Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d2fo00645f

Published on 30 May 2022. Downloaded on 7/3/2022 7:51:10 AM.

Impact of different processing methods on the phenolics and neuroprotective activity of *Fragaria ananassa* Duch. extracts in a D-galactose and aluminum chloride-induced rat model of aging

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Received 4th March 2022,
Accepted 28th May 2022

DOI: 10.1039/d2fo00645f

rsc.li/food-function

Age-related diseases, including dementia, are a major health concern affecting daily human life. Strawberry (*Fragaria ananassa* Duch.) is the most eaten fruit worldwide due to its exceptional aroma and flavor. However, it's rapid softening and decay limit its shelf-life. Freezing and boiling represent the well-known conservation methods to extend its shelf-life. Therefore, we aimed to discover the phytochemical content differences of fresh and processed strawberries associated with investigating and comparing their neuroprotective effects in a rat model of aging. Female Wistar rats were orally pretreated with fresh, boiled, and frozen *F. ananassa* methanolic extracts (250 mg kg^{-1}) for 2 weeks, and then these extracts were concomitantly exposed to D-galactose [65 mg kg^{-1}, subcutaneously (S/C)] and $AlCl_3$ (200 mg kg^{-1}, orally) for 6 weeks to develop aging-like symptoms. The results of UPLC/ESI-MS phytochemical profiling revealed 36 secondary metabolites, including phenolics, flavonoids, and their glycoside derivatives. Compared with boiled and frozen extracts, the fresh extract ameliorated the behavioral deficits including anxiety and cognitive dysfunction, upregulated brain HO-1 and Nrf2 levels, and markedly reduced caspase-3 and PPAR-γ levels. Moreover, LDH and miRNA-9, 124 and 132 protein expressions were reduced. The histological architecture of the brain hippocampus was restored and glial fibrillary acidic protein (GFAP) immunoexpression was downregulated. In conclusion, the fresh extract has neuroprotective activity that could have a promising role in ameliorating age-related neurodegeneration.

1. Introduction

Age-related diseases, including dementia, are a major health concern affecting daily human life.[1] Alzheimer's disease (AD) is the most common reason for dementia in the elderly over the age of 65. It is characterized by many symptoms beginning from forgetting recent events and conversations and ending with severe cognitive deficits and mental disabilities.[2] AD has very complex molecular mechanisms. The key event is the development of several amyloid plaques which cluster on blood vessels and the outer surface of brain neurons and the intracellular aggregation of microtubule-associated Tau proteins as neurofibrillary tangles. Tau proteins are a class of active structural proteins that become toxic insoluble protein aggregates implicated in the pathophysiology of AD.[3,4]

Tau proteins and amyloid β-peptide plaques (Aβ) have been identified as pathological hallmarks of Alzheimer's disease (AD) since this disease was first described. Both of these protein aggregates have a wide range of biochemical properties in common.[5] Post-translational modifications such as phosphorylation, truncation, and pyroglutamate formation have been observed in the AD brain for both of these proteins. Furthermore, Aβ has been shown to promote tau aggregation,

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which suggests that protein aggregation is a key initiator or promoter of this disease.[6] Other proteins, such as *N*-methyl-D-aspartate (NMDA) receptors, may influence the ability of Aβ to interact with tau protein aggregates causing more neuronal dysfunction and neurodegeneration.[7]

Oxidative injury is reported in several studies to be the central factor contributing to the pathogenesis of AD.[8,9] Other studies revealed the age-related decreased levels of heme oxygenase-1 (HO-1) in several brain regions. The end products of heme degradation, which include CO, Fe^{2+}, and BV (converted into bilirubin (BR) by biliverdin reductase), play a critical role in the regulation of cellular homeostasis. BR increases the reactivity of Kelch-like erythroid cell-derived protein (Keap1) with CNC homology-associated protein 1, resulting in the release of nuclear factor erythroid 2-related factor (Nrf2).[10] The role of the Keap1–Nrf2 system in protecting organisms from oxidative stress has been extensively studied in mammalian cells. The antioxidant, vasodilatory, anti-inflammatory, and anti-apoptotic properties of CO are well known. As a result, HO and its heme degradation products are effective oxidative stress modulators.[11] Importantly, Nrf2 transcription factor/heme oxygenase 1 (HO-1) is a primary regulator of brain cytoprotection as it activates the expression of various neuroprotective anti-oxidant, anti-inflammatory, and detoxifying proteins.[12]

Peroxisome proliferator-activated receptors (PPARs) are one of the nuclear hormone receptor superfamily ligand-activated transcription factors.[13] To control gene expression, PPARs bind to certain DNA sequence elements in the promoter region of target genes known as PPAR response elements.[14] There are three PPAR isotypes (PPARα, PPARβ/δ, and PPARγ) that are known to exist. In rats, PPAR is expressed in specific brain areas.[15] PPARγ is expressed at small amounts in the CNS in normal circumstances. On the other hand, PPARγ expression is elevated in some pathological conditions, such as cerebral ischemia and Alzheimer's disease.[16] PPAR levels were found to be elevated in the brains of Alzheimer's disease patients as reported by Kitamura and colleagues.[17] PPARγ mRNA levels were also found to be elevated in the brains of Alzheimer's disease patients in another study looking at gene expression.[18] As a result of these findings, PPAR has been established as a therapeutic target for Alzheimer's disease treatment based on its ability to modulate PPAR activity.

Since the current drugs for AD treatment are not completely effective and have side effects, there is a substantial need for novel drugs. Herbal medicine application is a promising approach that can act as an adjuvant or complementary agent, helping to lessen the progressive mental decline associated with AD.[19,20]

For the induction of cognitive impairment, an Alzheimer's-like rat model was established by the authors using D-galactose and aluminum chloride ($AlCl_3$) according to the methodology followed by Chiroma and colleagues.[21] They highlighted the altered cognitive functions, neuronal degeneration, and the increased expression of Tau protein in rats' brains after the administration of D-galactose 60/$AlCl_3$ 200 (mg per kg bw).

Strawberries (*Fragaria ananassa* Duch.) belonging to the genus *Fragaria* (Rosaceae) are consumed in high quantities globally due to their incomparable aroma and flavor.[22] They are distributed in many forms, including fresh, frozen and jam forms, either processed or derived.[23] *Fragaria ananassa* is considered a valuable supply of phenolics, involving flavonoids, anthocyanins, and ellagic and hydroxycinnamic acid derivatives. Additionally, it contains folate in large amounts, fatty acids, dietary fiber, and some other compounds, including nutritive components such as minerals, vitamins as ascorbic acid, and essential oils with a potent antioxidant effect.[23] These compounds exert anti-allergic, anti-inflammatory, anti-obesity, wound healing, antiplatelet aggregation, anti-hypertensive, anti-melanogenesis, anti-nephropathy, and anti-microbial effects. Moreover, strawberry intake protects against multiple chronic diseases such as dementia, Alzheimer's disease (AD), and cardiovascular disorders.[24,25]

Despite the importance of strawberries, their post-harvest life is relatively short due to their fragile structure associated with rapid changes in their phytochemical profile. Moreover, the general liking for strawberry fruit is closely linked to the quality attributes and attraction of customers. The complexity of the significant components of strawberries is considered an important factor affecting their marketing as it significantly modifies their flavor and taste.[23] Fresh strawberry fruits may lead to great economic losses as they are perishable and easily deteriorate. In addition, they have brief harvest seasons; therefore, they cannot be accessed around the year, leading to limited availability and consumption.

Freezing and boiling of strawberry fruits are preservation techniques that can expand the shelf-life and use of these fruits.[23,24] Freezing fruits is a common way of processing and preserving food.[26] Frozen fruits have the same aroma, taste and color as fresh fruits.[24,27] Although freezing could preserve sensory attributes, it may possibly cause changes or loss of bioactive compounds.[23,24] On the other hand, boiled fruit tissues are subject to oxygen and endogenous enzyme polyphenoloxidase activity that cause browning of the fruits resulting in the loss of thermolabile nutrients such as vitamin C and phenolic compounds. Changes in the color, flavor and juice texture may occur as well.[28] Commercial jams (heat exposed fruits) are consumed more owing to the new way of life. Thus, it is crucial to realize consumer demands with regard to phytoconstituent profiling, along with maintaining the potential health gains.

To our knowledge, the impact of heat treatments or freezing processes on strawberry fruits and the utilization of chromatographic analysis to detect the differences between fresh and treated samples have been barely investigated.[29–31] Hence, the aim of this study was to compare the outcomes of processing methods (freezing at −20 °C, and heat processing, *viz.* boiling at 100 °C) for preserving strawberry samples to maintain their sensory and chemical value that are considered the most important quality attributes by applying ultra-high-performance liquid chromatography (UHPLC/ESI-MS) method. UHPLC is one of the coupled chromatographic techniques profusely used in the profiling of plant phenolics. It affords quicker ana-

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lysis with better sensitivity and resolution enabling the detection of minor compounds, offering an extensive understanding of the profile of plant metabolites.[32,33] This is the first time for such a technique to be used for comparative study to determine the variations in the chemical profiles of processed strawberry fruits.

Then, the neuroprotective activities of fresh and processed strawberries were evaluated in a D-galactose and aluminum chloride induced rat model of aging. The aging model was confirmed using different measurements, including behavioral (anxiety-like behavior and cognitive behavior) and biochemical parameters (HO-1, Nrf2, and PPAR-γ), histopathological profile and immunohistochemical examination of brain tissues.

2. Materials and methods

2.1. Chemicals, reagents, and materials

LC-MS-grade deionized water and acetonitrile were obtained from Merck (Darmstadt, Germany). ACS grade formic acid was acquired from Sigma-Aldrich. Reagents and chemicals including D-galactose and $AlCl_3$ and the other reagents were obtained from Sigma Chemical Co. (USA).

2.2. Plant materials and preparation of the plant extracts

The fruits of *F. ananassa* were collected in March 2020, from the Horticulture Research Center, Ministry of Agriculture, Giza. Three kilograms of strawberry fruits were washed, and the bracts were removed. They were mixed and equally divided into three groups: fresh fruits, fruits for boiling and fruits for freezing. The first part was directly homogenized and then macerated in methanol (2 L) for one day after which methanol was filtered, and the strawberry was successively macerated 2 more times (2 L, each) until exhaustion. The methanol extracts were combined and evaporated under vacuum at a low temperature not exceeding 40 °C until dryness. The second group (1 kg) was placed into a stainless-steel pot and boiled in distilled water (700 mL) for 60 min while being covered with a lid. Following this procedure, the whole boiled fruits were then kept to cool, and then successively extracted with methanol, same as how the first group was extracted. The third part was frozen at −20 °C for 2 weeks and then extracted with methanol as previously described for the first group. All the dry extracts prepared were stored at −20 °C for further study.

2.3. UPLC/ESI-MS analysis of secondary metabolites

High Resolution Mass Spectrometry (HRMS) was conducted using a Waters Synapt G2 hybrid quadrupole-orthogonal acceleration time-of-flight configuration (Waters, Manchester, UK) operating in resolution mode ($M/\Delta M \geq 18\,000$), fitted with a Waters Acquity UPLC binary solvent chromatographic pump system. The column used was a reversed-phase Acquity BEH C18, 2.1 × 50 mm, 1.7-micron bead, and the analysis was completed under the subsequent gradient conditions: 0 to 1 min, 95% A (water containing 0.1% formic acid), 5% B (acetonitrile containing 0.1% formic acid); 1 to 16 min, 5 to 95% B; 16 to 18 min, 95% B; 18 to 20 min, 5% B; flow rate: 150 μL min^{-1}; injection volume: 3.1 μL (full loop injection). Mass calibration of the instrument was accomplished by means of sodium formate cluster ions, and an orthogonal Lock-Spray™ ESI probe was used with a lock mass calibrant, leucine-enkephalin. The pseudomolecular leucine-enkephalin ion at m/z = 554.2615 was used as the internal mass correction calibrant.

2.4. Principal component analysis (PCA) of the strawberry specimens based on their content of phytoconstituents

To find plausible correlations among the specimens depending on various pretreatments, the chemical components were analyzed by PCA using a covariance matrix involving 36 variables × 9 specimens (324 data). The software Minitab 17 (Minitab Ltd., Coventry, UK) was used for standardization of data values. Then Euclidean distance matrix was obtained according to Sneath and Sokal 1973[34] coupled with STATISTICA (StatSoft, Inc. 2003) to produce a two-dimensional score and loading plots. Thirty-six chemical metabolites, and a two-dimensional PCA biplot, comprising all specimens, fresh strawberries, and differently processed specimens, *viz.* those subjected to freezing and boiling treatments, *versus* compounds detected with UPLC, were used as inputs to compute eigenvalues using a covariance matrix.

2.5. Neuroprotective activity of strawberry fruit extracts

2.5.1. Animals. Female Wistar albino rats (150–200 g) were supplied by the Modern Veterinary Office for Laboratory Animals, Cairo, Egypt. The rats were maintained in groups at a constant temperature (23 ± 2 °C) and humidity (55 ± 1%) and under a light/dark (12/12 h) cycle. Animals were acclimatized for one week and were given a computed quantity of a balanced regular diet (50 g) and allowed free access to water *ad libitum*. Behavioral experiments were carried out in separate and isolated places. All the procedures and experimental design were approved by the research ethics committee for experimental and clinical studies at the Faculty of Pharmacy, Cairo University (REC-FOPCU). Protocol serial number: MP (2789).

2.5.2. Experimental groups. All the prepared extracts were used in a dose of 250 mg kg^{-1} in accordance with the Elmallah *et al.* study, where no indications of toxicity were noticed.[35] The dosage regime for inducing aging was adopted from Chiroma *et al.* (2018) with slight modifications.[36]

Rats were randomly distributed into five identical groups (n = 8) as follows:

Group I: control group; rats were gavaged with oral distilled water (vehicle) and injected with saline subcutaneously (S/C) for 8 weeks.

Group II: positive control group; rats were gavaged with water for 2 weeks and then co-administered with D-galactose (65 mg kg^{-1}, S/C) and $AlCl_3$ (200 mg kg^{-1}, orally) for 6 weeks.

Group III: rats were orally administered fresh extracts of *F. ananassa* (250 mg kg^{-1}) for 2 weeks and then co-administered with D-galactose (65 mg kg^{-1}, S/C) and $AlCl_3$ (200 mg kg^{-1}, orally) for 6 weeks.

Group IV: rats were orally administered boiled extracts of *F. ananassa* (250 mg kg^{-1}) for 2 weeks and then co-administered with D-galactose (65 mg kg^{-1}, S/C) and $AlCl_3$ (200 mg kg^{-1}, orally) for 6 weeks.

Group V: rats were orally administered frozen extracts of *F. ananassa* (250 mg kg^{-1}) for 2 weeks and then co-administered with D-galactose (65 mg kg^{-1}, S/C) and $AlCl_3$ (200 mg kg^{-1}, orally) for 6 weeks.

Twenty-four hours after the last dose of the vehicle, drug, and protective agents, all rats were subjected to behavioral testing to evaluate their anxiety levels, as well as cognitive behavior.

2.5.3. Behavioral testing. Rats were transferred to the behavioral examination room and were allowed to adapt to the room for 2 h before starting the behavioral test. They were handled gently to minimize stress.

2.5.4. Anxiety-like behavior. Anxiety-like behavior was evaluated in rats using the elevated plus-maze according to Hamdan *et al.* (2020) and Zaki *et al.* (2021).[37,38] Briefly, rats were positioned in the maze facing the open arm and permitted to wander for 5 min. Frequency of open and closed arm entries as well as duration in the open and closed arms were assessed. The more the animal enters the open arm associated with a longer duration, the less the anxious state of the animal.

2.5.5. Cognitive behavior. Short- and long-term memories were estimated using Y-maze and novel object recognition tests, respectively. In the Y-maze, the animals were allowed to discover the maze for 5 min and the number of arm entries and spontaneous alternation percentage were calculated.[39,40] While in the novel object recognition test, rats were acclimatized to the area for 5 min, and 24 h later, they were trained to explore two similar objects, and then 24 h later, they were tested with one common object and one new object. The measuring parameters were the discrimination ratio (DR) and recognition index (RI).[40] They were calculated from the following equations, respectively.

$$\mathrm{DR} = \text{New object exploration time} - \text{familiar object exploration time} / \text{New object exploration time} + \text{familiar object exploration time}$$

$$\mathrm{RI} = \text{New object exploration time} + \text{familiar object exploration time} / \text{New object exploration time}$$

2.5.6. Sample preparation. After the last behavioral test, rats were euthanized by cervical dislocation. Brains were immediately removed from the skulls, and then blotted and chilled. The brain tissues were quickly dried using filter paper and homogenized in ice-cold medium of 50 mM Tris/HCl (pH 7.4) to reach 10% (w/v). Total protein of the homogenized brain tissues was then evaluated according to Lowry *et al.*[41] Moreover, the brain tissues of rats in the different groups were dissected and immediately fixed in 10% neutral buffered formalin, and then processed in alcohols and xylenes followed by embedding in paraffin wax. 5 µm sections were cut and stained with hematoxylin and eosin for light microscopy.

2.5.7. Biochemical parameters: Enzyme-Linked Immuno-Sorbent Assay (ELISA) Kits for HO-1, Nrf2, and PPAR-γ. Briefly, brain tissue homogenates were prepared in 0.05 M phosphate buffer (pH 7) using a polytron homogenizer. The homogenates were centrifuged at 5000*g* for 20 min at 4 °C. The supernatant was frozen at −20 °C for the analysis of HO-1 (Biovision, USA), Nrf2 (Northwest Life Science Specialist, USA) and PPAR-γ (Cloud-Clone Corp., USA) according to the manufacturer's instructions. Results were normalized to the total protein in the tissues. An ELISA plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA) was used for the detection of color absorbance at an OD range of 490 to 630 nm.

2.5.8. Western blot analysis. For western blot analysis, the cortex, hippocampus and striatum tissue lysates were prepared in lysis buffer comprising 150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, pH 7.8, and Triton X-100 (1%) augmented with sodium pyrophosphate, sodium orthovanadate, a protease inhibitor cocktail, and PMSF. Anti-caspase 3 antibody was used (Merck KGaA, Germany). The intensity of protein bands was normalized to β-actin and quantified using ImageJ software. The data were expressed as a percentage of controls.

2.5.9. RNA extraction. Total RNA was isolated from the homogenized brain tissues using a Direct-zol RNA Miniprep Plus kit (cat# R2072, Zymo Research Corp., USA), and then the quality and quantity were evaluated using a Beckman dual spectrophotometer (USA).

2.5.10. Real-time PCR. A SuperScript IV One-Step RT-PCR kit (cat# 12594100, Thermo Fisher Scientific, Waltham, MA, USA) was applied for the reverse transcription of the isolated RNA followed by PCR. A forty-eight well plate StepOne instrument (Applied Biosystems, USA) was used in thermal profiling as follows: 10 min at 45 °C for reverse transcription, 2 min at 98 °C for RT inactivation and initial denaturation by 40 cycles of 10 s at 98 °C, 10 s at 55 °C and 30 s at 72 °C for the amplification step. After the RT-PCR run, the data were expressed as cycle threshold (Ct) for the target genes and housekeeping gene. Normalization for variation in the expression of each target microRNA, *miRNA 124*, *miRNA 132* and *miRNA 9* genes, was performed referring to the mean critical threshold (CT) expression value of the U6 housekeeping gene by the ΔΔCt method. The relative quantitation (RQ) of the target genes is computed according to the estimation of the $2^{-\Delta\Delta Ct}$ method (Table 1).

2.5.11. Immunohistochemistry of glial fibrillary acidic protein (GFAP) expression. Brain tissue sections were cut into adhesive slides, subjected to heat-induced retrieval, and blocked for endogenous peroxidases. After several washing steps, the slides were incubated with the primary antibody mouse monoclonal anti-GFAP (sc-166458, Santa Cruz Biotechnology) at a dilution of 1 : 100 in a humid chamber at 4 °C. Washing was carried out several times to remove excess antibody and then the tissue sections were incubated with goat anti-mouse HRP-labelled secondary antibody (Abcam, UK) at ambient temperature for 2 h. Color development and visualization were performed using a DAB-substrate kit (Pierce™ DAB Substrate Kit, Thermo Fisher Scientific).

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Table 1 Primer sequences used for RT-PCR

Genes	Forward	Reverse
miRNA 124	ATGTTCACAGCGGACCTTGAT	TTCACCGCGTGCCTTAATTG
miRNA 132	CGCGTCTCCAGGGCAAC	CGCAGCACGCCCACGCTC
miRNA 9	GGAAGCGAGTTGTTATCT	CATAAAGCTAGATAACCGA
U6	GGTCGGGCAGGAAAGAGGGC	GCTAATCTTCTCTGTATCGTTCC

Abbreviations: miRNA: micro RNA, U6: housekeeping gene (RNU6-1) snRNA.

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Negative control slides were attained by elimination of the primary antibody. The extent of positive immunostaining was calculated as the area percentage of expression using cellSens Dimension (Olympus software).

2.6. Statistical analysis

All results were tabulated and expressed as mean ± SD. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test during the interaction of two independent variables. Survival curves were plotted for the experimental groups and the number of surviving rats was compared using the chi-square test. Unpaired Student's *t*-test was used to detect the differences between two groups when appropriate. Data analysis was performed employing the Statistical Package for Social Sciences, version 17 (SPSS Software, SPSS Inc., Chicago, USA). $P < 0.05$ was considered significant.

3. Results

3.1. Metabolite identification using UPLC/ESI-MS

Secondary metabolites were detected using UPLC/ESI-QTOF-MS. Two main metabolite groups were detected: flavonoids and phenolics, in addition to other minor classes. The identified metabolites with their mass spectral information are provided in Table 2.

3.1.1. Phenolics and organic acids. Phenolics and organic acids were the highest abundant classes of the identified compounds denoted by 16 metabolites. Their abundance is observed within the elution region of Rt (0.64–8.02 min) (Fig. 1), being highly polar and eluted at an elevated water solvent composition. The first detected organic acid was in peak 2 with $[M - H]^-$ at *m*/*z* 191.019 ($C_6H_8O_7$), yielding product ions at *m*/*z* 173 $[M - H_2O - H]^-$ and *m*/*z* 111 $[M - 2H_2O - COO - H]^-$ and was identified as (iso)citric acid.[43] Caffeic acid hexoside was detected at peak 3 with $[M - H]^-$ at *m*/*z* 341.177 ($C_{15}H_{18}O_9$), yielding product ions at *m*/*z* 179 $[M - C_6H_{10}O_5 - H]^-$.[44] Peaks 10 and 15 showed a product ion at *m*/*z* 163 and 145, ascribed to the presence of coumaric acid, and were labelled as coumaric acid glycosides.[49] Peak 19 showed $[M - H]^-$ at *m*/*z* 355.101 ($C_{16}H_{20}O_9$), yielding product ions at *m*/*z* 193 $[M - C_6H_{10}O_5 - H]^-$ and was annotated as ferulic acid hexoside.[54] Sinapic acid hexoside was noticed in peak 29 with $[M - H]^-$ at *m*/*z* 385.153 ($C_{18}H_{26}O_9$), yielding product ions at *m*/*z* 223 $[M - C_6H_{10}O_5 - H]^-$ and *m*/*z* 179 $[M - C_6H_{10}O_5 - COO - H]^-$.[57] Several metabolites showed similar fragmentation patterns annotated for the presence of hexahydroxydiphenoyl (HHDP)-glucose and were labelled as its derivatives in peaks 6, 8, 13, and 18.[47] Likewise, peaks 17, 27, 31, 33 and 34 showed a product ion at *m*/*z* 169, ascribed to the presence of gallic acid, and were annotated as its derivatives.[53,55]

3.1.2. Flavonoids. Next to phenolic and organic acids, flavonoids represented the second most abundant metabolite class with 12 metabolites. Their abundance is visible within the elution region of Rt (2.34–8.67 s) (Fig. 1), being relatively less polar. Among them, several metabolites showed a product ion at *m*/*z* 285 ascribed to the presence of kaempferol aglycone and were labelled as kaempferol glycosides and derivatives; these metabolites were detected at peaks 12, 20, 22, 28 and 30.[51] Few metabolites at peaks 16 and 23 showed the characteristic product ion of quercetin at *m*/*z* 301 and hence were annotated as quercetin glycosides.[52] Likewise, peak 21 showed a product ion at *m*/*z* 315 characteristic of isorhamnetin aglycone and was annotated as isorhamnetin glucuronide. Rutin was detected at peak 36 with $[M - H]^-$ at *m*/*z* 609.119 ($C_{27}H_{30}O_{16}$), producing product ions at *m*/*z* 301 $[M - C_{12}H_{20}O_9 - H]^-$.[47]

Two flavan-3-ols and four proanthocyanidin metabolites were identified. Catechin and epicatechin were distinguished in peaks 5 and 9 with $[M - H]^-$ at *m*/*z* 289.067 and 289.092 ($C_{15}H_{14}O_6$), yielding the characteristic product ions at *m*/*z* 245, 125 and 109.[46] Likewise, peaks 7 and 25 with $[M - H]^-$ at *m*/*z* 577.138 ($C_{30}H_{26}O_{12}$) and 865.194 ($C_{45}H_{38}O_{18}$) showed product ions at *m*/*z* 289 ascribed to catechin or epicatechin and hence were labelled as their oligomers, namely, procyanidin dimer and trimer, respectively.[48] Furthermore, peaks 24 and 35 with $[M - H]^-$ at *m*/*z* 849.18 ($C_{45}H_{38}O_{17}$) and 561.295 ($C_{30}H_{26}O_{11}$) during fragmentation produced ions at *m*/*z* 289, which are characteristic of catechin and were identified as propelargonidin trimer and propelargonidin dimer, respectively.[50]

3.2. Principal component analysis (PCA)

According to their chemical constituents, PCA helped to differentiate between the different strawberry specimens. The PCA score plot in Fig. 2A and the loading plot in Fig. 2B show the relationships between the strawberry samples and the assigned constituents. The total of the first 2 principal components (PCs) represented 83.30% of data variability. PCA1 represents 45.60% variability and PCA2 represents 37.70% variability. The PCA graphs show a significant effect of the fruit pretreatment on the chemical composition of strawberries, developing three distinct groups. The data variability was generated mostly by

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Table 2 Metabolites putatively identified by UHPLC/ESI-MS analysis in fresh, frozen, and boiled strawberry fruit extracts

Peak no.	Ret. time	Tentative identification	Molecular formula	$[M-H]^-$	Fragments	Fresh	Frozen	Boiled	Ref.
1.	0.64	Chlorogenic acid	$C_{16}H_{18}O_9$	353.071	191, 179	+	+	+	42
2.	0.87	(Iso)citric acid	$C_6H_8O_7$	191.019	173, 111	+	+	+	43
3.	0.93	Caffeic acid hexoside	$C_{15}H_{18}O_9$	341.177	179	+	−	−	44
4.	1.23	Hydroxybenzoic acid hexoside	$C_{13}H_{16}O_8$	299.588	137	+	+	−	45
5.	2.34	Catechin	$C_{15}H_{14}O_6$	289.067	245, 125, 109	+	+	−	46
6.	2.83	Bis(hexahydroxydiphenoyl)-hexoside	$C_{34}H_{24}O_{22}$	783.066	481, 301	+	−	+	47
7.	3.12	Procyanidin dimer	$C_{30}H_{26}O_{12}$	577.138	407, 289, 245, 161, 125	+	+	+	48
8.	3.17	Hexahydroxydiphenoyl (HHDP)-hexoside	$C_{20}H_{18}O_{14}$	481.68	301	+	+	−	47
9.	3.25	Epicatechin	$C_{15}H_{14}O_6$	289.092	245, 125, 109	+	+	+	46
10.	3.33	Coumaric acid hexoside	$C_{15}H_{18}O_8$	325.089	163, 145	+	+	+	49
11.	3.36	Eutigoside A	$C_{23}H_{26}O_9$	445.154	309, 147	+	+	+	50
12.	3.6	Kaempferol hexoside	$C_{21}H_{20}O_{11}$	447.061	285	+	−	+	51
13.	3.98	Galloyl bis (HHDP) hexoside	$C_{41}H_{28}O_{26}$	935.087	467, 301	+	+	+	47
14.	4.36	Ellagic acid deoxyhexoside	$C_{20}H_{16}O_{12}$	447.059	300.9, 229	+	+	−	22
15.	4.58	*p*-Coumaroylhexose-4-*O*-hexoside	$C_{25}H_{28}O_{10}$	487.144	325, 163, 145	+	+	−	49
16.	4.6	Quercetin 3-*O*-glucuronide	$C_{21}H_{18}O_{13}$	477.062	301, 255, 178, 151	+	+	−	52
17.	4.76	Tri-galloyl glucose	$C_{27}H_{24}O_{18}$	635.090	169	+	−	−	53
18.	4.78	Galloyl HHDP glucose	$C_{27}H_{22}O_{18}$	633.213	301	+	+	−	47
19.	4.85	Ferulic acid hexoside	$C_{16}H_{20}O_9$	355.101	193	+	+	+	54
20.	5.02	Kaempferol glucuronide	$C_{21}H_{18}O_{12}$	461.072	285	+	+	+	51
21.	5.17	Isorhamnetin glucuronide	$C_{22}H_{20}O_{13}$	491.081	315, 301, 285, 229, 175, 151	+	−	+	52
22.	5.3	Kaempferol 3-acetylglucoside	$C_{23}H_{22}O_{12}$	489.094	285, 284	+	+	−	51
23.	5.47	Procyanidin trimer	$C_{45}H_{38}O_{18}$	865.194	577, 289	−	+	+	48
24.	5.53	Quercetin 3-*O*-glucoside	$C_{21}H_{20}O_{12}$	463.25	301	+	+	−	52
25.	5.63	Propelargonidin trimer (afz-cat-cat)	$C_{45}H_{38}O_{17}$	849.18	577, 289	+	−	−	55
26.	5.61	Lagerstannin A	$C_{34}H_{24}O_{23}$	799.012	755, 497, 301	−	+	+	56
27.	6.14	Galloyl-caffeoylhexoside	$C_{22}H_{22}O_{13}$	493.234	341, 179	+	+	+	55
28.	6.25	Kaempferol coumaroyl hexoside	$C_{30}H_{26}O_{13}$	593.126	447, 285, 187, 145	+	+	+	51
29.	6.54	Sinapic acid hexoside	$C_{18}H_{26}O_9$	385.153	223, 179, 125	+	+	+	57
30.	6.69	Kaempferol malonylhexoside	$C_{24}H_{22}O_{14}$	533.252	489, 285, 255	+	+	+	51
31.	7.23	Tri-galloyl glucose	$C_{27}H_{24}O_{18}$	635.027	465, 169	+	−	−	53
32.	7.77	Pelargonidin malonyl hexose	$C_{24}H_{23}O_{13}$	519.718	433, 271	−	+	−	55
33.	7.88	Galloylquinic acid	$C_{14}H_{16}O_{10}$	343.17	191, 169	+	−	+	53
34.	8.02	Galloyl glucose	$C_{13}H_{16}O_{10}$	331.9	313, 289, 287, 271, 235, 169	+	+	−	53
35.	8.09	Rutin	$C_{27}H_{30}O_{16}$	609.119	301	+	−	−	47
36.	8.67	Propelargonidin dimer (afz-cat)	$C_{30}H_{26}O_{11}$	561.295	289, 245	+	+	−	55

Ret. time = retention time, +: present in the sample, and −: absent from the sample.

the variances of galloyl-caffeoylhexose (values of eigenvectors: 0.18; 0.18), (iso)citric acid (0.21; 0.15) and caffeic acid hexose (0.21; 0.14) in the first PC, characteristic of raw strawberries.

Then, on the lower half of the loading plot, the following constituents are located, *viz.* pelargonidin malonyl hexose (values of eigenvectors: 0.05; −0.26), galloyl HHDP glucose (0.06; −0.26), procyanidin trimer (−0.06; −0.25) and quercetin 3-*O*-glucoside (0.11; −0.24), characterizing frozen strawberries in the second PC. The data for the boiled samples are located in the left part of the upper half of the score plot. They were mostly correlated with relatively higher contents of isorhamnetin glucuronide (values of eigenvectors: −0.19; 0.13), kaempferol coumaroyl hexose (−0.22; 0.11) and procyanidin dimer (−0.22; 0.09).

3.3. *In vivo* evaluation

3.3.1. The effect of the administration of different strawberry extracts on the anxiety-like behaviour and cognitive behaviour of D-galactose and $AlCl_3$-induced rats. The anxiety-like behavior in the positive control rats is expressed as a decrease in the open and closed arm entry as well as open arm duration associated with an increase in the closed arm duration compared to the healthy group. However, administration of fresh strawberry extracts reversed all of these effects as visualized by the decrease in the close arm duration and the increase in the open arm frequency and duration compared to the other groups. There was no significant difference among the frozen strawberry and boiled strawberry treated groups (Fig. 3).

Concerning the cognitive behavior of rats, the spatial working memory of the positive control rats was markedly decreased as expressed by a significant reduction in the number of arm entries and the ability of the rats to alternate between the three different arms (SAP%) compared to the healthy rats. Conversely, administration of fresh strawberries markedly increased the number of arm entries and SAP% com-

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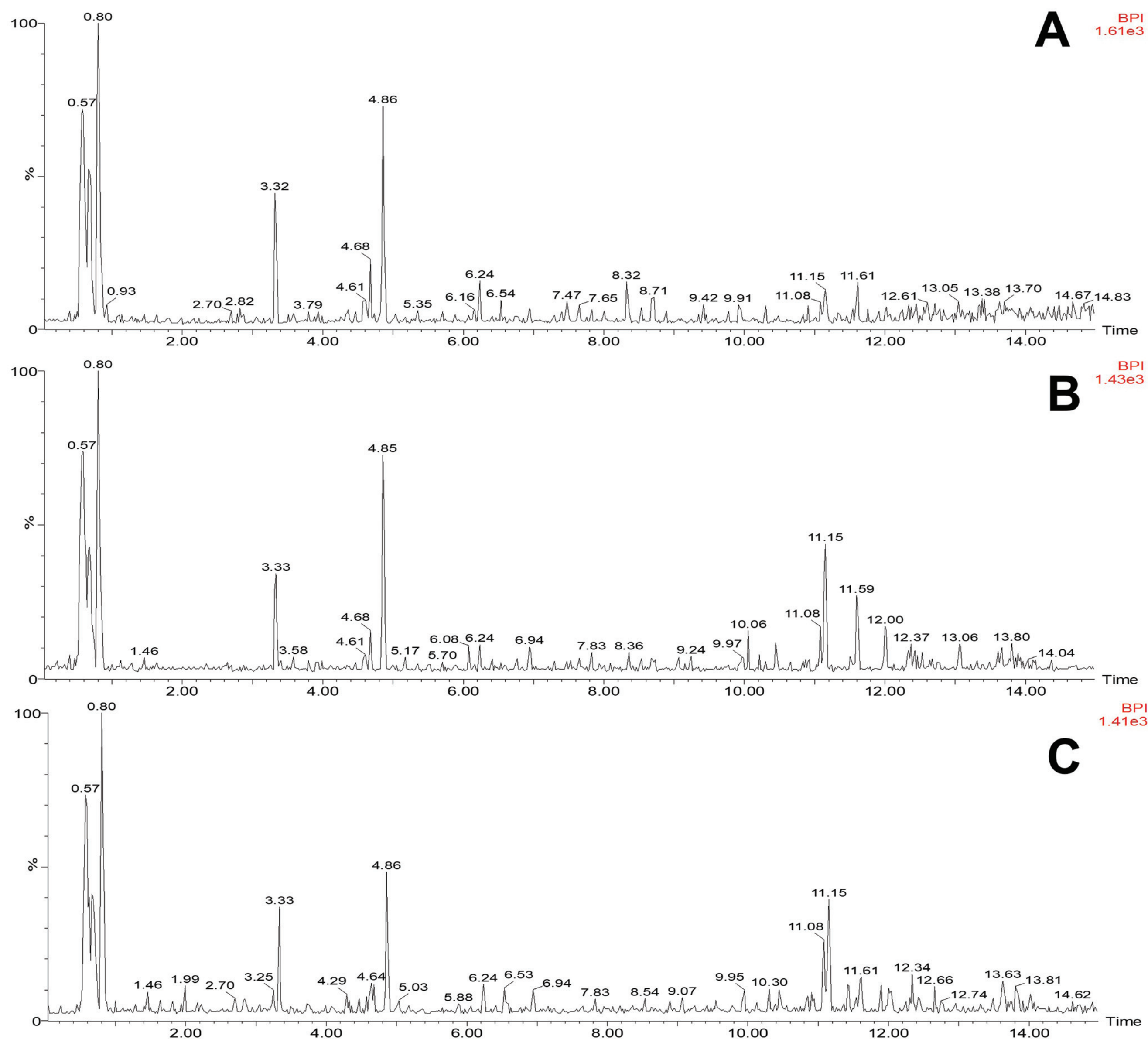

Fig. 1 Representative UPLC-MS base peak chromatograms of strawberry fruit extract samples in negative ionization mode. (A) Fresh strawberry, (B) frozen strawberry and (C) boiled strawberry.

pared with the other treatment groups (Fig. 4). Concerning the long-term memory evaluated in the novel object recognition test, the positive control rats exhibited a significant decrease in the memory indices, including the exploration time duration, discrimination ratio, and recognition index compared with the healthy rats. In contrast, the administration of strawberries (fresh, frozen, and boiled) improved the memory indices (Fig. 5). Notably, fresh strawberries resulted in superior memory improvement compared to the other treatment groups.

3.3.2. The effect of the administration of different strawberry extracts on HO-1 and Nrf2-mediated mitochondrial function in the animals. The current study revealed that the levels of HO-1 in the positive control group were significantly reduced compared to those of the healthy group. The three strawberry extracts enhanced the HO-1 levels significantly as compared with the positive control, while the fresh and frozen extracts were capable of returning the HO-1 levels to normal levels (Fig. 6a).

Amounts of Nrf2 were significantly lowered in the positive control group compared to the healthy one. The three strawberry extracts elevated Nrf2 significantly as compared with the positive control. Furthermore, the fresh extracts efficiently returned the Nrf2 levels to normal levels (Fig. 6b).

3.3.3. Effect of the administration of different strawberry extracts on PPAR-γ expression. It was indicated that the significantly elevated levels of PPAR-γ in the positive control group were downregulated by the three different strawberry extracts. However, the PPAR-γ level in the fresh extract group was the

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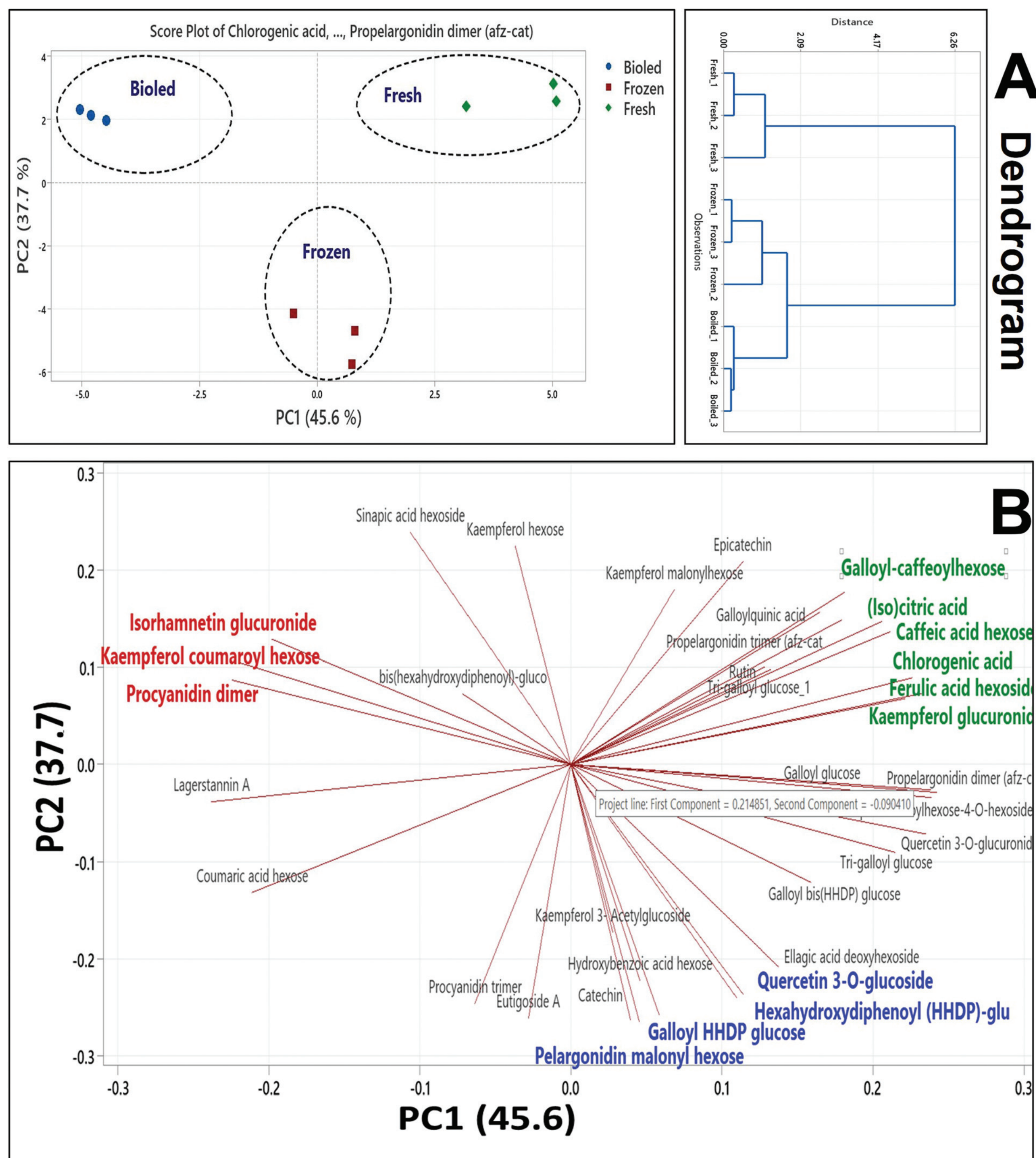

Fig. 2 (A) Dendogram and score plot representing the distribution of fresh and processed samples of strawberry, *viz*. frozen and boiled. (B) Loading plot showing the variable correlation within the matrix.

only one that was similar to the level of the normal healthy group, as shown in Fig. 6c.

3.3.4. Antiapoptotic effect of the administration of different strawberry extracts. It was highlighted that caspase 3 protein expression was significantly elevated in the positive control group, and downregulated in the three pretreatment groups. Both fresh and frozen extracts efficiently normalized the caspase 3 levels (Fig. 6d and e).

3.3.5. Effect of the administration of different strawberry extracts on neuronal injury through the PCR expression of LDH and miRNAs. The PCR results revealed that the RNA expression of LDH was significantly upregulated in the brain tissues of the positive control rats compared to the healthy group. The three pretreated groups efficiently reduced LDH levels, while the group pretreated with the fresh extract was the only one with normal levels of LDH (Fig. 7a and d).

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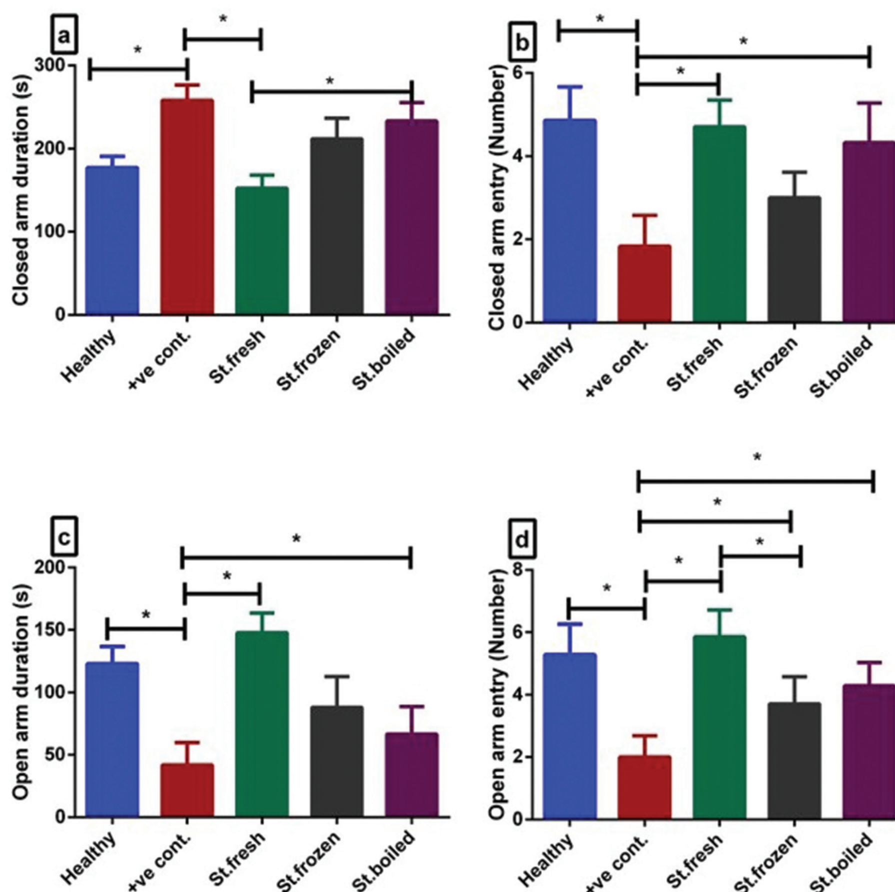

Fig. 3 Effect of Egyptian *Fragaria ananassa* extracts on the anxiety-like behavior of rats in the elevated plus-maze. (a) Close arm duration, (b) close arm entry (c) open arm duration, and (d) open arm entry. Data are expressed as mean ± SD, one-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

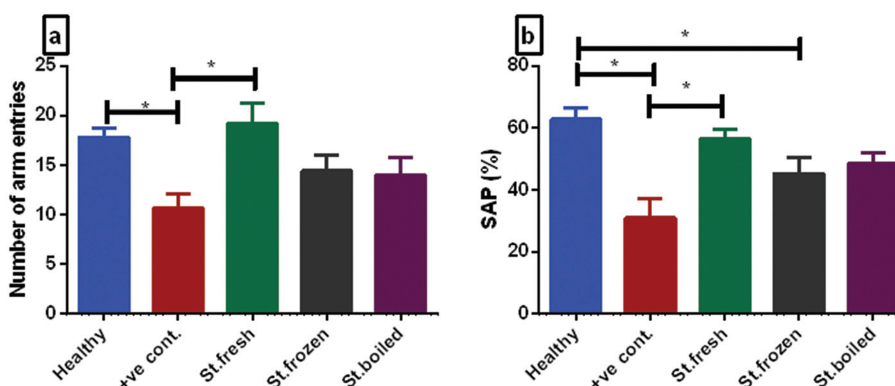

Fig. 4 Effect of Egyptian *Fragaria ananassa* extracts on the spatial working memory of rats in the Y-maze. (a) Number of arm entries, and (b) spontaneous alternation percentage (SAP%). Data are expressed as mean ± SD, one-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

The miRNA 9, miRNA 124 and miRNA 132 expression was significantly elevated in the positive control group. Pretreatment with any of the strawberry extracts showed significantly lower levels of miRNA when compared with the positive control. Both fresh and frozen extract pretreatments efficiently normalized the expression of miRNA 9 and 124,

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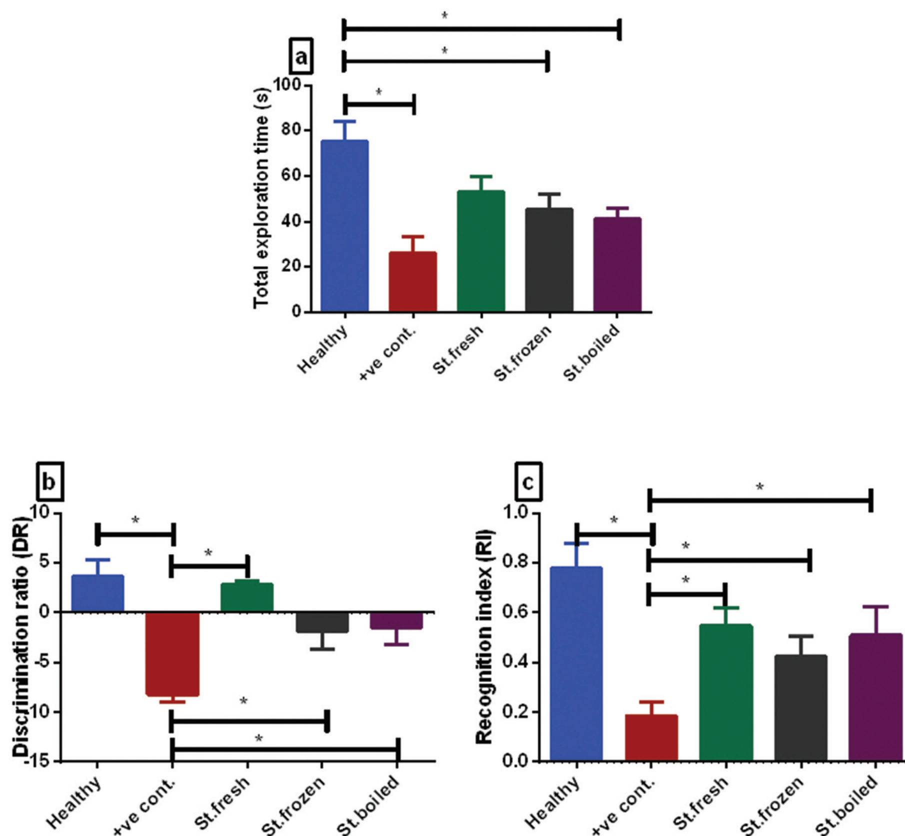

Fig. 5 Effect of Egyptian *Fragaria ananassa* extracts on the recognition memory of rats in the novel object recognition test. (a) Total exploration time, (b) the discrimination ratio (DR), and (c) the recognition index (RI). Data are expressed as mean ± SD, one-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

while only the fresh extract pretreatment succeeded in normalizing the miRNA 132 expression (Fig. 7b–d).

3.3.6. Histopathological examination of the brain tissues. The healthy group showed a normal histological structure of the cerebral cortex. Compared to the healthy group, the cerebral cortex of the positive control group exhibited marked pathological changes that were illustrated by shrunken degenerated neurons with acidophilic cytoplasm and hyperchromatic nuclei. Thickening of cortical blood vessels was noticed in some examined sections associated with vasculitis. Marked protection was noticed in the fresh strawberry group, which showed numerous apparently intact neurons with few cells showing neuronal degeneration and neuronophagia. The frozen strawberry group showed a comparable effect to that of the fresh strawberry group; however, thickening of blood vessels was a prominent alteration in some instances. Regarding the boiled strawberry group, neurodegenerative changes were commonly detected accompanied by astrocytosis in some sections (Fig. 8).

Examination of the hippocampus in the positive control group revealed numerous degenerated neurons scattered in the CA1, CA2, CA4, and DG regions. Meanwhile, the treated groups showed intact neurons in different regions of the hippocampus. Congested blood vessels were frequently detected in the boiled strawberry group (Fig. 9). Concerning the cerebellar sections of the different groups, the healthy group showed a normal histological structure of the molecular and granular layers and the Purkinje cells. Several necrosed Purkinje cells were detected in the positive control group. The absence of pathological alterations was noticed in the fresh strawberry group. Meanwhile, a fewer number of necrosed Purkinje cells were recorded in the frozen and boiled strawberry groups (Fig. 10).

3.3.7. GFAP expression. The immune expression of GFAP in the cerebral cortex and hippocampus is illustrated in Fig. 11; significantly higher values of brain GFAP expression were noticed in the positive control group in comparison with the other experimental groups. The fresh strawberry group showed the greatest significant reduction in GFAP expression in both cerebral cortex and hippocampus, followed by the frozen and boiled strawberry groups. A significant decrease was observed in the frozen strawberry group when compared to the boiled strawberry group.

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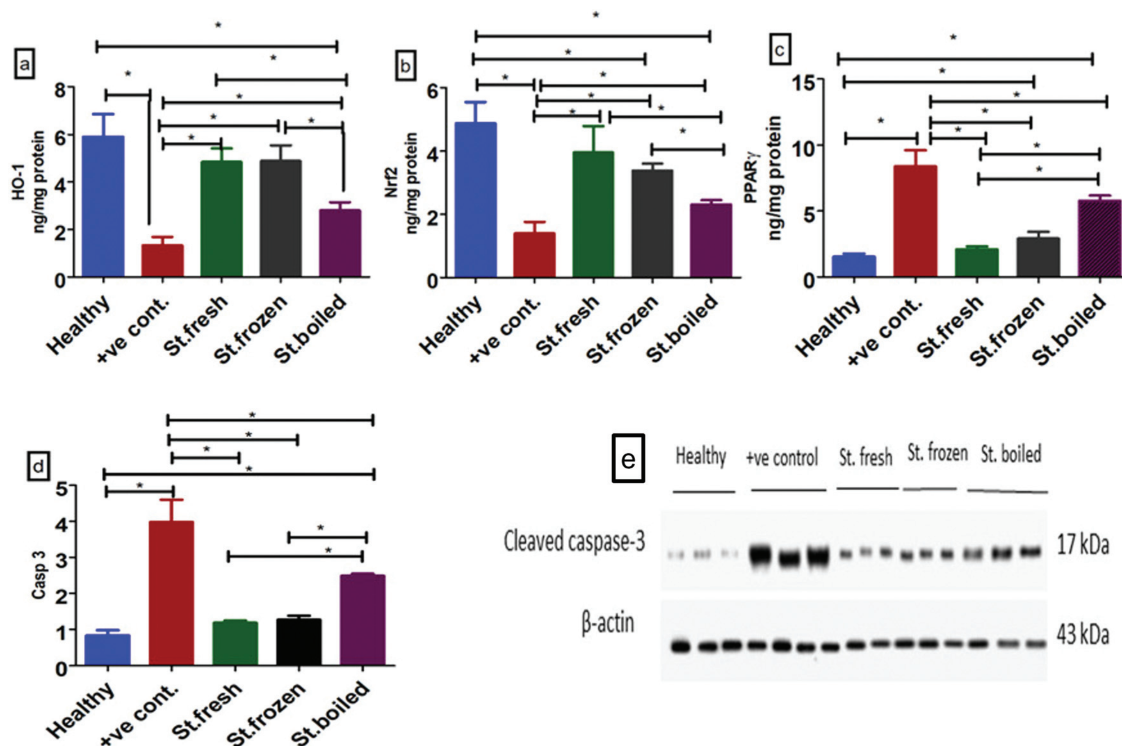

Fig. 6 Effect of Egyptian *Fragaria ananassa* extracts on the levels of (a) heme oxygenase-1 (HO-1), (b) nuclear factor erythroid 2-related factor 2 (Nrf2), (c) proliferating cell nuclear antigen (PPAR-γ) markers and (d) the caspase-3 (Casp 3) marker and protein band of D-galactose and $AlCl_3$-induced AD in rats. Data are expressed as mean ± SD, one-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

4. Discussion

The goal of this work was to compare the chemical profiles of fresh, frozen, and boiled strawberry fruit extracts in order to determine a better manufacturing technique that can match the market demands as well as to investigate the neuroprotective potential of fresh and processed extracts of *Fragaria ananassa* fruits in D-galactose and $AlCl_3$-induced aging in rats.

UPLC/ESI-qTOF-MS analysis led to the annotation of 36 metabolites in different ratios in the examined specimens. Fresh fruits were distinguished by the presence of caffeic acid hexose, propelargonidin trimer, tri-galloyl glucose, and rutin in this sample only, in addition to galloyl-caffeoylhexose, (iso) citric acid, chlorogenic acid, ferulic acid hexoside and kaempferol glucuronide, as detected from PCA. From this, we assumed that retaining of the constituents in strawberry fruits differed according to the mode of processing, *viz.* heating and freezing. Our study classified the strawberry samples into three groups depending on the secondary metabolite characteristics for each sample category: fresh (comprising most of the chemical compounds detected, represented by phenolics, organic acids, flavonoids and cyanidines) and frozen (sharing most constituents with the fresh strawberry specimens, *viz.* quercetin 3-*O*-glucoside, *p*-coumaroylhexose-4-*O*-hexoside and propelargonidin dimer), and lastly, the boiled samples were located far from the fresh ones but still shared a few constituents with them, *viz.* sinapic acid hexoside, kaempferol malonyl hexoside and kaempferol hexoside. Accordingly, from the obtained UPLC/ESI-MS results (Table 2) and the PCA (Fig. 2A and B), concerning the chemical classes of the processed strawberry specimens, the frozen sample retained almost a high content of secondary metabolites of the strawberry fruits, representing a good level of phytoconstituent strength in agreement with the literature.[23] However, the boiling process revealed an intermediate loss of constituents.

Our current study showed that co-administration of D-galactose and $AlCl_3$ causes an increment in anxiety-like behavior and cognitive deficits, including working and long-term memories in positive control AD rats. The elevated plus maze is a widely used test to detect the anxiety-like behavior in AD rats.[58] The reduction in the open arm entry and duration observed in AD rats is an indicator of increased anxious state of AD rats. However, *Fragaria ananassa* fresh extract was able to normalize the anxious state of the AD rats; this may be attributed to the chemical profile of the fresh extract, including several polyphenols such as catechin, epicatechin, rutin, proanthocyanidin, quercetin glycosides, and chlorogenic acid mostly found in fresh samples which have strong neuroprotective properties as observed here.[59] Moreover, caffeic acid present in the fresh strawberry sample was proved to have a

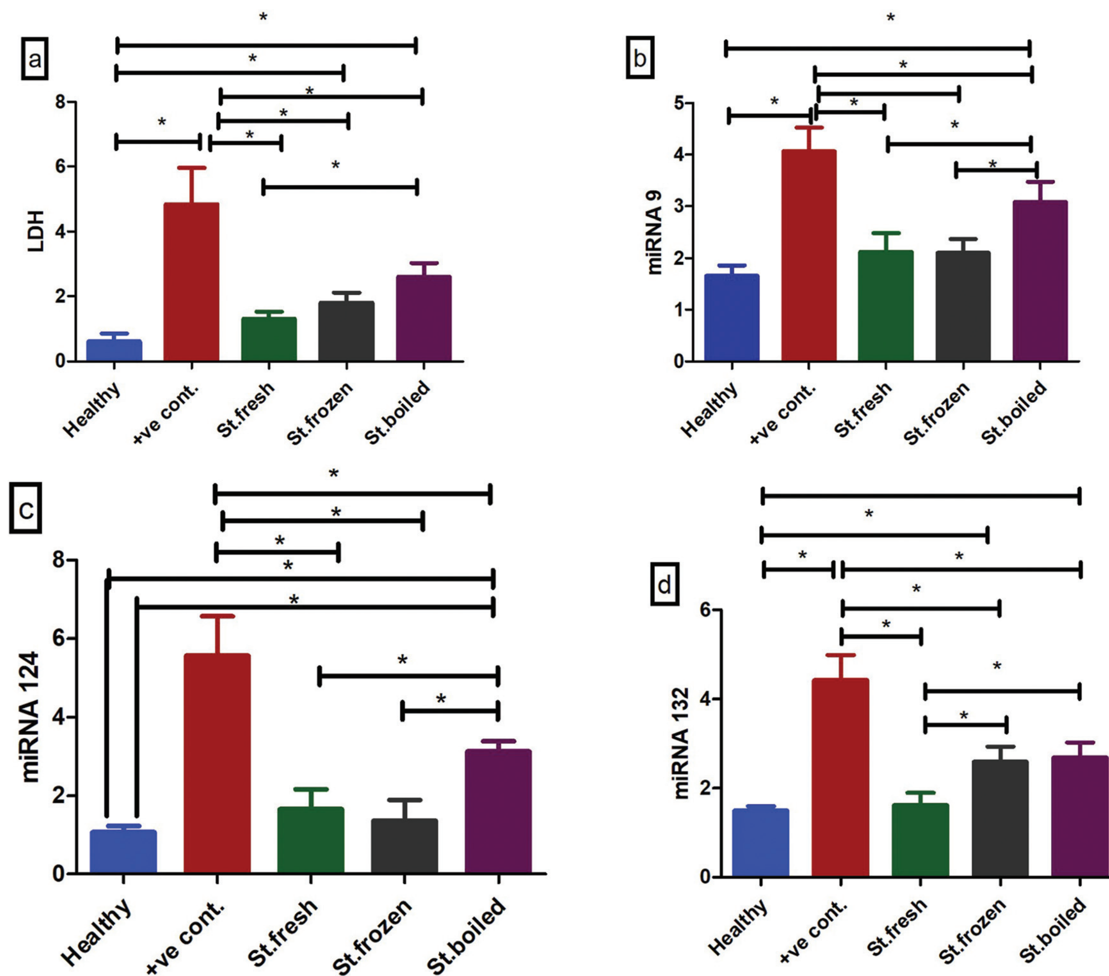

Fig. 7 Effect of Egyptian *Fragaria ananassa* extracts on the levels of (a) lactate dehydrogenase (LDH), (b) micro-RNA (miRNA-9), (c) miRNA-124 and (d) miRNA-132 determined by real-time PCR of D-galactose and $AlCl_3$-induced AD in rats. Data are expressed as mean ± SD, one-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

protective role in Alzheimer's disease.[60] In addition, pelargonidin has been linked with a lowered risk of Alzheimer's dementia in the elderly.[61] Conversely, *Fragaria ananassa* frozen and boiled extract treated rats showed an increase in their anxious state, and this was likely due to the loss of some phytoconstituents during processing. Spatial working memory was tested using Y-maze, a commonly used hippocampal dependent test.[39] AD rats exhibited a spatial working memory decline evidenced by a decrease in the ability of rats to distinguish the three different arms, known as spontaneous alternation; this finding was confirmed in a previous study.[39] Moreover, novel object recognition test is utilized to measure the recognition memory in AD rats.[58] AD rats showed a decrement in the total exploration time, discrimination ratio and recognition index as previously reported.[39,62,63] Conversely, *Fragaria ananassa* fresh extract administration was able to significantly improve the cognitive dysfunction.

D-Galactose and $AlCl_3$ exposure were also found to be associated with the downregulation of the Nrf2-mediated pathway and HO-1, a byproduct of this pathway. Nrf2 plays a critical role in maintaining intracellular redox homeostasis. It enhances the expression of cytoprotective genes and the antioxidant capacity of mammalian cells. Certain bioactive compounds have been shown to reduce cell stress and hence accelerate neovascularization, cell proliferation, and tissue restoration by advancing Nrf2 activation.[64–66] Our results showed that in D-galactose and $AlCl_3$ exposed rats, the levels of both HO-1 and Nrf2 were reduced. Interestingly, the fresh extract of *Fragaria ananassa* reversed these perturbations and raised the HO-1 and Nrf2 levels; this effect was followed by the frozen sample extract being capable of normalizing the HO-1 but not the Nrf2 levels. These findings showed the potential beneficial role of the fresh extract as a neuroprotective agent.

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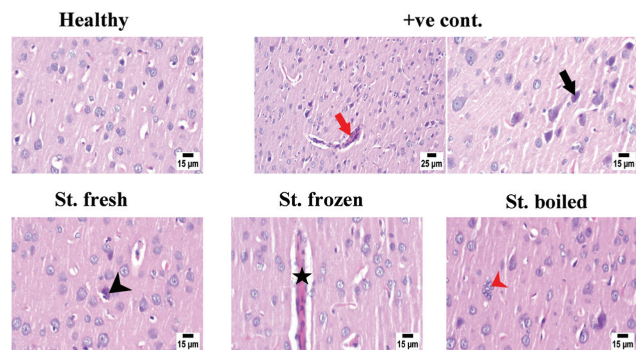

Fig. 8 Photomicrograph of the H&E sections from the cerebral cortex in the brains of the different groups. The healthy group showing normal intact neurons. The +ve cont. group showing vasculitis (red arrow) and degenerated neurons with acidophilic cytoplasm (black arrow). The St. fresh group showing numerous intact neurons with some extent of neuronal degeneration and neuronophagia (black arrow head). The St. frozen group showing congested blood vessels (star). The St. boiled group showing focal astrocytosis (red arrow head). +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

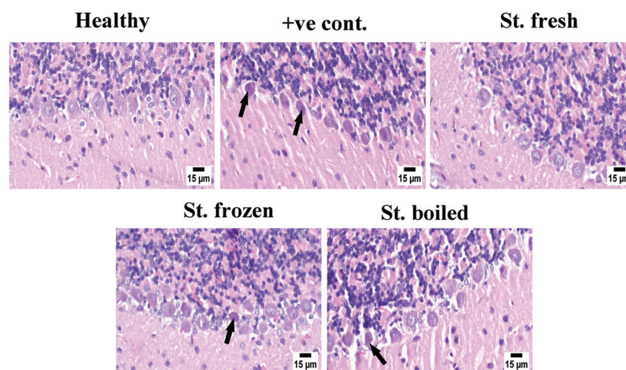

Fig. 10 Photomicrograph of the H&E sections from the cerebellum in the brains of the different groups. The healthy group showing a normal cerebellum. The +ve cont. group showing necrosis of numerous Purkinje cells (arrows). The St. fresh group showing apparently normal Purkinje cells. The St. frozen and St. boiled groups showing fewer Purkinje cell deaths (arrow). +ve cont. = positive control AD rats, St. fresh = fresh strawberry extract, St. frozen = frozen strawberry extract, St. boiled = boiled strawberry extract group.

Under physiological conditions, PPAR-γ is expressed at low levels in the brain.[18] This finding was proven in the current study through the observation of low expression of PPAR-γ in the healthy group. Several recent studies showed that PPAR-γ agonist administration may reduce neurodegeneration in AD patients and that can explain the elevated levels of PPAR-γ in the +ve control group as a compensatory action in the

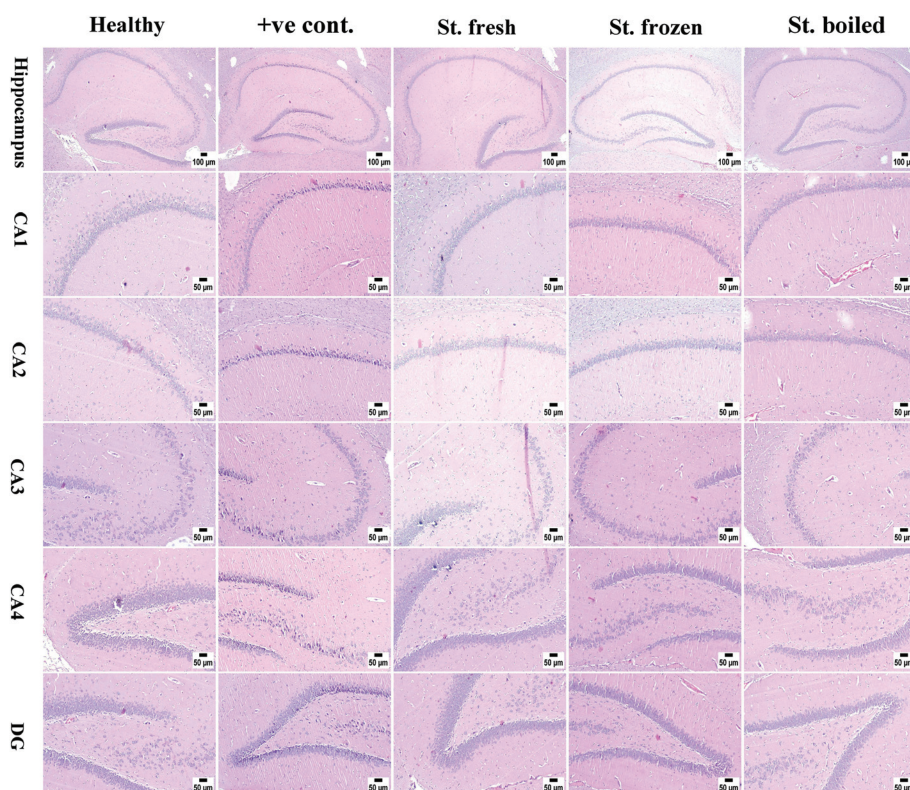

Fig. 9 Photomicrograph of the H&E sections from the hippocampus in the brains of the different groups. The healthy group showing normal intact neurons in different hippocampal regions. The +ve cont. group showing degenerated neurons in CA1, CA2, CA4 and DG. The St. fresh and St. frozen groups showing apparently a normal hippocampus. The St. boiled group showing congested blood vessels adjacent to the CA1 neurons. +ve cont. = positive control AD rats, St. fresh = fresh strawberry extract, St. frozen = frozen strawberry extract, St. boiled = boiled strawberry extract group.

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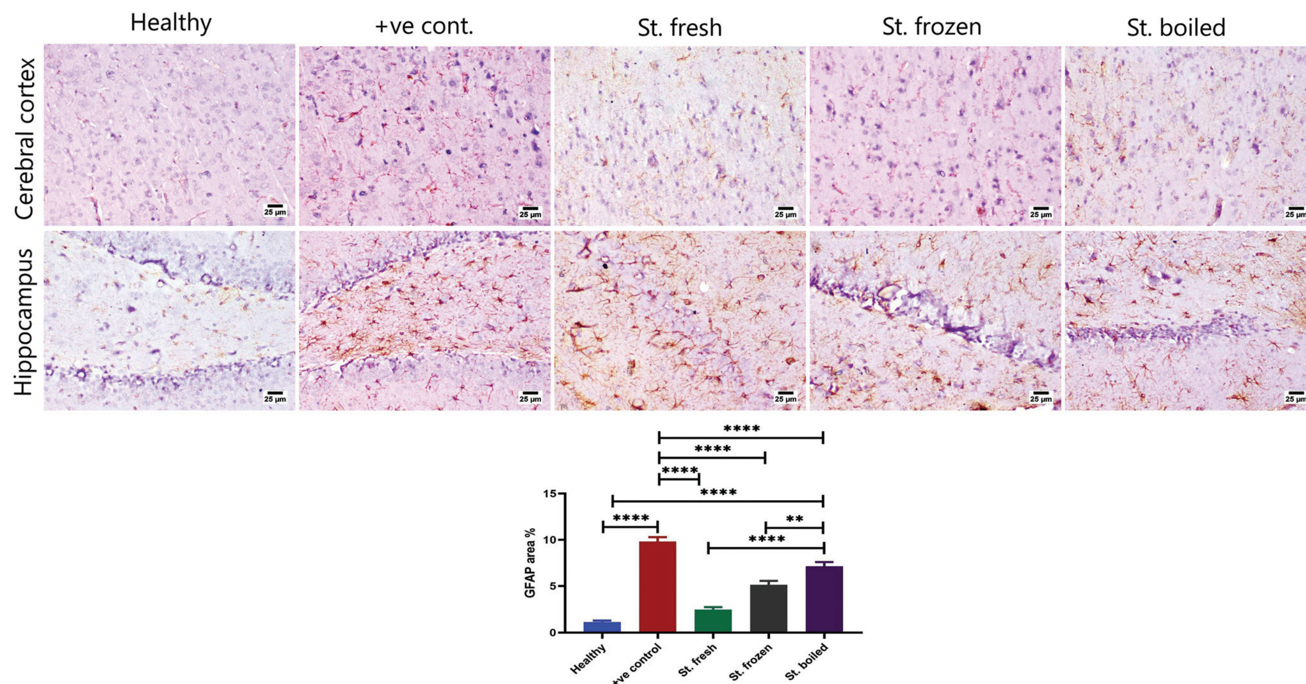

Fig. 11 Immune expression of GFAP in the brain tissue (immunostaining), the +ve control group showing increased GFAP expression in both cerebral cortex and hippocampus, all treated groups showing decreased GFAP expression, and the chart showing quantification of GFAP expression as area %; data were presented as means ± SD. One-way ANOVA followed by Tukey's *post hoc* test for eight rats in each group. Statistical differences between groups are denoted by *, $P \leq 0.05$. +ve cont. = positive control rat group, St. fresh = fresh strawberry extract group, St. frozen = frozen strawberry extract group, St. boiled = boiled strawberry extract group.

brain.[67,68] When a pretreatment is added, brain neurodegeneration was reduced, leading to a reduction in PPAR-γ levels to approach normal levels. These findings proved the promising anti-inflammatory neuroprotective effect of different strawberry extracts, especially the fresh extract which led to normal PPAR-γ levels.

The brain tissues are considered severely damaged during AD development; this tissue damage is proven by distinct neuronal degeneration. LDH activity is utilized as a tissue health indicator, and any variation in its levels is an indication of disruptions in the neuronal tissue composition.[69] Significant increases in LDH levels in (D-gal + $AlCl_3$) induced rats were prevented when fresh strawberry extract was administered by preventing the enzyme leakage of neuronal cell membranes. Our results are similar to those of the studies performed by Shunan *et al.*[70] and Zhao *et al.*,[69] illustrating the protective effect of betalain and syringic acid against $AlCl_3$ generated AD in albino rats, respectively. Our results can also clarify the general amelioration of rats' brain tissue damage upon strawberry pre-treatment, rationalizing its healing ability against D-galactose + $AlCl_3$ induced injuries.

Recent research has emphasized the importance of microRNAs (miRNAs) in the development of Alzheimer's disease (AD). Numerous miRNAs have been implicated in the development of AD *via* a variety of mechanisms, including their effect on inflammatory responses. Tan and colleagues revealed that miR-9 was up-regulated in the sera of AD patients compared to those of normal controls.[71] Our findings were in the same line demonstrating that +ve control rats showed the highest levels of miR-9, which were downregulated in all pretreated groups. On the other hand, Kiko and colleagues showed no significant difference in the levels of miR-9 in AD patients and normal controls.[72] A previous study reported the estimation of miR-9 in the serum or cerebrospinal fluid and that could explain the different results; however, more studies are needed to be conducted for confirmation.

Al-Rawaf and colleagues reported that the levels of miR-124 significantly increased in the serum of mild cognitive impairment (MCI) patients compared to controls, showing the association of elevated levels and neuropathology, apoptosis, and oxidative stress. MCI is an intermediate phase between the normal aging decline of cognitive decline and the more serious progressive decline of dementia.[73] Additionally, miR-132 has been shown in recent studies to stimulate the phosphorylation of Tau protein through targeting glycosyltransferase-like domain-containing protein 1 (GTDC-1) and forkhead box Q1, and consequently neuronal apoptosis is induced.[74] Notably, the current study demonstrated that the fresh strawberry fruit extract may act as a neuroprotective agent by normalizing miR-132 expression.[74,75]

Dysregulation of apoptosis can lead to neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, which are distinguished by neuron loss, often *via* excessive apoptosis.[76,77] For assessing the magnitude of apoptosis in the

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brain tissues of rats subjected to D-galactose and $AlCl_3$ and the protective impacts of strawberry fruits, the expression of caspase-3 was evaluated by western blotting. Expression analyses of the brains of different groups of rats indicated that there was a four-fold increase in caspase-3 expression in the rats exposed to D-galactose and $AlCl_3$ compared with the rats in the control group. Comparable results in mice were described previously by Yang.[78] Co-administration of strawberry extracts to rats subjected to D-galactose and $AlCl_3$ downregulated the protein expression of caspase-3 and notably both fresh and frozen extracts normalized caspase-3 levels in the brain tissue, reflecting a promising protective effect of these extracts on brain tissues.[79]

Astrocytes show a marked increased number when compared to brain neuron cells and show several significant roles in healthy brain function.[80] Concerning neurodegenerative diseases, astrocytes undergo morphological and functional alterations including hypertrophy or proliferation, which result in an increased expression of GFAP.[81] A previous study showed the effect of D-galactose on the upregulation of GFAP in affected brain tissues.[82]

5. Conclusion

Fragaria ananassa Duch. is a remarkable supply of bioactive compounds that may play a role as a candidate in treating neurodegenerative diseases. Fresh strawberry rapidly deteriorates, leading to mighty economic losses; hence, processing techniques, *viz*. freezing and boiling, play an important role in the strawberry industry. The fresh sample still retains most of the phenolic compounds and phytoconsituents as revealed by UPLC-QTOF/MS-MS and chemometric analysis. A proven well-known neurodegenerative aging model was applied to evaluate the effect of different strawberry extracts on the cognitive functions of the experimental rats and consequently predict the potential effect of the tested preparations on their health promotion and age-related neurodegeneration. The results revealed some variations in the activity of each preparation, which were all processing dependent. Generally, the fresh fruits gave superior results, followed by the frozen preparation and then the boiled one.

Abbreviations

+ve cont.	Positive control rat group
AD	Alzheimer's disease
ACS	American Chemical Society
ANOVA	One-way analysis of variance
BV	Biliverdin
BR	Bilirubin
DR	Discrimination ratio
ELISA	Enzyme-linked immuno-sorbent assay
GFAP	Glial fibrillary acidic protein
H&E	Hematoxylin and eosin
HO-1	Heme oxygenase-1
HRMS	High resolution mass spectrometry
Keap1	Kelch-like erythroid cell-derived protein 1
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
MAPK1	Mitogen-activated protein kinase 1
miRNA	microRNA
Nrf2	Nuclear factor erythroid 2
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RI	Recognition index
RT-PCR	Reverse transcription polymerase chain reaction
SAP%	Spontaneous alternation percentage
S/C	Subcutaneous
St. boiled	Boiled strawberry extract group
St. fresh	Fresh strawberry extract group
St. frozen	Frozen strawberry extract group
UPLC/ESI-MS	Ultrahigh-performance liquid chromatography coupled with electrospray ionization mass spectrometry detection

Conflicts of interest

The authors have confirmed that there are no conflicting interests.

Funding

The authors received no specific funding for this work.

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