Protective effects of *Abelmoschus moschatus* seed extract on neurotransmitter system of developing brain of Wistar rats with gestational and post-natal exposure of sodium fluoride

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**Abstract**

**Aims:** Fluoride known environmental pollutant and also neurotoxicant and functions through oxidative stress and excitotoxicity mechanisms in brain. Balanced levels of neurotransmitters (NTs) are essential for healthy condition while their imbalanced status leads to illnesses and associated abnormalities. The present study focused on maternal as well as post-natal exposure of fluoride and its effects on NTs and protective effects of *Abelmoschus moschatus* seed extract through regulation of NTs system. **Materials and Methods:** The pregnant Wistar rats were randomly categorized into six groups of five animals each. Group I is of control rats which received normal tap water. Group II is sodium fluoride (NaF) exposed group with 20 ppm (or 20 mg/kg body wt.) in their drinking water. Group III and Group IV rats were treated with *A. moschatus* aqueous (AMAE) and ethanolic (AMEE) extract (at the rate of 300 mg/kg body wt./day/rat), respectively, along with NaF water (20 ppm). Group V and Group VI rats were treated with AMAE and AMEE (300 mg/kg body wt./day/rat) respectively. On 1st, 7th, 14th, 21st, and 30th day (postpartum days), the pups were sacrificed to assess NTs levels of brain of all experimental groups. **Results:** The epinephrine and glutamate levels were increased, while nor-epinephrine, dopamine, serotonin, and acetylcholine levels were decreased significantly ($P < 0.001$) in NaF-fed rats with respect to control group and were restored on the treatment of AMAE and AMEE toward control. In addition, monoamine oxidase (MAO-A and MAO-B) activity also increased in NaF intoxicated rats than the control, and its activity was reverted to normal in NaF-received rats along with AMAE and AMEE. **Discussion:** These findings suggested that NaF exposure during developmental stages alter the NTs levels where as their levels are regulated on the treatment of *A. moschatus* toward NaF. AMEE showed better efficacy over AMAE. **Conclusion:** It can be concluded that the seed extract of *Abelmoschus* has therapeutically significant efficacy in protection from NaF toxicity.

**Key words:** *Abelmoschus*, acetylcholine, excitotoxicity, fluorosis, monoamine oxidase, neurotransmitter

**INTRODUCTION**

Excessive intake of fluoride causes toxic effects on soft tissues and organs, giving rise to a vast array of symptoms and pathological changes in addition to the illusory effects on skeleton and teeth.[1] Interestingly, an association between excessive consumption of fluoride and dysfunction of the central nervous system has been known. Chronic exposure of pregnant rats to high concentrations of sodium fluoride (NaF) has been reported to induce disturbances in the development of brain in offspring of rats,[2] and fluoride is known to cross the placenta[3,4] and the blood–brain barrier[5] and accumulate in fetal brain tissue. Moreover, it is also transferred to the feeding pups from their mother’s through milk.[6] Once it enter into the brain, it can cause adverse effects on the brain cell structure, metabolism, enzymes, the oxidant-antioxidant status and on neurotransmitters (NTs) and thus overall metabolism of brain.[7,8] In addition, impairment of motor

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coordinated, learning, and memory as a result of prenatal exposure to NaF (5 mg/kg) was found.\[9\]

NTs released at synaptic junctions and regulate many physiological functions with a wide variety of processes including emotions, fear, pleasure, joy, anger, mood, memory, cognition, attention, concentration, alertness, energy, appetite, cravings, sleep, and the perception of pain through receptors. Balanced status in the levels of NTs is essential for healthy lives. NTs affect each other, and an imbalance of one NT may affect balance of another. High levels of fluoride exposure during developing period have an adverse effect on levels of fetal NTs.\[10\] Fluoride administration resulted in a significant decrease in acetylcholine (ACH) esterase in the brains of rats\[11\] and mice.\[12\] Altered NTs levels and their abnormal functioning on NaF administration are resulted in the decreased learning and memory ability.\[13\] Chronic fluoride poisoning causes a drop in the level of transmitters, such as 5-hydroxytryptamine (5-HT) and norepinephrine (NE), and also the density of NE-specialized α1-receptors, leading to abnormalities in nerve function.\[14\] Yu et al.\[15\] reported that the lowered levels of NE, and elevated levels of epinephrine in endemic fluoride exposed brains of fetuses. A significant decrease in the level of NE, dopamine (DA), and 5-HT in fluoride-exposed animals has earlier been reported.\[16\] It is also, reported that the decrease in NE, and ACh and elevated levels of epinephrine in NaF exposed rat.\[16\]

Recently research reports showed the therapeutic benefits of plants secondary metabolites particularly phenolic compounds. Such polyphenolic products such as curcumin,\[17\] silymarin,\[18\] quercetin,\[19\] and resveratrol\[20\] in addition to vitamins, Ca, Mg, etc., were found to have beneficial effects treated against NaF-induced oxidative stress. In our earlier study, quercetin had shown protection against NaF toxicity on brain.\[20\] The possible mechanism of these natural products to treat against NaF is their reducing potential of free radicals production. One such plant with constituent with potential of anti-oxidant and possible neuroprotective properties is *A. moschatus*. *A. moschatus*, an aromatic and medicinal plant popularly known as ornamental okra, the seed extract has showed the occurrence of flavonoids, phenols, saponins, carbohydrates, terpenoids, myrcetin, alkaloids, and steroids.\[21\] Phenolic compounds such as oligomeric catechins and flavonol derivatives richly deposited in Okra pods, while the epidermis polyphenolic profile is composed predominantly by hydroxycinnamic and quercetin derivatives.\[22\] Okra flour has anti-oxidant properties.\[23\] Neuroprotective effect of total flavonol of *Abelmoschus* against cerebral ischemia injured rats through inhibition of lipid peroxidation and stimulation of NO release was considerable.\[24\] Neuronal functions, restoration of learning and memory deficits in mice treated with dexamethasone were reverted on administration of *A. esculentus* extract.\[25\] In our earlier study, we reported that the neuroprotective effects of seed extract of *A. moschatus* against NaF-induced behavior and neurodegenerative alterations in brain in adult rats\[26\] and protective effects on body weight, brain weight, brain-somatic index along with oxidative stress in gestationally intoxicated fluoride rats.\[27\] *A. moschatus* medik seems to be a promising one for strengthening memory, anticholinesterase activity, and antioxidant property and it may be protective candidate for relating neurodegenerative disorders such as dementia and Alzheimer’s disease.\[28\] Hence, this study is undertaken to reports the protective nature of aqueous and ethanolic extract of *A. moschatus* against fluoride-induced changes in the levels of NTs and MAO activity during pre- and post-natal fluoride exposure which provide critical data on fate of NTs in growth and maturation of brain.

**MATERIALS AND METHODS**

All experiments were performed on healthy, developing Wistar rats. All animals received humane care in compliance with the guidelines of the CPCSEA. The experimental protocols were approved by the Institutional Animal Ethical Committee and conducted according to the CPCSEA guidelines on the use and care of experimental animals. Before dosing, they were acclimatized for 7 days to light from 6:00 AM to 6:00 PM alternating with 12 h dark. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25°C ± 2°C. Rats were allowed with standard chow diet throughout the experiment and water *ad-libitum*. Totally 30 females Wistar rats and 15 males were categorized into separate cages as in 2:1 ratio for breeding. Once they conceived, females were randomized into six groups of five animals each and treated as below for pre-natal (gestation - 21 days) and post-natal (30 days) period.

1. Group I – Control rats (untreated) received normal tap water.
2. Group II – Rats received NaF (20 ppm) in their drinking water.
3. Group III – Rats received NaF (20 ppm) + *A. moschatus* aqueous seed extract (AMAE) at the rate of 300 mg/kg body weight/rat/day.
4. Group IV – Rats received NaF (20 ppm) + *A. moschatus* ethanolic seed extract (AMEE) at the rate of 300 mg/kg body weight/rat/day.
5. Group V – Rats received AMAE at the rate of 300 mg/kg body weight/rat/day.
6. Group VI – Rats received AMEE at the rate of 300 mg/kg body weight/rat/day.

After the treatment, all rat pups from each group were sacrificed at different age groups such as post-natal Day 1, 7, 14, 21, and 30. Brain was removed and used for the estimation of NTs.

**Brain Catecholamines Level**

Brain tissue samples were weighed and homogenized in acidicified butanol. DA, epinephrine, NE, and 5-HT were estimated on spectrofluorometric method.\[29\]
Sample Preparation

Brain tissue was homogenated with 0.25M sucrose solution. The homogenate was centrifuged and to the supernatant 1 ml of acid butanol was added, mixed well, and centrifuged again. Butanol layer was collected, mixed with 2.5 ml of heptane and 1.5ml of double-distilled water. The contents were centrifuged, the aqueous layer was collected and mixed with 200 mg of acid alumina and 1 ml of 2M sodium acetate. pH 8 was adjusted with 1N NaOH and again centrifuged. Supernatant was used for serotonin assay and precipitate (acid alumina) was used for catecholamines (epinephrine, norepinephrine, and DA) assay.

Serotonin Assay

A volume of 1.5 ml of supernatant was added to reaction mixture (0.1 ml of 100 mM cysteine, 0.5 ml of 0.1 N Hcl, 1% O-phthalaldehyde, OPT - in acetone-free methanol). 0.1 ml of sodium metaperiodate was added after 20 min incubation at room temperature. The quantification was done on spectrofluorometer with excitation 410 nm and emission 510 nm.

Catecholamines Assay

Epinephrine

Acid alumina was washed with double-distilled water twice, mixed with 0.2N glacial acetic acid. Centrifuged the contents and to the supernatant 0.1 ml of 100 mM EDTA (pH 6.3) and 0.1 ml of 100 mM iodine was added. Finally, on addition of 0.2 ml of sodium sulfite, measured on spectrofluorometer for epinephrine with excitation 410 nm, emission 500 nm.

NE

After completion of experiment for epinephrine, tubes were kept in hot water bath for 2 min and cooled the tubes at room temperature. The nor-epinephrine excitation 387 nm, and emission 487 nm was measured.

DA

Again the tubes were allowed to hot water bath for 5 min and cooled down to room temperature. Measured on spectrofluorometer with excitation 310 nm, emission 410 nm for DA.

Glutamate (Glu) assay

Glu was assessed by the modified method of Raju et al. Brain tissue homogenate was prepared with 1 ml of 3% perchloric acid and centrifuged it 0.2 ml of 1% ninhydrin solution was added to the supernatant then followed by cooling to 0°C. Then, the contents were mixed with reaction mixture (0.4 ml of guanidine carbonate, 1 ml of 100 mM lead acetate, 0.5 ml of 0.1N NaOH, 6ml of distilled water). The values were noted down at 420 nm with UV-visible spectrophotometer on addition of 2 ml of 1% 2,4-dinitrophenylhydrazine (dissolved in 0.01N Hcl in ice-cold condition).

ACH estimation

ACh estimation is carried out by the method of Hestrin. Reaction mixture was prepared with alkaline hydroxylamine reagent by mixing equal volumes of 2M hydroxylamine hydrochloride (freshly prepared) and 3.5N sodium hydroxide. A volume of 2 ml of reaction mixture was added to the 1 ml of tissue supernatant and adjust pH to 1–1.4 with 1 ml of acid. On addition of 1 ml of 0.37M (in 0.1N Hcl) ferric chloride purple-brown color was developed and which was measured at 540 nm on spectrophotometer.

Monoamine oxidase (MAO) activity

MAO activity was estimated in brain by the method of Tipton et al.

Sample preparation

Tissue homogenate was centrifuged and supernatant was collected into separate tubes and again centrifuged. Precipitate was used for the assay of MAOs activity.

MAO-A

To the supernatant (250 µl), 250 µl of serotonin and 250 µl of buffer were added and leave at room temperature for 20 min. After that, 200 µl of 1M Hcl and 5 ml of butyl acetate were added. The organic phase layer was read at 280 nm on spectrophotometer.

MAO-B

For the assay of MAO-B, the same method of MAO-A was repeated but in the final step 5 ml of cyclohexane was added and organic phase layer was measured at 280 nm on spectrophotometer.

Statistical analysis

Data are expressed as means ± standard error of the mean. Data comparisons were carried out using one-way analysis of variance followed by t-test to compare means between the different treatment groups. Differences between all possible pair-wise comparisons were tested and *P* < 0.01 is considered statistically significant.

RESULTS

Serotonin (5-HT)

Decreased 5-HT level [Figure 1] in the brain tissue of NaF intoxicated pups was observed when compared to control.
group of pups. The percent of decreased in 5-HT levels is gradually increased from day 1 to day 30. On the treatment of AMAE and AMEE against fluoride, the restored levels of 5-HT were observed.

Monoamine NTs

**Epinephrine**

An increase in the levels of epinephrine was found in brain tissue of NaF-treated rat pups [Figure 2] and its levels were progressively increased from day 1 to day 30 compared to same age controls, but significant from day 1 to day 14. Epinephrine levels are reverted on the treatment of AMAE and AMEE along with NaF combination. AMAE showed better efficacy over AMEE.

**NE**

The NE levels were decreased in the brain of NaF-administered rats when compared to control group of same-aged pups [Figure 3]. The per cent of decrease in NE levels were progressively declined with increasing age. The reverted levels of NE were observed on AMAE and AMEE treatment toward NaF.

**DA**

Decreased levels of DA [Figure 4] were observed in day 1 (−28.07%), day 7 (−23.33%), day 14 (−24.19%), day 21 (−17.18%), and day 30 (−20.77%) aged pups of NaF intoxicated when compared to control group of respective aged pups. DA levels were gradually reduced with increasing age. In AMAE+NaF and AMEE+NaF-treated pups showed the decreased levels of DA as compared to NaF alone treated.

**Glu**

Increased Glu levels [Figure 5] in the brain tissue of NaF intoxicated pups were observed when compared to control group of pups. The per cent of increase in Glu levels were sequentially dropped from day 1 NaF fed rats to day 30 NaF rats when compared to corresponding age of control group. These levels were recovered in AMAE and AMEE treated toward NaF.

**ACh**

Progressively decreased levels of ACh [Figure 6] from day 1 to day 30 pups of NaF intoxicated was observed when
compared to control group of respective age. In AMAE and AMEE treated groups against NaF exposed pups showed the retrieved levels of ACh. AMEE-treated showed better efficacy over AMAE.

**MAO**

**MAO-A**

The results shown in the Figure 7 indicated the increased activity of brain MAO-A in NaF administered rats when compared to control group of same age. The increase in the activity of MAO-A was declined with growing age. The MAO-A activity was reversed on administration of AMAE and AMEE.

**MAO-B**

The results shown in the Figure 8 indicated the increased activity of brain MAO-B in NaF administered rats of day 1 old by 29.54% when compared to control group of same age. And also increased MAO-B activity was observed in day 7, 14, 21, and 30 aged pups of NaF intoxicated by 25.58%, 26.66%, 24.44%, and 17.77%, respectively, with compared to respective aged control group of pups. Its activity was reduced on treating with AMAE and AMEE.

![Figure 4](image1.png)

*Figure 4: Effect of *Abelmoschus moschatus* seed extract on dopamine levels in brain tissue of rat pups exposed to sodium fluoride. Data presented as mean ± standard error of the mean (*N* = 5). Data exposed to one-way analysis of variance and *t*-test to determine the statistical differences between groups at significance levels of *P* < 0.01*

![Figure 5](image2.png)

*Figure 5: Effect of *Abelmoschus moschatus* seed extract on glutamate levels in brain tissue of rat pups exposed to sodium fluoride. Data presented as mean ± standard error of the mean (*N* = 5). Data exposed to one-way analysis of variance and *t*-test to determine the statistical differences between groups. Significant data are *P* < 0.01*

![Figure 6](image3.png)

*Figure 6: Effect of *Abelmoschus moschatus* seed extract on acetylcholine levels in brain tissue of rat pups exposed to sodium fluoride. Data presented as mean ± standard error of the mean (*N* = 5). Data exposed to one-way analysis of variance and *t*-test to determine the statistical differences between groups. Significant data are *P* < 0.01*

![Figure 7](image4.png)

*Figure 7: Effect of *Abelmoschus moschatus* seed extract on monoamine oxidase-A activity in brain tissue of rat pups exposed to sodium fluoride. Data presented as mean ± standard error of the mean (*N* = 5). Data exposed to one-way analysis of variance and *t*-test to determine the statistical differences between groups. All the data satisfied the *P* < 0.01*
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**DISCUSSION**

Excessive exposure to fluoride for longer durations or at high doses of fluoride even for shorter periods results in the development of fluorosis. In the case of animals, with intake high levels of fluoride through their drinking water, food, or any other source, its levels not only increases in blood and urine but also get deposit in soft tissues such as brain, liver, kidney, and heart among other organs. The fluoride cross blood–brain barrier, accumulate in brain tissue and alters the levels of biogenic amines and NTs. The developing brain was found to be particularly vulnerable to neurotoxic effects of fluoride because of the BBB in the embryo, fetus, and newborn is poorly developed, leaky, or even absent, rendering the developing brain more vulnerable to fluoride entering the fetal circulation from the mother. In this study, alterations in the levels of NTs and monoamine oxidase activity were noticed in rats receiving 20 ppm fluoride through their drinking water during pre- and post-natal development for a period of 51 days. The monoamine NTs 5-HT, DA, and NE play a pivotal role in cognition, learning, reinforcement, pleasure, and physical motivation. Many of the regions of brain receive their inputs including striatum, neocortex, amygdala, and hippocampus. The DA input to the striatum is provided by a very dense network of axon terminals arising from cell bodies in the midbrain–substantia nigra pars compacta and ventral tegmental area. Due to depleted levels of it, the earlier mentioned behaviors were altered and also severely depleted levels lead to Parkinson’s disease (PD). Experimental lowering of serotonin has little effect on pre-frontally mediated tasks, but does impair learning. Striatal DA levels modulate the behavioral reactivity of the organism. In the present study, decrease in DA and NE levels was noticed in NaF received rats than the control, while their levels were reverted to normal in AMAE- and AMEE-treated rats against NaF. Due to decreased DA levels which we observed in NaF exposed rats, may underlie the uncoordinated body movements and they were restored on AMAE and AMEE treatment. These results were in agreement with previously reported results of Mesram et al. showing quercetin protective effects against NaF. The declined levels of DA and NE may either be due to the diminished activity of enzymes involved in their synthesis like tyrosine hydroxylase, DOPA and DA β-hydroxylase or to the boosted release of catechol-O-methyl transferase (COMT) caused by increased neuronal activity. COMT is responsible for half of DA decline. A second set of enzymes, the MAOs, catalyze the oxidation of monoamine NTs, including serotonin, DA, and NA from their location in the mitochondrial outer membrane. The A isoform (MAO-A) is more abundant in catecholaminergic neurons, while MAO-B is more abundant in serotonergic neurons. The increased activity of MAOs catalyses enzymatic breakdown of DA into 3,4-dihydroxy phenyl acetic acid. Thus, the decreased concentration of DA was resulted on fluoride exposure. In this report, elevated epinephrine levels were witnessed in NaF-administered group of rats. The reverted DA and NE levels were found on supplementation of Vitamin E, curcumin, quercetin. AMAE and AMEE pose quercetin, its derivatives and anti-oxidants and hence help in reverting DA and NE levels. The DA and NE levels were progressively decreased from day 1 to day 14 NaF intoxicated rats and decrease in the levels of DA and NE gradually declined in day 21 and day 30 of NaF-fed rats. The decrease in 5-HT levels in NaF exposed rats than the control is similar to the obtained results. NaF+AMAE- and NaF+AMEE-treated rats displayed restoration to the normal level of 5-HT. 5-HT progressively decreased in NaF exposed rats from day 1 to day 14 whereas progressiveness of decrease was declined in day 21 and 30.

Glu is principal excitatory amino NT within the vertebrate nervous system, which makes most of the contribution of total excitatory NT in brain, particularly in hippocampus. It is the region of the brain where learning and memory processes takes place. The role of Glu in synaptic transmission and synaptic plasticity, considered to be the basis of learning and memory, has been recently studied and characterized in the hippocampus, thus it enhances the formation of LTP in the central nervous system. Fluoride intoxication...
resulted in elevation Glu which was reverted to normal in NaF+AMAE- and NaF+AMEE-treated rats. Free radicals from fluoride cause increase in Glu which eventually results in cell death. As Glu is an excitatory NT, it gets activated on fluoride toxicity. The elevated levels of Glu thus alter the LTP and finally behavioral changes. Since, AMAE and AMEE reduce the free radical production from fluoride, maintain normal Glu levels. Thus, seed extract of *Abelmoschus* restores Glu concentration and finally leads to normal function of the rat. The NaF-treated rats showed depleted levels of ACh significantly as compared to the control. The simultaneous AMAE and AMEE treatment toward NaF intoxication resulted in reclamation of ACh levels in cerebral cortex of developing rats. The diminished ACh levels in NaF treated rats also reported in the studies of Ashani et al. and Mesram et al. quercetin was administered as antidote to the rats who received NaF, delivered significant reversal of ACh levels in comparison to those of NaF alone treated rats.

MAO, the enzyme responsible for the oxidative metabolism of the monoamine NTs such as DA, noradrenaline, adrenaline, and 5-HT accompanied by the release of NH₃ and H₂O₂. The two MAO isoenzymes, A and B; MAO-A has a higher affinity for 5-HT, NE, and DA. In contrast, MAO-B prefers phenylethylamine as substrate although it can degrade 5-HT, NE, and DA in the absence of MAO-A. Oxidation of DA by MAO can lead to production of neurotoxic compounds such as hydrogen peroxide, semiquinones, hydroxyl radicals, and superoxide. The formation of these radicals can forestall potent OH formation through the Fenton reaction and induce subsequent lipid peroxidation and neurodegeneration. Increased in activity of MAOs (MAOs: MAO-A and MAO-B) and elevated levels of free radicals are major pathogenic factors in neurodegenerative disorders. Therefore, inhibition of MAO activity and free radical scavenging might be important for the treatment or prevention of neurodegenerative disorders. An earlier study supported a distinct relationship between MAOs activity, free radicals, and the development of neurodegenerative diseases. Increased oxidative deamination of DA enhances the production of hydrogen peroxide, which finally converted to harmful cytotoxic hydroxyl free radicals. It has been suggested that excessive oxidative deamination of DA and the resultant boosted oxidative stress may be involved in the pathogenesis of PD. Inhibition of deamination-induced oxidative stress by blocking MAO activity and inhibiting the formation of free radicals might have a beneficial effect on neurodegenerative disorders. Naturally occurring flavonoids have been reported to pose several activities, for instance, antioxidant activity. These altered biogenic amines responded favorably toward AMAE and AMEE supplementation suggesting involvement of free radicals in impaired neurotransmission. Therefore, flavonoids have attracted attention because of their beneficial effects and which quercetin, isoquercetin, rutin, and quercetin, (isolated from the leaves of *Melastoma candidum* D. Don.) showed an inhibitory effect on MAO-B. This suggests that the flavonoids possess both MAO-B inhibitory and free radical scavenging activities, and hopefully may be used for preventing some neurodegenerative diseases in the future.

Fluoride enhance the production of free radicals which responsible for oxidative stress and excitotoxicity. As a result Glu and epinephrine levels and MAOs activity were increased while epinephrine, DA, serotonin and ACh levels were decreased in NaF administered rats. Plant flavonoids, quercetin, curcumin and their derivative pose more number of –OH groups (poly hydroxyl) having anti-oxidative properties. Hence, these compounds reduce the production of free radicals. By the presence of those components in extract of *Abelmoschus* seed nullify the production of free radicals from NaF as reported in our previous reports and also protect brain from NaF-induced neuroderangement by maintaining balanced status of NTs.

**CONCLUSION**

Pre- and post-natal exposure of fluoride cause inimical effects on developing brain of pups. Since developing pups are deficient or absence in well-formed blood–brain barrier, fluoride get easy access into the brain. Once after the entry, it get accumulated and outturns in free radicals which alter the NTs system. This effect is more severe in day 1 to day 14 post-natal pups may because of earlier mentioned reason and the effect is gradually reduced in day 21 to day 30 pups. All the alterations produced as an aftermath of fluoride intoxication was reverted back to normal on treatment with AMAE and AMEE. Seed extract contains curcumin, quercetin, and its derivatives which facilitate in the elimination of free radicals thus restoring NTs to their normal ranges. Hence, we conclude seed extract of *A. moschatus* have therapeutic values in particular in restoring NTs levels in the brain against fluoride toxicity, in addition to protective effects against oxidative stress and neurodegeneration as reported in our earlier reports.

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