figure 5d). DS I treated group revealed AD showed mid-stage intervention with the absence of neurofibrillary tangles (Figure 5e), while DS II treated group showed AD early-stage interventions characterized by an absence of β-amyloid plaques and neurofibrillary tangles (Figure 5f). So, DS II was superior to DS I in the prevention of changes in brain tissues due to STZ as a model for induction AD in mice.

Discussion

In the current research, the protective effects of two DS were studied in STZ-induced AD in mice. In the present study, STZ was used as an animal model for induction of AD in mice by ICV injection. Injection of STZ leads to deterioration of encephalic glucose and energy metabolism, increased AChE activity, hyperphosphorylation of tau proteins, deposition of amyloid-β plaque, oxidative stress, and inflammation (48). All these changes due to STZ injection lead to cognitive deficits (49). AD is the most common cause of dementia worldwide, characterized by the deposition of β-amyloid plaque and neurofibrillary tangles (1). In the current research, the induction of AD by STZ led to cognitive impairment as shown from changes observed in the behavior assessment (Y-maze, Morris water maze, and novel object recognition) compared with the normal control. Also, STZ led to the deposition of β-amyloid plaque and neurofibrillary tangles, especially in the hippocampus, as observed from the histopathological examination of brain tissue. The present results are in accordance with the results of many previous studies (48-50). All these changes in the behavior and histopathological assessments in mice are associated with biochemical changes as observed by elevation of AChE activity, oxidative stress (elevation of lipid peroxidation and reduction in antioxidant enzymes), and inflammation (elevation of TNF-α) in brain tissues. It was reported previously that reactive oxygen species play an important role in the age-related neurodegeneration process and cognitive decline (51). Inflammation enhances neuron damage, which leads to cognitive impairments (52). Pretreatment of mice with DS I or DS II showed improvement in all the studied behavior tests, which is an indicator for the reduction of cognitive memory impairment in association with improvement in the histopathology of brain tissues. Improvement in memory retardation and histopathological examination was associated with enhancement in all biochemical parameters in brain tissues. Reduction of oxidative stress in brain tissue observed in the present study, due to administration of DS I or DS II, may be attributed to their antioxidant activities as shown in the present results. The studied DS showed antiradical activity against DPPH radical and reducing power activity. This antioxidant activity noticed in the studied DS may be attributed to phytochemical compounds (betalanins, anthocyanins and phenolic compounds) and omega-3 fatty acids present in the prepared DS. The DS I contained anthocyanin-rich extract of purple carrot; anthocyanins have antioxidant activities (53,54). The DS II was rich in betalanins, which

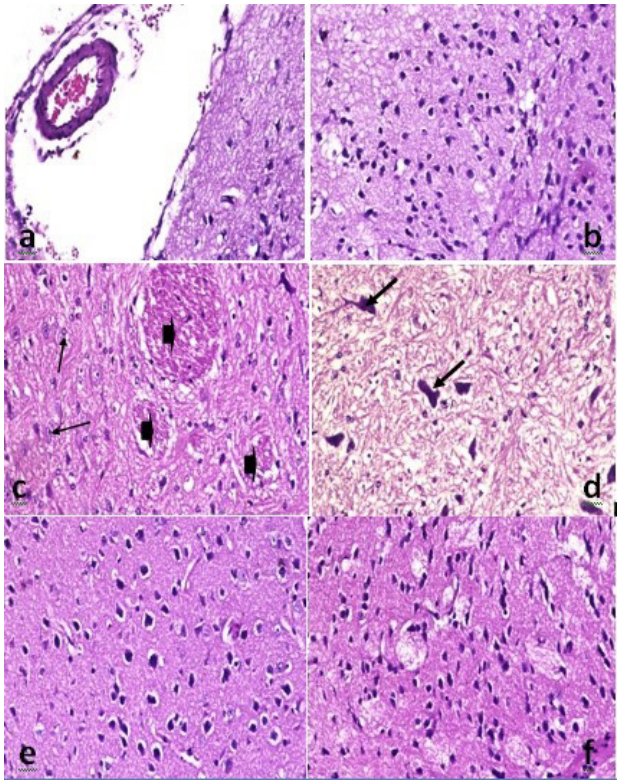

Figure 5. Micrographs of mice brain from different groups (H&E ×400). (a) Normal control group showed normal white matter. (b) Normal control group showed normal grey matter. (c) Alzheimer disease control group showed amyloid plaques (arrows head) and granulovacuolar degeneration (arrows). (d) Alzheimer's disease control group showed neurofibrillary tangles (arrows). (e) Dietary supplement I group showed mid-stage intervention; note the absence of neurofibrillary tangles. (f) Dietary supplement II group showed early-stage and mid-stage interventions; note the absence of amyloid plaques and neurofibrillary tangles.

proved previously antioxidant activity against DPPH radical and also active in preventing lipid peroxidation (55). Also, both DS showed a reduction of TNF-α as an inflammatory marker in brain tissue. This reduction in inflammation may be attributed to the presence of omega-3 fatty acids and phytochemicals in the studied DS from flaxseed oil. Alpha-linolenic acid, an omega-3 fatty acid, was present in DS I and DS II by 50.8%. Omega-3 fatty acids possess anti-inflammatory effect (56,57). Improved oxidative stress and inflammation in brain tissues of mice pretreated with DS I or DS II before STZ injection was associated with improved histopathology of brain tissues through a reduction in β-amyloid deposition and neurofibrillary tangles. Omega-3 fatty acids are beneficial to improve cognitive function in very mild AD and major depressive disorder (57). Phenolic compounds, polyphenols, and flavonoids from fruits and vegetables modulate tau hyperphosphorylation and β-amyloid aggregation in animal models of AD (58). It was reported previously that anthocyanins possessed a neurodegenerative protective effect (54). Beetroot extract could prevent cognitive dysfunction and enhance memory function (59) due to the presence of betalains and dietary nitrate (60).

Conclusion

In the present study, both DS showed protective effects against STZ induced AD in mice. DS II was superior in this concern. The protective effects of both DS against STZ memory impairment may be attributed to the presence of anthocyanins, betalains, and omega-3 fatty acids and their antioxidant and anti-inflammatory activities.

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Authors' contributions

DM designed all the experimental works, prepared plants extracts and analyzed all the phytochemicals, fatty acids profile, antioxidant activity, wrote the final manuscript, and reviewed the final version of the manuscript. ME studied the behavioral of the mice and contributed in writing of the manuscript. The histological examination was done by SSA. RM did all animal interventions (animal experiment and blood and tissue analysis), made the statistical analysis of the results, final tables of the manuscript and contributed in writing the manuscript. The paper has been 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. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and approved by the Ethics Committee at Cairo University. All efforts were made to minimize animal suffering and discomfort. This study has been carried out as a part of internal project No. 12050203 in the National Research Centre, Cairo, Egypt. This project was approved by the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt with approval number 19176.

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