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Supplementary data

Poly(ginger oil) organoparticles as a promising neuroprotective agent: *in vivo* evidence from an aluminum chloride-induced neurodegenerative model in rat

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Table S1. Plants reported to have anticholinesterase activity and potential effects on memory, Alzheimer's disease, and dementia. (The references cited in Supplementary Table S1 were included in the main text and reference list).

Herbal/plant	Mode of action	References
	Memory impairment	
Valeriana officinalis L.		[1,2]
Abrus precatorius L.		[3]
Bryophyllum pinnatum L. .		[3]
Centella asiatica (L.) Urb.		[4–7]
Convolvulus pluricaulis		[8–13]
Dioscorea mangenotiana J. Miege		[3]
Evolvulus alsinoides L.		[11]
Ficus exasperate Vahl.		[14,15]
Ficus racemosa		[15]
Jatropha curcas L.		[15]
Musa sapientum L.		[3]
Myristica fragrans Houtt.		[16]
Tinospora cordifolia		[17–19]
Salvia officinalis L.		[20,21]
Spondia smombin L		[3]
	Alzheimer's disease and dementia	
Ocimum basilicum L.		[3]
Cissampelos pareira		[22]
Juglans regia L.		[23]
Cuminum cyminum L.		[24]
Crocus sativus		[25,26]
Melissa officinalis L.		[27–29]
Rosmarinus officinalis L.		[30]
Piper nigrum L.		[31]
Ginkgo biloba L.		[32–35]
Ginkgo ginseng		[35]
Emblica officinalis Gaertn.		[36,37]

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Table S1. *Cont.*

Herbal/plant	Mode of action	References
	Anticholinesterase activity	
Brassica alba (L.) Rabenh.		[38]
Brassica nigra (L.) W. D. J. Koch.		[38]
Bacopa monnieri		[39–43]
Camellia sinensis (L.) Kuntze.		[44]
Celastrus paniculatus		[44–47]
Cinchona officinalis L.		[23]
Citrus aurantifolia L.		[23]
Commiphora whighitti		[17,48,49]
Coriandrum sativum L.		[50]
Curcuma longa		[51–53]
Evolvulus alsinoides		[1]
Ferula assafoetida L.		[38]
Glycyrrhiza glabra		[54,55]
Humulus lupulus L.		[38]
Juglans regia L.		[38]
Juniperus sabina L.		[56–59]
Ilex paraguariensis		[56–59]
Lepidium meyenii		[60–62]
Moringa oleifera		[63–65]
Myristica fragrans		[66–69]
Magnolia officinalis		[70–79]
Nardostachys jatamansi		[80–82]
Panax ginseng		[83–86]
Punica granatum L.		[87,88]
Rheum officinale L.		[38]
Salix alba L.		[38]
Zizyphus vulgaris L.		[3]
Zingiber officinale		[89–91]
Withania somnifera		[92,93]

1. Tissue homogenization

Tissue samples were weighed on ice and homogenized in a general tissue homogenization buffer (GDH; 50 mM KH_2PO_4, 10 mM EDTA, pH 7.4). Briefly, 500 mg of tissue was initially homogenized with 1 mL of GDH buffer using a mechanical homogenizer while maintaining the samples on ice. An additional 1 mL of buffer was then added, and homogenization was continued using an ultrasonic homogenizer under cooling conditions to minimize heat generation. The samples were finally adjusted to a total volume of 5 mL with GDH buffer. Homogenates were centrifuged at 9500 rpm for 30 min at 4 °C, and the resulting supernatants were collected and stored at −80 °C until analysis [1].

2. Preparation of erythrocyte samples

Blood samples collected into EDTA-containing tubes were used for erythrocyte preparation. Briefly, 1 mL of whole blood was mixed with 2 mL of 0.9% NaCl and centrifuged at 3000 rpm for 15 min. The supernatant was removed, and the washing step was repeated when necessary. The resulting erythrocyte pellet was resuspended in 2 mL of GDH buffer and stored at −80 °C until analysis [1].

3. Glutathione determination

GSH levels were determined using a DTNB-based spectrophotometric method. For erythrocyte samples, 200 µL of EDTA-treated blood was diluted with 1.8 mL of distilled water to induce hemolysis and then

mixed with 3 mL of precipitating solution containing EDTA, NaCl, and metaphosphoric acid. After 5 min, the samples were filtered, and 2 mL of the filtrate was mixed with 8 mL of phosphate buffer (pH 7.2). The samples were incubated at 60 °C for 10 min, cooled to room temperature, and reacted with DTNB. Absorbance was measured at 412 nm.

For tissue samples, 1 mL of supernatant was mixed with 5 mL of phosphate buffer and incubated at 60 °C for 10 min. After cooling to room temperature, 1 mL of DTNB solution was added, and absorbance was measured at 412 nm. A phosphate-buffer blank was used for background correction [1].

4. Glutathione s-transferase activity

GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate and reduced glutathione (GSH) as the co-substrate. The reaction mixture contained PBS, CDNB, GSH, and tissue supernatant. Absorbance changes were monitored at 340 nm at 15- or 30-s intervals for 1.5–3 min. Appropriate sample and reagent blanks were included in the measurements [1].

5. Glutathione reductase activity

glutathione reductase (GR) activity was determined by monitoring the NADPH-dependent reduction of oxidized glutathione (GSSG). The reaction mixture consisted of phosphate buffer, NADPH, GSSG, and tissue supernatant. The decrease in absorbance was monitored at 340 nm at 30-s intervals for 5 min. The phosphate buffer contained EDTA and was adjusted to pH 7.2. NADPH and GSSG solutions were prepared separately and added to the reaction mixture immediately before measurement.

All spectrophotometric measurements were performed under controlled conditions, and the same procedure was applied to the experimental groups to ensure comparability among samples [1].

6. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity in tissue homogenates was determined according to the method originally described by Paglia and Valentine (1967) and modified by Wendel (1981) (Turan and Çelik; 2016). The assay is based on the reduction of hydrogen peroxide (H_2O_2) by reduced glutathione (GSH), resulting in the formation of oxidized glutathione (GSSG). GSSG is subsequently reduced back to GSH by GR in an NADPH-dependent reaction. The oxidation of NADPH during this coupled reaction results in a decrease in absorbance, which was monitored spectrophotometrically at 366 nm.

The reaction buffer consisted of potassium phosphate buffer (KPi; pH 7.0) containing EDTA and sodium azide. Fresh GSH (10 mM), NADPH (2.5 mM), GR (6 U/mL), and H_2O_2 (12 mM) solutions were prepared immediately before the assay. The H_2O_2 solution was protected from light until use.

For the assay, the blank contained 0.6 mL KPi buffer, 0.1 mL GSH, 0.1 mL NADPH, 0.1 mL GR, and 0.1 mL H_2O_2. For tissue samples, 0.4 mL KPi buffer, 0.2 mL tissue homogenate, 0.1 mL GSH, 0.1 mL NADPH, 0.1 mL GR, and 0.1 mL H_2O_2 were combined. The reaction was initiated by addition of H_2O_2, and the decrease in absorbance was recorded at 366 nm at 1-min intervals for 5 min. The change in absorbance of the sample was corrected against the corresponding blank. GPx activity was calculated using the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}) and expressed according to the amount of tissue protein used in the assay [1].

7. Catalase activity

Catalase (CAT) activity was determined in tissue supernatants and erythrocyte preparations using a spectrophotometric method based on the decomposition of hydrogen peroxide (H_2O_2), Catalase catalyzes the conversion of H_2O_2 to water and molecular oxygen, and the decrease in H_2O_2 concentration was monitored by measuring the decrease in absorbance at 240 nm.

The assay was performed using 50 mM phosphate buffer and freshly prepared H_2O_2 substrate solution. The H_2O_2 substrate was prepared from a 35% H_2O_2 stock solution using phosphate buffer and was prepared freshly before each assay. The reaction was initiated by addition of the sample to the H_2O_2-containing reaction mixture.

For the measurement, 2.99 mL of H_2O_2-containing phosphate buffer was combined with 0.01 mL of tissue supernatant or erythrocyte preparation. The corresponding blank contained phosphate buffer and H_2O_2 without biological sample. Following sample addition, the cuvette was immediately mixed and the absorbance was monitored at 240 nm. Absorbance changes were recorded at 15-s intervals for 5 min, and the linear decrease in absorbance over a 1-min interval was used for calculation of CAT activity [1].

8. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined in tissue homogenates using a spectrophotometric method based on the inhibition of the photochemical reduction reaction in the presence of riboflavin. The assay was performed using phosphate buffer containing EDTA, riboflavin, and *o*-dianisidine dihydrochloride. A series of SOD standards was prepared to establish a calibration curve, and the enzyme activity of the tissue samples was determined from the corresponding standard curve.

The SOD assay buffer consisted of 50 mM phosphate buffer containing 0.1 mM EDTA and was adjusted to pH 7.8. A riboflavin solution was prepared at a final concentration of 0.2 mM in phosphate buffer adjusted to pH 7.5. An *o*-dianisidine·2HCl solution was prepared at a concentration of 6 mM and freshly prepared on the day of analysis. A lyophilized SOD preparation was dissolved in cold distilled water to obtain a stock solution of 120 U/L and stored at 4 °C until use. Working SOD standards of 2, 4, 6, 8, and 10 U/L were prepared by serial dilution of the stock solution with cold distilled water. The resulting standard series was used to generate the calibration curve for SOD activity determination.

Tissue homogenates, reagent blanks, and SOD standards were prepared according to the assay protocol. The reaction mixtures contained SOD assay buffer, *o*-dianisidine·2HCl, riboflavin, and either tissue homogenate or SOD standard, as appropriate. The riboflavin solution was added at 30-s intervals to minimize differences in reaction initiation time. Immediately after addition, each tube was thoroughly mixed using a vortex mixer, and the initial absorbance was measured at 460 nm against the reagent blank.

Following the initial measurement, the reaction tubes were exposed to a fluorescent light source for 8 min under the conditions specified for the assay. After incubation, the absorbance was measured again at 460 nm against the corresponding blank. The difference between the absorbance values measured at 0 and 8 min was calculated for each standard and sample [1].

9. Malondialdehyde measurement

Malondialdehyde (MDA), as an indicator of lipid peroxidation, was determined in tissue supernatants and erythrocyte preparations using a thiobarbituric acid (TBA)-based spectrophotometric method. The assay was based on the formation of a colored MDA–TBA reaction product following heating of the samples under acidic reaction conditions. Absorbance was measured spectrophotometrically at 532 nm.

For sample preparation, tissue supernatant was mixed with phosphate buffer, butylated hydroxytoluene (BHT), and trichloroacetic acid (TCA). The mixture was vortexed and kept at −20 °C for 2 h to facilitate protein precipitation and to minimize further lipid peroxidation. Following incubation, the samples were allowed to thaw and centrifuged under refrigerated conditions. The resulting supernatant was collected for the subsequent derivatization step.

An aliquot of the supernatant was transferred to a clean tube and mixed with EDTA and thiobarbituric acid (TBA) solution. The reaction mixture was vortexed and immediately incubated at 90 °C for 15 min in a water bath. After incubation, the samples were cooled to room temperature, and their absorbance was measured at 532 nm.

The same procedure was applied to erythrocyte samples. In addition to the measurement at 532 nm, erythrocyte samples were also measured at 600 nm to account for nonspecific background absorbance. Reagent blanks were prepared by replacing the biological sample with phosphate buffer and were used for correction of the corresponding absorbance measurements.

The phosphate buffer was prepared using NaCl, Na_2HPO_4, and NaH_2PO_4 and adjusted to pH 7.4. BHT was prepared in ethanol, whereas TCA was prepared in distilled water. EDTA and TBA solutions were prepared separately, with TBA dissolved in 0.05 N NaOH. Fresh reagent solutions were prepared as required according to the assay protocol.

MDA levels were determined from the corrected absorbance values obtained after the TBA reaction. The results were normalized to the protein concentration of the corresponding tissue samples and expressed according to the units used for the biochemical analyses [1].

10. Total antioxidant status

Total antioxidant status (TAS) was determined using an ABTS radical cation-based spectrophotometric method. In this assay, ABTS is oxidized to the relatively stable ABTS radical cation (ABTS•+) in an acidic medium. Antioxidants present in the sample inhibit the color intensity of ABTS•+ in proportion to their total antioxidant capacity. The decrease in absorbance was therefore used as an indirect measure of the total antioxidant capacity of the sample.

The assay was performed using acetate buffer solutions at pH 5.8 and pH 3.6. The ABTS•+ reagent was prepared in the acidic acetate buffer in the presence of hydrogen peroxide and allowed to equilibrate before use. Trolox was used as the calibration standard. Trolox stock solution was prepared at 30 mM, and working standards of 0.1, 0.5, 1, 2, and 3 mM were freshly prepared on the day of analysis.

For the assay, 5 µL of serum or tissue sample was combined with 200 µL of Reagent 1 and 20 µL of Reagent 2 containing ABTS•+. The initial absorbance was recorded before the reaction, and the final absorbance was measured after a 5-min incubation period. Absorbance measurements were performed at 660 nm. The change in absorbance was calculated by subtracting the initial absorbance from the final

absorbance, and TAS values were obtained using the Trolox calibration curve. Results were expressed as mmol Trolox equivalent/L [2].

11. Total oxidant status

Total oxidant status (TOS) was determined using a colorimetric spectrophotometric method based on the oxidation of ferrous ions to ferric ions in the presence of oxidizing substances in the sample. The resulting ferric ions form a colored complex with *o*-dianisidine, and the intensity of the color is proportional to the total oxidant content of the sample.

For the TOS assay, a Clark and Lubs solution containing KCl and hydrochloric acid was prepared and adjusted to pH 1.8. *o*-Dianisidine dihydrochloride and ferrous ammonium sulfate were incorporated into this solution to prepare the first reagent. Hydrogen peroxide solution was used as the second reagent. The H_2O_2 concentration was verified spectrophotometrically at 240 nm before use.

For each measurement, 5 µL of sample was mixed with 200 µL of Reagent 1 and 10 µL of Reagent 2. The initial absorbance was measured before addition of Reagent 2, and the final absorbance was recorded after the reaction reached a stable plateau, approximately 3–4 min after mixing. Absorbance was measured at 444 nm. A linear calibration procedure was used, and the change in absorbance was calculated as the difference between the final and initial measurements.

TOS values were calculated relative to the corresponding standard and expressed in the units used for the assay calibration [2].

12. Oxidative stress index

The oxidative stress index (OSI) was calculated from the ratio of TOS to TAS according to the previously described approach. Because TOS and TAS were expressed in different units, TAS values were converted from mmol Trolox equivalent/L to µmol Trolox equivalent/L before calculation.

The OSI was calculated using the following Equation (1):

$$\text{OSI (arbitrary units)} = [\text{TOS (µmol Trolox equivalent/L)} / \text{TAS (µmol Trolox equivalent/L)}] \times 100 \quad (1)$$

Higher OSI values indicate a greater imbalance between oxidant and antioxidant status [2].

References

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