Protective effects of gingerol on streptozotocin-induced sporadic Alzheimer's disease: emphasis on inhibition of β-amyloid, COX-2, alpha-, beta - secretases and APH1a.

Ali M. El Halawany[1], Nesrine S. EL Sayed[2,3], Hossam, M. Abdallah[1,4,*], and Riham Salah El Dine[1]

Supporting information

1.1. Plant material

Seeds of *Aframomummelegueta* K. Schum were purchased from Harraz herbal store, Cairo, Egypt. The plant was authenticated by Assistant Prof. Dr. Sherif El-Khanagry, Agriculture Museum, El-Dokki, Cairo, Egypt. A voucher specimen (AM 1307) was deposited in the herbarium of Department of Natural Product, Faculty of pharmacy, King Abdulaziz University.

1.2. Identification of gingerol

Gingerol was isolated as yellowish liquid. ^{1}H-NMR ($CDCl_3$) δ 6.82 (1H, d, J = 8.0 Hz, H-5'), 6.67 (1H,d, J = 1.6 Hz, H-2'), 6.52 (1H, dd, J = 1.6, 8.0 Hz, H-6'), 4.05 (1H, m, H-5), 3.86 (3H, s, -OCH3), 2.82 (2H, t, J =7.2 , H-1), 2.72 (2H, t, J = 7.2 , H-2), 2.55 (2H, dd, J = 2.4, 17.0 Hz, H-4a), 2.47 (1H, dd, J = 8.8, 17.0 Hz, H-4b), 1.49–1.24 (8H, m, H-6~9), 0.87 (3H, t, J = 6.4 Hz, H-10); ^{13}C-NMR ($CDCl_3$) δ 211.5, 146.5, 143.9, 132.6, 120.7, 114.4, 110.9, 67.7, 55.8, 49.3, 45.4, 36.4, 31.7, 29.3, 25.1, 22.6, 14.0; ESIMS (positive ion mode) m/z 295 $[M+1]^+$, 277 $[M+1-H_2O]^+$, 259 $[M+1-2H_2O]^+$.

1.3. Extraction, isolation and identification of gingerol

Dried ground seeds of *A. melegueta*(2 Kg) were extracted with methanol by cold maceration until exhaustion. The pooled and vacuum dried methanol extract (130 g) was suspended in water (500 ml) and partitioned successively with n-hexane (1L x 3) and $CHCl_3$ (1L x 3). The

collected hexane and chloroform soluble fractions were evaporated, separately, under vacuum to get hexane fraction (29 g) and a chloroform fraction (65 g). The $CHCl_3$-fraction was chromatographed on a silica gel column (70 cm x 8 cm) and gradiently eluted with n-hexane-EtOAc (5~20% v/v). The pooled fractions containing gingerol (6.5 g) was applied to silica gel column (15 cm x 3 cm) and eluted with n-hexane-EtOAc (9:1 v/v) to yield gingerol (4 g).

The identity of the compound was determined by comparing its ^{1}H and ^{13}C-NMR data with reported ones (Supplementary Figs. 1 and 2). The purity of the compound was verified using HPLC consisted of an Agilent 1200 system, a solvent delivery module, a quaternary pump, an auto-sampler, a diode array detector (DAD) (Agilent Technology, Baden-Wuttemberg, Germany), Agilent Zorbax XDB-C18 column (80 A°, 150 mm length × 4.6 mm, i.d., 5 µm), using 0.1 % TFA in H_2O as solvent A and MeOH as solvent B with gradient elution starting from 30 % until 90 % MeOH during 15 min, and monitored at 254 and 280 nm. Using the previous conditions, the purity of the isolated compound was 97.0 % (Supplementary Fig. 3).

2. Biological study

2.1. STZ-induced SAD

SAD was induced in mice by intracerebroventricular (ICV) injection of STZ (3 mg/kg) dissolved in saline (Mehla et al., 2013). Mice were anesthetized in order to avoid the risk of penetrating the cerebral vein running along the midline. A downward pressure above the ears was performed to stabilize the head. The needle was introduced directly through the skin and skull into the lateral ventricle which was targeted by visualizing an equilateral triangle between the eyes and the center of the skull to locate the bregma. Then, the needle was inserted about 1 mm lateral to this point. Injections were performed into the right or left ventricle randomly, made with A hypodermic needle of 0.4 mm external diameter attached to a 10 µl Hamilton microlitre syringe was used to apply the ICV injections.

2.2. Morris Water Maze

The Morris Water Maze (MWM) test was performed to assess the spatial reference learning and memory as described previously (Morris, 1984).Each mouse was exposed to two sequential training trials on each day with at least 15 minutes gap between the trials, for four days. Each mouse was quietly positioned in the water between quadrants, opposite the wall of the pool. The drop location was different for each trial. The maximum time for each trial was 120 seconds (2 minutes). If the mouse was capable to find the hidden platform during the 120 seconds, it was permitted to stay there for 20 more seconds before it was removed from the pool. Though, if the mouse failed to find the hidden platform during the 2 minutes, it was gently directed onto the platform and allowed to remain there for 20 seconds. The Mean escape latency time (MEL) is well-defined as the time needed by each mouse to allocate the concealed platform and it was noted for each mouse during each trial during the four days. After that, the average of the two trials done together on each day was considered. Day 4 mean escape latency time (MEL) was distinguished as an index of acquisition or learning. On the fifth day, a probe-trial session was performed, 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, the quadrant where the hidden platform was previously located, was recorded as an index of retrieval or memory.

2.3. Ymi Test

The Y-maze was shaped by including remarkable intra-maze signs (Ymi). In this prompted version, one vast woody painted question (unique in relation to those utilized as a part of the object recognition test and sufficiently heavy to keep the mice from moving them) was set toward the end of every arm.These objects could be recognized by surface and in addition by their paint plot. These objects were available during both and testing. A mouse was situated in the start arm and permitted to find the start and different arms for 15 min during the training stage while the novel arm was hindered with dark Plexiglas. Prior to the training and testing of every mouse, the corncob bedding was blended on the maze floor. The maze was cleaned and turned amongst preparing and testing periods to prevent the utilization of smell signals in the maze route. The terms novel, start and other indicate the spatial areas of the arms with respect

to extra maze cues. Likewise, objects at the ends of the arms stayed in the same spatial locations relative to the extra-maze cues for training and testing. Following a 4-hour interval, the mouse was set into the start area for testing and was left to investigate every one of the three arms for 5 min. If memory and novelty-seeking behaviour persisted intact, mice were probably going to enter the novel arm more than the other arm, because of the natural propensity of mice to investigate novelty. An entry was scored when a mouse's front paws crossed into an arm. The start arm was excluded in the investigation in light of the fact that the mice were put at the start of the testing experiment, which means that it was naturally biased and not orthogonal to the novel and other arms**.**

2.4. ELISA measurement of amyloid β -42

The mean of Aβ-42 concentration present in mouse brains was calculated by mouse Aβ-42 ELISA kit (Anaspec, Germany)(Mehta et al., 2000). Centrifugation of brain samples was done at 16,000 g for 20 min at 4ºC. The supernatant of each sample was diluted to bring the final guanidine concentration to 0.1M using Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween-20. The supernatant was kept on ice until assay of amyloid beta 42 levels.

2.5. Estimation of APH1a in brain homogenate

Quantitative determination of mouse gamma secretase subunit APH-1a concentrations in brain homogenate was done using mouse gamma-secretase subunit APH-1a ELISA kit (Catalog No: E0406m, EIAab, China). Addition of the samples was done to the provided microtiter plate which was coated with a biotin-conjugated polyclonal antibody which is specific for APH-1a and avidin conjugated to Horseradish Peroxidase (HRP) and left for 2 hours incubated at 37ºC. Then a TMB substrate is added to each well and only those wells that contain APH-1a, biotin conjugated antibody and enzyme-conjugated avidin showed a variation in the colour. Addition of sulphuric acid to the enzyme-substrate will stop the reaction and the colour change was measured spectrophotometrically at a wavelength of 450 nm. Calculation of the concentration

of APH-1a in the samples was done by comparing the optical density (OD) of the samples to the standard curve.

2.6. Estimation of alpha-secretase in brain homogenate

The total activity of α-secretase or tumor necrosis factor alpha converting enzyme (TACE) present in mouse brain homogenates was determined using a commercially available Sensolyte 520 TACE (α-secretase) activity kit (Anaspec, CA, USA), according to the provided instructions. Each brain tissue was homogenized in the provided assay buffer containing 0.1% (v/v) Triton-X100, incubated for 15 min. at 4°C then centrifuged and the supernatants were used. The alpha- secretase activity was determined by mixing 50 µl of supernatant with 50 µl of TACE substrate. Incubation of the mixture was performed in the dark at 37°C for 1 hour followed by addition of 50 µl of stop solution. Then to maintain the continuous measurement of enzyme activity, a FRET peptide substrate was used. The FRET pair is composed of 5-FAM (5-carboxyfluorescein) and QXL™ 520. In the intact FRET peptide, quenching of the fluorescence of 5-FAM is done by QXL™ 520. The fluorescence of 5-FAM is recovered through cleavage of the FRET peptide by the active enzyme, and then monitored constantly at excitation/emission = 490 nm/520 nm. The level of α-secretase enzymatic activity was proportional to fluorescence with the intensity of fluorescence which was expressed as fluorescence units.

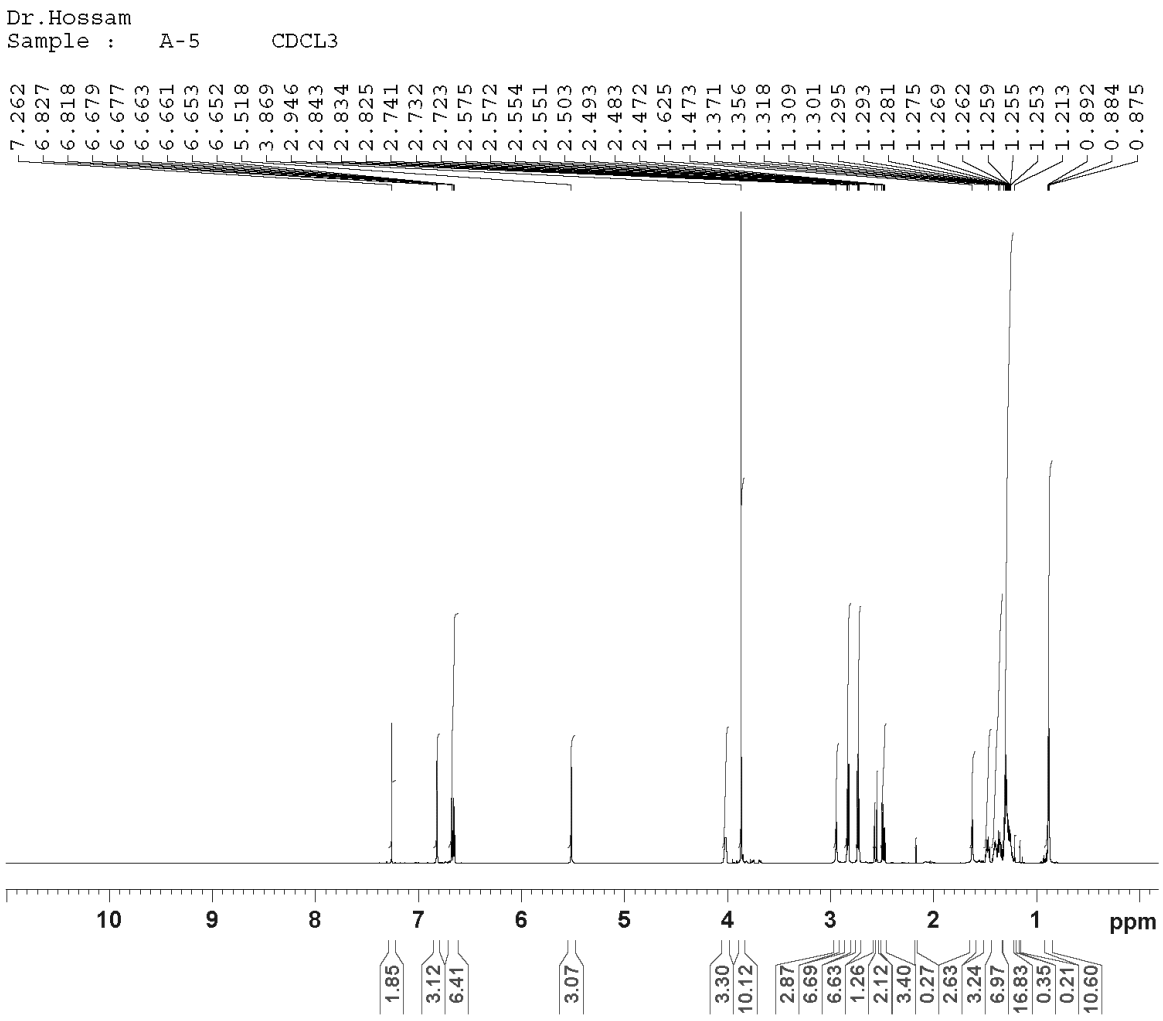
2.7. Estimation of beta-secretase in brain homogenate

In this assay, a beta-secretase-specific peptide was used and conjugated to two reporter molecules EDANS and DABCYL. Then, the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety in the un-cleaved form. The physical cleavage of the peptide by β-secretase separates EDANS and DABCYL resulting into release of a fluorescent signal. The level of secretase enzymatic activity in the brain samples is proportional to the level of fluorescence intensity. For the determination of the β-secretase activity, 50 µl of sample were added to 50 µl of reaction buffer and 2 µl BACE substrate. The reaction mixture was then incubated at room temperature for 1 hr in the dark. A Fluostar galaxy fluorometer (excitation at

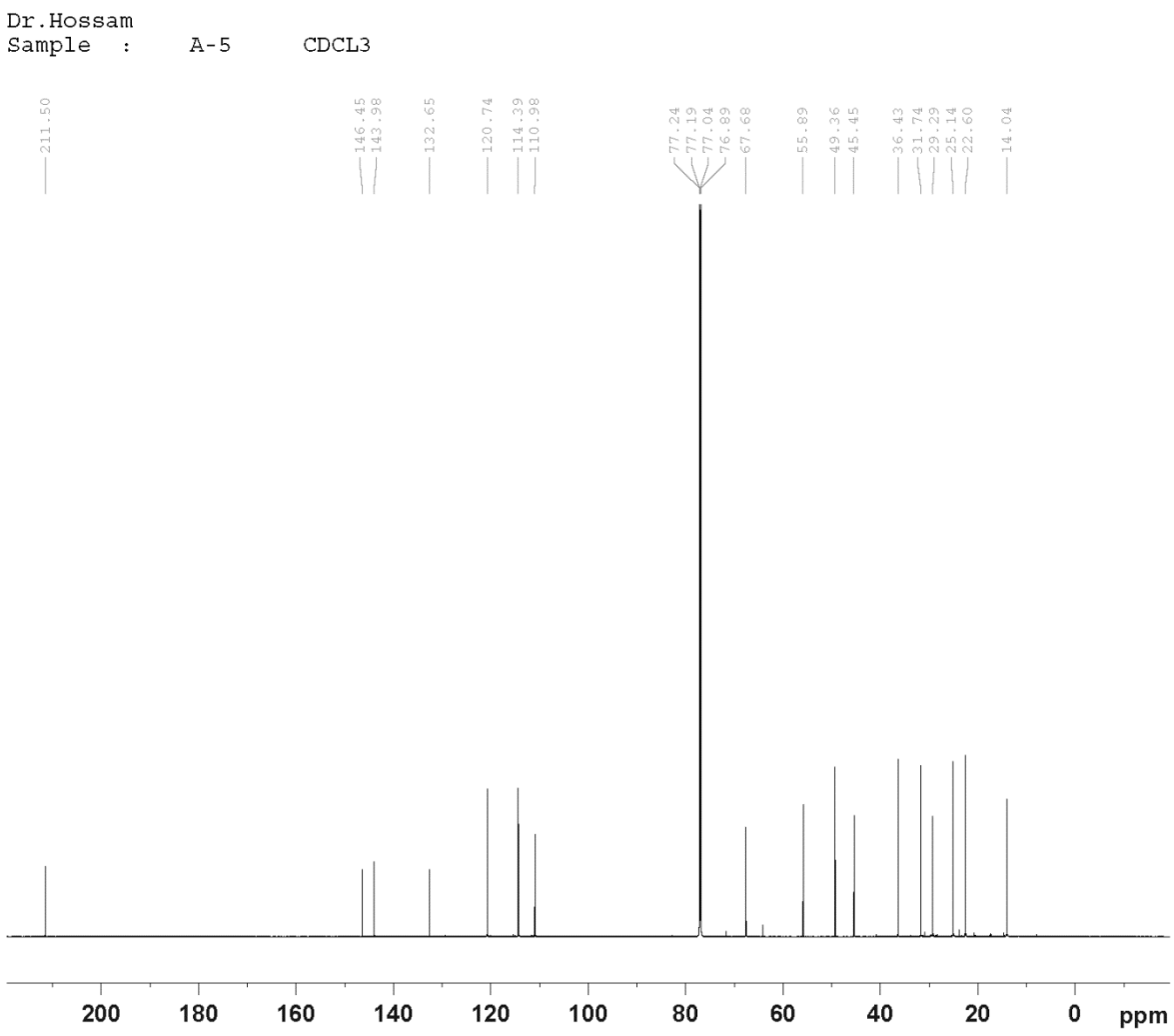
355 nm and emission at 510 nm was used to measure the fluorescence. A linear relation was found between the enzyme activity and the increase of fluorescence, then the activity was expressed as fluorescence units.

2.8. Immunohistochemistry for COX-2 deposition in the brain

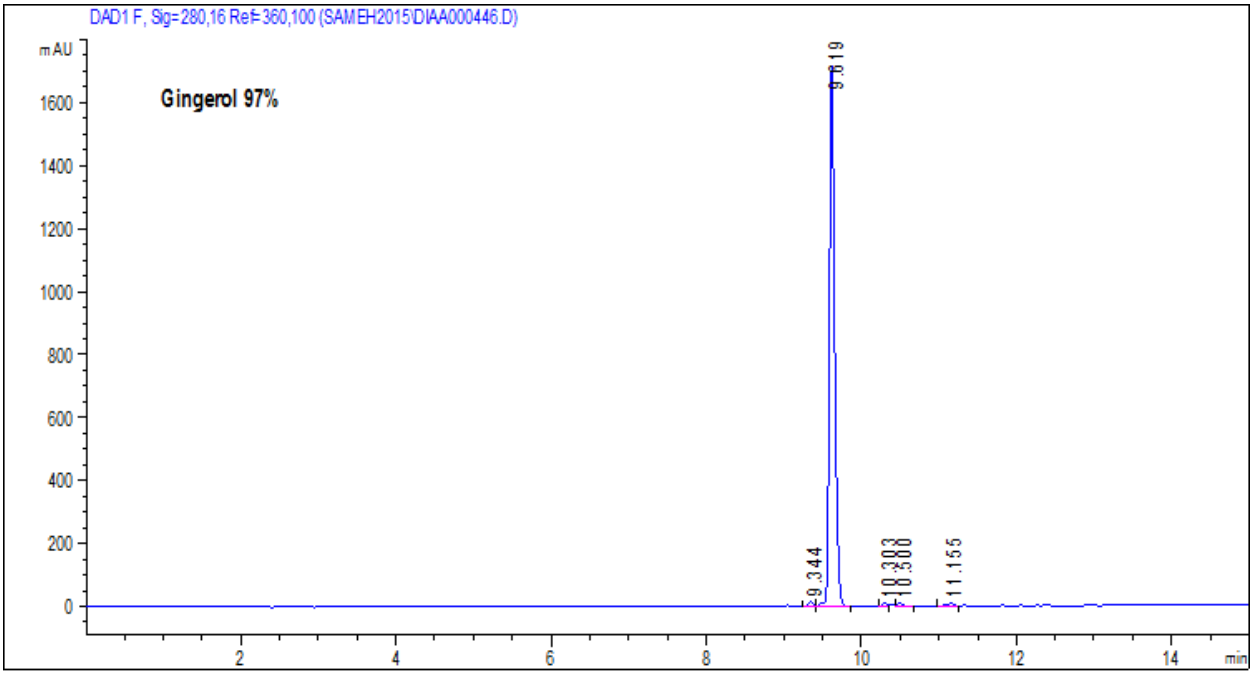
According to Ramos-Vara et al (Ramos-Vara et al., 2008), fixation of the brains from each group was done in 4% formaldehyde, then tissues were dehydrated for 15 minutes in 80% ethanol; then 20 minutes in 95% ethanol; three times, 20 min. each, in 100% ethanol; and finally, three times, 20 min. each, in xylenes. Tissues were then fixed in paraffin, sectioned 5- to 8-µm thick, and the samples were placed on poly-L-lysine-coated slides. After that, sections were air dried; the slides were incubated for 2 hours at 58°C. Two staining dishes were filled with 200 ml of trilogy solution; the slide rack was added in one of them. The two staining dishes were then put in electric pressure cooker (filled with 700 ml of deionized water) for 15 min. Fifteen minutes later, the slide rack was transferred into another hot rinse containing staining dish for de-paraffinization and rehydration of slides for 5 min. Then the slides were rinsed in TBS buffer. Staining started with adding 200 ml of peroxide block (reduce nonspecific background by endogenous peroxide) for 10 min. The slides were then rinsed with TBS buffer and any extra liquid was rubbed away. Super block was used to block nonspecific staining and slides were washed with TBS buffer. Then the primary antibody was added onto the slides, and was allowed to incubate for 15 to 30 min. The slides were rinsed with TBS buffer and any excess liquid was wiped away. Econo Tek Biontinylated antipolyvalent was added onto slides and left to incubate for 30 min. The slides were then washed with TBS buffer. 200 µl of DAB Chromogen were added to DAB substrate to be mixed, the mixture was added to slides and was incubated for 5 to 15 min. A counter stain (Hematoxylin) is added to slides for visualization, it was incubated on slides for one min. The slides were finally washed with TBS buffer. To cover slip, the slides were run through graded alcohol 50%, 95% and 100% each for 10 min. then through xylene solution 10 min. each, three times. Mounting medium was added onto slides followed by cover slips and were left to dry.



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Supplementary Table S1. Effect of gingerol (10mg/kg and 20 mg/kg) on the mean escape latency in Morris water maze

Days	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
1st day MEL(s)	40.60±2.04	40.32±1.27	39.92±1.02
2nd day MEL(s)	22.7±0.87	21.55±1.21	22.92±1.04
3rd day MEL(s)	18.9±0.55	19.18±0.86	18.32±0.88
4th day MEL(s)	12.625±1.16	12.64±0.91	12.75±0.94

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to the behavioral tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S2.Effect of gingerol (10 and 20 mg/kg) on the Morris water maze probe test

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
Mean Time spent in target quadrant(s)	38.10 ± 2.571	37.48±1.90	38.77 ±2.01

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally

injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to the behavioral tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S3. Effect of gingerol (10 and 20 mg/kg) on the novel arm count in the intra-maze cue version of the Y-maze

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
Novel arm Count	7.778 ± 0.5958	8.122 ± 0.4690	8.004 ± 0.5236

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to the behavioral tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S4.Effect of gingerol (10 and 20 mg/kg) on mean mouse amyloid beta 42 concentration using ELISA assay

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
Mean amyloid beta 42 (pg/ml)	1.863 ± 0.118	1.729 ± 0.161	1.772 ± 0.147

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was

carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S5. Effect of gingerol (10 and 20 mg/kg) on mean mouse APH-1a concentration using ELISA assay

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
Mean APH-1a (ng/ml)	19.78 ± 1.26	20.11 ± 1.03	20.28 ± 1.15

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S6. Effect of gingerol(10 and 20 mg/kg) on mean mouse α -secretase concentration using ELISA assay

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
Mean α-secretase (µM)	1.06 ± 0.09	0.921 ± 0.07	0.925 ± 0.06

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was

carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.

Supplementary Table S7. Effect of gingerol(10 and 20 mg/kg) on mean mouse β –secretase cleavage activity using ELISA assay

Group	Saline (untreated)	Gingerol (10mg/kg)	Gingerol (20mg/kg)
RFU/µg protein	126.95 ± 4.09	127.06 ± 4.18	127.88 ± 4.55

Animals were divided into 3 groups. Group saline received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with saline for seven days. Group gingerol (10mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (10mg/kg) for seven days. Group gingerol (20mg/kg) received a single ICV dose of saline; then, after 2 weeks, animals were intraperitoneally injected with gingerol (20mg/kg) for seven days. Animals from the 3 groups were subjected to tests after the 3rd week of injections. Each result represents the mean value for 10 mice ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by the Tukey-Kramer multiple comparison test with the aid of GraphPad Prism Software version 5.