Investigation on the role of *Spirulina platensis* in ameliorating behavioural changes, thyroid dysfunction and oxidative stress in offspring of pregnant rats exposed to fluoride

David Banji\(^a\),*\, Otilia J.F. Banji \(^a\), N. Gouri Pratusha \(^a\), A.R. Annamalai \(^b\)

\(^a\) Department of Pharmacology and Toxicology, Nalanda College of Pharmacy, Charlapally, Nalgonda 508001, AP, India  
\(^b\) Department of Pharmacology, Rajah Muthai Medical College, Annamalai University, Annamalainagar, Tamilnadu, India

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**A B S T R A C T**

The study investigated the role of *Spirulina platensis* in reversing sodium fluoride-induced thyroid, neurodevelopment and oxidative alterations in offspring of pregnant rats. The total antioxidant activity, phycocyanins, and β carotene content were quantified in *Spirulina*. Thirty female pregnant rats were allocated to six groups and treatment initiated orally from embryonic day (ED) 6 to postnatal day (PND) 15. Treatment groups included control, *Spirulina* alone, sodium fluoride (20 mg/kg) alone, and sodium fluoride along with *Spirulina* (250 and 500 mg/kg). Serum fluoride levels were determined on ED 20 and PND 11. Offspring were subjected to behavioural testing, estimation of thyroid levels, oxidative measurements in brain mitochondrial fraction and histological evaluation of the cerebellum. Fluoride-induced alterations in thyroid hormones, behaviour and increased oxidative stress. *Spirulina* augmented the displacement of fluoride, facilitated antioxidant formation, improved behaviour and protected Purkinje cells. Supplementation *Spirulina* during pregnancy could reduce the risk of fluoride toxicity in offspring.

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1. Introduction

Fluorine, a strongly electronegative member of the halogen group is one of the most reactive elements, forming fluoride ions in solution (Hem, 1989). Groundwater contains variable concentration of fluoride depending upon the nature of the rocks and the occurrence of fluoride-bearing minerals. Dissolution of fluorite, apatite and topaz from local bedrock raises the level of fluoride in groundwater. Endemic fluorosis continues to be a challenge and a widely studied national health problem in India. Nalgonda, a district in Andhra Pradesh in southern India is seriously affected with endemic fluorosis, with levels of fluoride ranging from 0.1 to 8.8 mg/l in ground water (Brindha, Rajesh, Murugan, & Elango, 2011). This has led to the innovation of a local technology called Nalgonda technique, which is being utilised by developing countries to defluoridate water (Ayoob, Gupta, & Bhat, 2008); however, this technique is suitable only for small communities. Thyroid gland is particularly sensitive to the deleterious effects of fluoride (Bouaziz, Soussa, Guermazi, & Zeghal, 2005; Shashi, 1988; Susheela, Bhatnagar, Vig, & Mondal, 2005). Follicular epithelial cells of the thyroid gland undergo structural and functional changes on exposure to fluorides (Wang et al., 2005) characterised by a decline in the colloidal content, vacuolation and damage to the endoplasmic reticulum (Assmaa, Manal, & Iman, 2012). All this can disrupt the synthesis of thyroid hormones (Bouaziz et al., 2004). Balanced thyroid hormone status is essential during pregnancy as it facilitates differentiation and maturation of the brain. Fluoride can induce neuroendocrinal changes, triggering developmental disability in offspring.

Natural antioxidants, such as β carotene, tocopherol, chlorophyll, and flavonoids, have gained tremendous attention, owing to their ability to support the physiological system against oxidative stress. *Spirulina platensis* (*Spirulina*), a single celled spiral-shaped blue-green alga (Family Oscillatoriaceae) referred to as a “super food” is nutritionally rich in chlorophyll, β carotene, phycocyanin, and minerals (Annapurna, Deosthale, & Banjii, 1991). *Spirulina* strengthens the immune system, and is considered as the poor man’s HIV/AIDS anti-retroviral therapy (Teas, Herbert, Fitton, & Zimba, 2004). Chronic treatment with blueberry, spinach and *Spirulina* prevented ischaemia/reperfusion-induced apoptosis (Wang et al., 2005) and treatment of aged rats with *Spirulina* produced considerable improvement in motor abilities (Bickford et al., 2000).

*Spirulina* has been evaluated for various activities including neuroprotection in rodents (Bickford, Shukitt-Hale, & Joseph, 1999; Strömberg, Gemma, Vila, & Paula, 2005). It is extensively used the world over as a natural food supplement without being subjected to further extraction. We, therefore, presumed that the
plethora of nutrients and antioxidants in *Spirulina* might facilitate the displacement of fluoride from tissues and minimise toxicity. To the best of our knowledge, no study has been undertaken so far to examine the protection exerted by *Spirulina* against fluoride-induced thyroid toxicity and its impact on neurodevelopment. Keeping this in perspective, we have investigated the role of *Spirulina* in ameliorating fluoride-induced thyroid toxicity, behavioural alterations, oxidative changes and histopathological changes in the cerebellum.

2. Methods

2.1. Drugs and chemicals

Sodium fluoride was procured from Universal Laboratories Pvt. Ltd, Mumbai; *Spirulina*, a dark blue green alga, as a spray dried powder was purchased from Parry Nutraceuticals, Chennai, India having a composition of proteins (65.38%), phycocyanin (15.37%), mineral (7.95%), total carotenoids (4.3 mg/g), and *β*-carotene (1.67 mg/g); 2–2 diphenyl-1-picrylhydrazyl (DPPH), glutathione and corticosterone were purchased from Sigma–Aldrich (St Louis, MO). ELISA kits for the estimation of rat-specific TSH were obtained from GenXbio Health Sciences (P) Ltd., New Delhi, India. All other chemicals used were of analytical grade.

2.2. In vitro evaluation

2.2.1. Quantitative determination of phycobiliproteins

*Spirulina* powder (1 g) was suspended in 100 ml of sodium–phosphate buffer (0.1 M, pH 7.0, containing 1 mM sodium azide), and sonicated for 60 s. It was then subjected to repeated freezing at −20 °C and thawing at room temperature in the dark to facilitate the extraction of phycobiliproteins. The mixture was subsequently centrifuged at 10,000g for 30 min at 4 °C and the phycobiliprotein containing clear supernatant was collected. The absorbance of phycobiliprotein-containing supernatant was measured on a UV–vis spectrophotometer at 620, 652, and 666 nm in triplicate and averaged to determine the concentrations of C-phycocyanin (CPC), allophycocyanin (APC), and phycobiliprotein (PE), respectively, using the following equations (Bennett & Bogorad, 1973):

\[
\text{CPC (mg/ml)} = \frac{A_{620} - 0.474 (A_{652})}{5.34}
\]

\[
\text{APC (mg/ml)} = \frac{A_{652} - 0.208 (A_{620})}{5.09}
\]

\[
\text{PE (mg/ml)} = \frac{A_{652} - 2.41 (PC) - 0.849 (APC)}{9.62}
\]

2.2.2. Determination of total carotenoids

Methanolic solutions of *Spirulina* (5 mg/ml) were prepared by sonicating powdered *Spirulina* with 70% aqueous methanol for 15 min. The extract was filtered and the methanolic solutions of *Spirulina* (5 mg/ml) were analysed in triplicate for the presence of total carotenoids by recording the absorbance at 470 nm, using a UV/vis spectrophotometer. The concentration of chlorophyll a, and chlorophyll b was also determined in the same extract at 653 and 666 nm respectively. The content of total carotenoids, chlorophyll a, and chlorophyll b was calculated based on the formula proposed by Lichtenthaler and Wellburn (1985). The concentration of chlorophyll a and b was utilised to calculate the total carotenoid content.

Chlorophyll a = 15.65 $A_{666}$ - 7.340 $A_{653}$

Chlorophyll b = 27.05 $A_{653}$ - 11.21 $A_{666}$

Total carotenoids (mg/l) = 1000 $A_{420}$ – 2.860 Ca – 129.2 Cb/245 where Ca indicates Chlorophyll a and Cb corresponds to chlorophyll b.

2.2.3. Determination of *β*-carotene/linoleic acid assay

*β*-Carotene/linoleic acid were determined by the method of Dapkevicius, Venskus, Van Beek, and Linssen (1998). *Spirulina* extract (5 mg/ml) was prepared by shaking the powder with 0.4% (w/v) Tween 40 solution followed by centrifugation at 600g for 10 min. Butylated hydroxy toluene (5 mg/ml) was also shaken with 0.4% (w/v) Tween 40 solution. *β*-Carotene (0.5 mg) was dissolved in 1 ml of chloroform, 25 µl of linoleic acid and 0.8 ml Tween 20 were added to the above mixture. The chloroform extract was evaporated under vacuum; and 100 ml of distilled water was added to the residue. Aliquots (250 µl) of the *β*-carotene/linoleic acid emulsion was transferred to tubes to which *Spirulina* extract or BHT (500 µl) was added. The test tubes were incubated for 2 h at 50 °C together with the control sample. The absorbance was measured at the beginning (t = 0 min) and after the experiment (t = 120 min) at 470 nm. Standard used was butylated hydroxytoluene which was also subjected to the same procedure. All determinations were carried out in triplicate and averaged. The antioxidant activity (AA) was calculated as percentage inhibition of oxidation using the following equation:

\[
\text{%AA} = \frac{[1 - (A_s - A_t)]}{(A_s - A_c)} \times 100
\]

2.2.4. Determination of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical scavenging activity

*Spirulina* extract (5 mg/ml) was prepared by sonicating *Spirulina* powder with methanol for 15 min followed by centrifugation at 600g for 10 min. To 1 ml of the supernatant, 5 ml of 0.004% methanolic solution of DPPH were added and the absorbance was measured at 517 nm after 30 min. Control (without any additive) and standard (BHT) were also subjected to the same procedure for comparison (Burits & Bucar, 2000a, 2000b). The ability to scavenge DPPH radicals was calculated using the following equation:

DPPH scavenging effect (%) = 100 × ($A_t - A_c$)/$A_t$

where $A_t$ is the absorbance of control, and $A_c$ is the absorbance of test sample.

2.3. Animals, dose selection and experimental design

Healthy adult Wistar rats (180–200 g) were obtained from the National Institute of Nutrition, Hyderabad. They were kept in polypropylene cages, housed in a room at 22 ± 2 °C on alternative 12 h light–dark cycle, fed with standard chow diet (National Institute of Nutrition), and provided with water ad libitum. After 1 week of acclimation, one male and two females were placed together in the cage for mating. The females were separated and moved to separate cages after the appearance of the vaginal plug. All the experiments were carried out in accordance with the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India and have been approved by the Institutional Animal Ethical Committee (IAEC) bearing number NCP/IAEC/approved/23/2011 dated 20.09.2011.

Sodium fluoride was administered in a dose of 20 mg/kg based on earlier reports (Paul, Ekambaram, & Jayakumar, 1998). The doses of *S. platensis* selected were 250 and 500 mg/kg based on previous findings (Simsek, Karadeniz, Kalkan, Keles, & Unal, 2009).

Pregnant animals were randomised into six groups with each group consisting of six animals. They were housed separately from gestational day 17 and the litters were culled to eight
pups/mother. All the treatments were initiated from embryonic day (ED) six and continued after parturition up to postnatal (PND) 15 by the oral route. Group 1 animals received deionised water orally; group 2 animals were given *Spirulina* alone (250 mg/kg body weight) in deionised water; group 3 animals were given sodium fluoride (20 mg/kg body weight) dissolved in deionised water; group 4 animals received *Spirulina* (250 mg/kg body weight) and sodium fluoride (20 mg/kg body weight) dissolved separately in deionised water, and group 5 animals were given *Spirulina* (500 mg/kg body weight) and sodium fluoride (20 mg/kg body weight).

2.4. Determination of fluoride concentration

Serum fluoride levels were determined by collecting 0.5 ml blood from the tail vein of the dam on ED 20 (corresponding to the 15th day of treatment) and PND 11 (corresponding to the 30th day of treatment). Fluoride levels were determined using a potentiometer with an ion selective electrode (Orion 9609, Orion Research Inc., Allentown, PA).

2.5. Behavioural models

After the last treatment on PND 15, pups were subjected to behavioural testing.

2.5.1. Mid-air righting

On PND 15–19, pups were grasped by the scruff of the neck with the ventral side up and all the four paws extended 30 cm above a padded surface. Ability to right was scored positive if the pup landed on all four paws. A score of two out of three successful mid-air righting attempts was recorded as ability to right on each day (Cheh, Millonig, Roselli, & Ming, 2006).

2.5.2. Rota-rod test

Rota-rod apparatus consisted of a horizontal rod (5 cm diameter, 30 cm long with partitions for testing three animals at a time) which moved on its axis at a speed adjusted to 40 rpm. The rationale of this test was that the animal was forced to stand on the moving rod and animals having defective motor function would drop off from the moving rod to a tray placed below the rod. A test period of 90 s was allowed to each treated animal and the endurance time was determined by measuring the time between placing the rat on the moving rod and the time taken to fall down (Schneider & Przewlochki, 2005).

2.5.3. Elevated plus maze (EPM)

The apparatus consisted of an elevated, plus-shaped runway elevated 66 cm above the floor. Two open arms and two closed arms, measuring 23 cm each, emerged from a central open platform. The height of the walls of the closed arms was 20 cm. Testing was conducted in a room lit only with dim red light. The animals were placed in the centre of the EPM, where the four arms cross each other, facing a closed arm. The time spent and number of entries in the open or closed arms was recorded by a blind observer. An entry was scored when both front paws were placed in an arm. The test duration was 5 min (Carcoba, Santiago, Moss, & Cabeza, 2008).

2.5.4. Open field test

The open field comprised of a square wooden arena measuring 90 × 90 × 25 cm with the floor divided by black lines into 36 small squares (15 × 15 cm). All testing was conducted between 09:00 and 15:00 h. All treatments groups were tested on the same day in a random order. Rats were gently placed in a corner of the arena and exploratory measures were recorded (Kalueff, Keisala, Minasyan, Kuuslah, & Tuohimaa, 2006). The time spent freezing and rearing, as a measure for vertical activity; wherein the number of times an animal stood erect on its hind legs with its forelegs in the air or leaning against the wall of the open field were recorded. After the 3 min test session, the rat was returned to its home cage.

2.6. Collection of blood and determination of blood hormone levels

On completion of the behavioural studies, pups were anaesthetized with pentobarbital (30 mg/kg, i.p. injection) and blood was withdrawn by retro-orbital puncture into heparinised tubes. Plasma free T3 (FT3), free T4 (FT4) was determined by commercially available radio immunoassay kits and thyroid stimulating hormone (TSH) levels were determined by ELISA using rat specific TSH kits obtained from GENXbio Health Systems (Delhi, India) according to the manufacturer’s instructions.

2.7. Body weight and organosomatic index (OSI)

Each animal was weighed 30 days after birth and the weight of whole brain was recorded. From these values the OSI was calculated by the following formula:

\[
\text{OSI} = \frac{\text{weight(g) of the organ}}{\text{total body weight(g) at day 30}} \times 100
\]

2.8. Isolation of brain and preparation of mitochondrial fraction

After withdrawal of blood, the animals were decapitated, the brain isolated, and placed in beakers containing chilled isolation media at 4 °C, in order to prepare the mitochondria fraction (Kau-shal, Dave, & Katyre 1999). The isolation medium contained 0.25 M sucrose, 10 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA and 250 µg BSA/ml. The tissues were washed repeatedly with the isolation medium to remove adhering blood and 10% (w/v) homogenate was prepared using a Potter homogenizer. The nuclei and cell debris were centrifuged at 650g for 10 min. The supernatant was subjected to further centrifugation at 7500g for 10 min. The mitochondrial pellet was washed by suspending gently in the isolation medium and by re-sedimenting at 7500g for 10 min. Finally, the mitochondria were suspended in the fresh isolation medium.

2.8.1. Total protein

The amount of protein in the mitochondrial fraction was estimated by the method of Lowry, Rosebrough, Farr, and Randall (1951). To 0.1 ml of diluted (1/10) mitochondrial fraction, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added, and kept at room temperature for 10 min. Then 0.5 ml of Folin’s reagent were added and the mixture was allowed to stand for 20 min. The colour developed was read at 640 nm and the levels of protein were expressed as mg/g tissue.

2.8.2. TBARS levels

Lipid peroxidation was measured by the method of Ohkawa, Onishi, and Yagi (1979). Brain mitochondrial fraction was incubated with 8.1% sodium dodecyl sulfate (w/v) for 10 min followed by the addition of 20% acetic acid (pH 3.5). Reaction mixture was incubated with 0.6% thiobarbituric acid (w/v) for 1 h in a boiling water bath. The pink coloured chromogen was extracted in butanol:pyridine solution (15:1) and read at 532 nm.

2.8.3. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was measured by the method of Misra and Fridovich (1972). To the mitochondrial fraction, 0.05 M carbonate buffer (pH 10.2) containing 0.1 mM EDTA and 30 mM epinephrine in 0.05% acetic acid were added and the activity was...
measured at 480 nm for 4 min. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equivalent to one unit and expressed in terms of U/g tissue.

2.8.4. Catalase (CAT) activity determination

CAT activity in supernatant was determined according to the method of Aebi (1984) by monitoring the initial rate of disappearance of H₂O₂ in a cuvette containing 10.5 mm H₂O₂ in 1 ml of 50 mM phosphate buffer (pH 7, 25 °C), at 240 nm in a spectrophotometer. Activities were expressed as nmol/mg protein.

2.8.5. Estimation of glutathione

Glutathione (GSH) was estimated by the method of Moron, Deipriere, and Mannervik (1979). To the supernatant (0.5 ml), 2 ml of 5,5′-dithio-bis-2-nitro benzoic acid (DTNB) was added and the volume made up to 3 ml with phosphate buffer. The formation of a yellow coloured complex due to the reduction of DTNB was taken into consideration and the level was measured spectrophotometrically at 412 nm. The molar absorption value of GSH was taken into consideration and the level was expressed as nmol/mg protein.

2.8.6. Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured by the procedure of Flohe and Gunzler (1984). One millilitre of reaction mixture contained 0.3 ml of mitochondrial fraction, 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of GSH (2 mM), 0.1 ml of sodium azide (10 mM), and 0.1 ml of H₂O₂ (1 mM). After incubation at 37 °C for 15 min, reaction was terminated by the addition of 0.5 ml of 5% TCA. Tubes were centrifuged again at 1500 g for 5 min and supernatant was collected. To 0.1 ml of supernatant, 0.2 ml of phosphate buffer (0.1 M, pH 7.4), and 0.7 ml of DTNB (0.4 mg/ml) were added, mixed, and absorbance was recorded at 420 nm.

2.9. Histopathology

Animals were sacrificed immediately after behavioural study by cervical dislocation on PND 20. Whole brains were isolated and placed in 10% neutral formalin solution processed and embedded in paraffin. 7-μm thick sagittal sections of cerebellum were stained with haematoxylin and eosin (H&E), and analysed using a light microscope (Labomed L-4) for histopathological changes and photomicrographs of sections were taken using a camera (DCE-2, India) with image driving software.

3. Statistical analysis

Data for in vitro measurements are expressed as mean ± S.D. Data for in vivo measurements were expressed as mean ± S.E.M. Mid-air righting was analysed by χ²-square test. Analysis of anxiety measures, rota rod performance and biochemical parameters were assessed by ANOVA followed by Dunnett post hoc test. A criterion of p < 0.05 was accepted as statistically significant. Computer program Graph Pad InStat version 3.10 for Windows 2009 (Graph Pad Software) was used for data analysis.

4. Results

4.1. In vitro studies

The PE, CPC and APC contents in S. platensis were found to be 1.1 ± 0.03, 14.8 ± 1.2, and 2.3 ± 0.19 (% w/w), respectively (Table 1). Total carotenoid content was found to be 3.5 ± 0.22 mg/g (Table 1). The percentage antioxidant activity and % DPPH-scavenging ability of S. platensis were 42 ± 0.54 and 14.1 ± 0.88 (Table 2).

4.2. Behavioural parameters

4.2.1. Mid-air righting

On fluoride exposure, the number of animals capable of mid-air righting was less compared with the control. Treatment with Spirulina significantly increased the ability of the animals to right from PND 15 (χ² (4) = 13.6, p < 0.05), PND 16 (χ² (4) = 15.7, p < 0.05), PND 17 (χ² (4) = 18.4, p < 0.05), PND 18 (χ² (4) = 19.8, p < 0.05), and PND 19 (χ² (4) = 20.2, p < 0.05) (Table 3).

4.2.2. Rota rod

Fig. 1 depicts the significant decline in endurance time of fluoride-exposed animals compared with the control (p < 0.05). Spirulina alone elicited a similar response as the control. Treatment with Spirulina produced marked improvement in endurance time compared with the fluoride-treated group (F₄,₂₀ = 36.82, p < 0.05).

4.2.3. Elevated plus maze

The tendency to enter and spend time in the open arms significantly decreased in fluoride-exposed animals compared with the control (p < 0.05). The number of entries and time spent in the open arms did not differ between the Spirulina-alone-treated group and the control. However, treatment of fluoride-exposed animals with Spirulina improved open arm exploration (F₄,₂₀ = 1.80, p < 0.05) and enhanced the time spent in the open arms (F₄,₂₀ = 0.75, p < 0.05), compared with the fluoride-treated group (Table 4).

4.2.4. Open field test

In the open-field test, fluoride-exposed animals spent more time freezing (Fig. 2), which was significant compared with the control (p < 0.05). A marginal increase in rearing was observed on fluoride exposure. Spirulina-alone group did not show any difference in response compared with the control. On the other hand, a significant reduction in time spent freezing (F₄,₂₀ = 31.64, p < 0.05) and rearing (F₄,₂₀ = 20.56, p < 0.05) (Fig. 3) was observed on Spirulina treatment compared with the control.

4.3. Fluoride levels in serum

As depicted in Table 5, fluoride levels in serum were significantly higher in the fluoride-treated animals, compared to the control (p < 0.05). On treatment with both doses of Spirulina, the level of fluoride significantly decreased, compared with the fluoride-alone group (p < 0.05).

4.4. Blood hormone levels

A decline in the serum levels of FT₃ and FT₄ was observed in the fluoride-treated group compared with the control (p < 0.05). Supplementation with 500 mg/kg of Spirulina elevated the levels of FT₃ (Fig. 4), FT4 (Fig. 5), and TSH compared with the fluoride-alone group (p < 0.05) (Fig. 6).

4.5. Organosomatic index

Rat offspring exposed to fluoride exhibited a reduction in the organosomatic index compared with the control (p < 0.05). Treatment with Spirulina produced increment both in the body weight and brain weight, resulting in a rise in the organosomatic index compared with the fluoride-treated group (F₄,₂₀ = 3.36, p < 0.05). A difference between the control and Spirulina-alone group was not observed (Table 5).
4.6. Oxidative parameters

An increase in lipid peroxidation, decrease in glutathione and antioxidant enzymes was observed with fluoride treatment, compared with the control (p < 0.05). Supplementation of fluoride-exposed animals with Spirulina produced an increase in the levels of GSH (F_{4,20} = 570, p < 0.05), GPx (F_{4,20} = 66.95, p < 0.05), catalase (F_{4,20} = 314, p < 0.05) and SOD (F_{4,20} = 6.86, p < 0.05). Decrease in the levels of lipid peroxides were also observed compared with the fluoride-alone group (F_{4,20} = 185, p < 0.05) (Table 6).

4.7. Total protein

A decline in the level of total protein was observed in the fluoride-alone group which was significant compared with the control. At both the doses, Spirulina produced an elevation in the levels of total protein compared with the fluoride-alone group (F_{4,20} = 1195, p < 0.05). A marginal increase in protein content was detected with Spirulina alone compared with the control (Table 6).

4.8. Histopathology

Fig. 7 depicts sagittal sections of the cerebellum having distinct Purkinje cells (P) with a dense granular layer in the control group (A) and Spirulina-alone group (B). Fluoride produced degeneration of the Purkinje cells of the cerebellum and decrease in the granule cells (C). Treatment of fluoride exposed animals with 250 mg/kg and 500 mg/kg of Spirulina (D; E), resulted in protection of the architecture of the Purkinje cells and a populated granule layer.

5. Discussion

The present study evaluated the impact of Spirulina on thyroid function, oxidative stress, and neurobehavioural profile of the offspring of rats prenatally exposed to fluoride from embryonic day 6 to PND 15. In rodents, the foetus is predominantly dependent on maternal thyroid function from ED 1 to 17, and on maternal as well as foetal thyroid function from ED 17 to PND 0 (Ausó et al., 2004; Porterfield & Hendrich, 1993). As early foetal brain development depends on maternal thyroid hormones and later relies on its own thyroid function, the time-line of exposure selected in our studies extended from ED 6 to PND 15.

The organosomatic index was found to be significantly lower in fluoride-exposed animals. Toxic effects of fluoride on the physio-

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Table 1
Quantitative evaluation of phycocyanins, phycobiliproteins, and total carotenoids in Spirulina platensis.

<table>
<thead>
<tr>
<th>C-phycocyanin (%)</th>
<th>Allophycocyanin (%)</th>
<th>Phycobiliproteins (%)</th>
<th>Total carotenoids (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.8 ± 1.20</td>
<td>2.3 ± 0.19</td>
<td>1.1 ± 0.03</td>
<td>3.5 ± 0.22</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of three determinations.

Table 2
Total antioxidant activity and DPPH scavenging ability of Spirulina platensis.

<table>
<thead>
<tr>
<th></th>
<th>% Antioxidant activity</th>
<th>% DPPH scavenging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirulina platensis</td>
<td>42 ± 0.54</td>
<td>14.1 ± 0.88</td>
</tr>
<tr>
<td>BHT</td>
<td>85.2 ± 2.3</td>
<td>20.4 ± 1.2</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. of three determinations. BHT: Butylated hydroxytoluene.

Table 3
Effect of Spirulina platensis on mid-air righting in sodium fluoride induced toxicity rat offspring.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. capable of righting in mid air</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PND 17</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>5.0 ± 0.42</td>
</tr>
<tr>
<td>2</td>
<td>Spirulina (250 mg/kg)</td>
<td>5.0 ± 0.36b</td>
</tr>
<tr>
<td>3</td>
<td>Sodium fluoride (20 mg/kg)</td>
<td>2.0 ± 0.15a</td>
</tr>
<tr>
<td>4</td>
<td>Sod flu + Spirulina (250 mg/kg)</td>
<td>3.0 ± 0.20</td>
</tr>
<tr>
<td>5</td>
<td>Sod flu + Spirulina (500 mg/kg)</td>
<td>4.0 ± 0.32ab</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M., n = 6.
* p < 0.05 compared with the control group.
** p < 0.05 compared with the fluoride alone group. Sod flu: Sodium fluoride.

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Fig. 1. Effect of Spirulina platensis on rotarod endurance in offspring of fluoride-exposed rats. Values are mean ± S.E.M., n = 6, *p < 0.05 compared with control, **p < 0.05 compared with the fluoride-alone treated group.
logical system appear to be reversed on treatment with *Spirulina* as evidenced by a rise in the organosomatic index. Further, *Spirulina* treated animals exhibited lower levels of fluoride in serum. This suggests that *Spirulina* minimised accumulation of fluorides, either by serving as a biosorbent or by enhancing their displacement from the body.

Behavioural changes observed in paradigms such as the EPM, and open field is typically a consequence of fluoride exposure.

**Table 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of entries in open arms</th>
<th>% Time spent in open arms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.3 ± 0.74</td>
<td>75.72 ± 4.8</td>
</tr>
<tr>
<td><em>Spirulina</em> alone (250 mg/kg)</td>
<td>8.6 ± 0.49</td>
<td>77.88 ± 5.07</td>
</tr>
<tr>
<td>Sodium fluoride (20 mg/kg)</td>
<td>7.3 ± 0.55</td>
<td>62.73 ± 3.18</td>
</tr>
<tr>
<td>NaF + <em>Spirulina</em> (250 mg/kg)</td>
<td>7.1 ± 0.68</td>
<td>69.90 ± 1.99</td>
</tr>
<tr>
<td>NaF + <em>Spirulina</em> (500 mg/kg)</td>
<td>8.2 ± 0.60</td>
<td>72.1 ± 4.4</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M, *n* = 6.

*a* *p* < 0.05 compared with the control group.

*b* *p* < 0.05 compared with the fluoride alone group. NaF: Sodium fluoride.

**Fig. 2.** Effect of *Spirulina platensis* on open-field performance in offspring of fluoride-exposed rats. Values are mean ± S.E.M., *n* = 6, *a* *p* < 0.05 compared with control, *b* *p* < 0.05 compared with the fluoride-alone treated group.

**Fig. 3.** Effect of *Spirulina platensis* on rearing in the open field in offspring of fluoride-exposed rats. Values are mean ± S.E.M., *n* = 6, *a* *p* < 0.05 compared with control, *b* *p* < 0.05 compared with the fluoride-alone treated group.
Animals inherently explore novel environments which are altered in anxiety (Ohl, 2003). Time spent freezing increased; whereas open arm entries decreased in the fluoride-exposed group. Treatment with *Spirulina* resulted in anti-anxiety action as evidenced by an increase in the number of open arm entries, and reduction in the time spent freezing. Fluoride-induced aberrant behaviour was reversed with *Spirulina* perceivably by preventing alterations in neural organisation or by modulating hormone levels.

Adequate development of motor skills can occur only when the sensory and motor organisation work in consonance. Mid-air righting was disturbed in fluoride-treated animals, suggesting that the fine balance between sensory and motor function was disturbed. The rotarod test is commonly employed in the context of assessing motor function, endurance and balance (Jones & Roberts, 1968); therefore, testing animals on the rotarod provides an insight on motor performance. Furthermore, the cerebellum plays a pivotal role in these functions.

Table 5
**Effect of Spirulina platensis on organosomatic index and fluoride levels in serum in fluoride-induced toxicity in rats.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organosomatic index</th>
<th>Fluoride levels in serum (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ED 20</td>
</tr>
<tr>
<td>Control</td>
<td>2.23 ± 0.011</td>
<td>0.12 ± 0.001</td>
</tr>
<tr>
<td>Spirulina alone (250 mg/kg)</td>
<td>2.3 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10 ± 0.001</td>
</tr>
<tr>
<td>Sodium fluoride (20 mg/kg)</td>
<td>2.07 ± 0.022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.04&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF + Spirulina (250 mg/kg)</td>
<td>2.15 ± 0.013</td>
<td>0.27 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF + Spirulina (500 mg/kg)</td>
<td>2.2 ± 0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.24&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M, n = 6.

<sup>a</sup> p < 0.05 compared with the control group.

<sup>b</sup> p < 0.05 compared with the fluoride alone group. NaF: Sodium fluoride.

Fig. 4. Effect of *Spirulina platensis* on T3 levels in offspring of fluoride exposed rats. Values are mean ± S.E.M., n = 6,<sup>a</sup>p < 0.05 compared with control,<sup>b</sup>p < 0.05 compared with the fluoride-alone treated group.

Fig. 5. Effect of *Spirulina platensis* on T4 levels in offspring of fluoride exposed rats. Values are mean ± S.E.M., n = 6,<sup>a</sup>p < 0.05 compared with control,<sup>b</sup>p < 0.05 compared with the fluoride-alone treated group.
role in modulating motor function and coordination. Endurance time of the fluoride-exposed rats declined, presumably due to its negative impact on the cerebellum. On the contrary, Spirulina-treated rats exhibited improvement in endurance time, demonstrating that the higher centres were protected from fluoride-induced damage.

The mechanisms of fluoride-induced toxicity are manifold; however, in the ionised form, it alters the metabolism of oxygen and induces the formation of free radicals. Fluoride toxicity is believed to be largely mediated through oxidative stress (Bhanuprakash et al., 2003; Bouaziz, Croute, Boudawara, Soleilhavoup, & Zeghal, 2007; Gao, Liu, & Guan, 2009; Ghiselli, Serafini, Natella, & Scaccini, 2000; Guan et al., 1998; Shivarajashankara, Shivarashankara, Bhat, & Rao, 2002; Shivarajashankara, Shivarashankara, Rao, & Bhat, 2001; Yur, Belge, Mert, & Yoruk, 2003). It has been discerned that oxidative stress is the core mechanism by which fluorides damage hepatic tissue, which is effectively mitigated with antioxidants, such as quercetin and gallic acid (Nabavi, Nabavi, Eslami, & Moghaddam, 2012; Nabavi et al., 2013). As fluorides target endogenous antioxidants like N-acetylcysteine and glutathione (GSH), they can lower the viability of neuronal cells (Anuradha, Kanno, & Hirano, 2000). Moreover, the probability of oxidative damage to the brain is high, owing to the presence of polyunsaturated fatty acids and its utilisation of abundant oxygen. Further, revival of neuronal tissue is low; thus, increasing its susceptibility to oxidative stress. We observed increased TBARS formation and lowered levels of GSH, SOD, catalase, and glutathione peroxidase on fluoride treatment. Oxidative stress induced by fluoride could disrupt the integrity of the neuronal cell membrane leading to behavioural impairment. Spirulina was found to modulate the levels of GSH and glutathione peroxidase particularly at high dose, thereby facilitating the conversion of toxic hydroperoxides to non-toxic products.

Consumption of fluoride through drinking water triggers damage to soft tissues, such as the brain and thyroid gland, causing tremendous distress (Mullenix, Denbesten, Schunior, & Kernan, 1995; Sharma & Chinoy, 1998). Brain development is largely regulated by thyroid hormones (Dussault & Ruel, 1987; Farwell, Dubord-Tomasetti, Pietrzykowski, Stachelek, & Leonard, 2005; Koibuchi & Chin, 2000; Porterfield & Hendrich, 1993; Thompson & Potter, 2000), which also facilitate the maturation of granule neurons (Heisenberg, Thoenen, & Lindholm, 1992), and protect them from apoptosis (Miller, Romano, & Cotman, 1995). Deficiency of thyroid hormones during the sensitive period of neurogenesis and neuronal migration can inflict irremediable damage to various structures, leading to death of granule cells (Dubuis, Sanchez-Mengay, & Burger, 1992; Trabelsi, Guermazi, & Zeghal, 2001), and blunt dendritic arborisation of Purkinje cells (Ruiz-Marcos, Sanchez-Toscano, Obregon, Escobar, & Morreale de Escobar, 1982; Vincent, Legrand, Rabie, & Legrand, 1982). Our studies revealed that the administration of fluoride produced significant decline in the levels of FT3, FT4 and TSH in the offspring, which is in consonance with several earlier findings (Bouaziz et al., 2004; Trabelsi et al., 2001). The decline in the levels of thyroid hormones could be

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**Table 6**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/g tissue)</th>
<th>Glutathione (nmol/mg protein)</th>
<th>Glutathione peroxidase (nmol/mg protein)</th>
<th>Superoxide dismutase (U/g tissue)</th>
<th>Catalase (mU/mg protein)</th>
<th>Total protein (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5 ± 0.17</td>
<td>269 ± 3.3</td>
<td>4.53 ± 0.12</td>
<td>17.0 ± 0.29</td>
<td>467 ± 7.17</td>
<td>49.6 ± 2.54</td>
</tr>
<tr>
<td>Spirulina alone (250 mg/kg)</td>
<td>3.3 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>270 ± 3.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.39 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.4 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>471 ± 9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.2 ± 2.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sodium fluoride (20 mg/kg)</td>
<td>9.55 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.3 ± 3.136&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.72 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.28 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164 ± 6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.7 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF + Spirulina (250 mg/kg)</td>
<td>5.57 ± 0.25&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>204 ± 1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.03 ± 0.09&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>16.7 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>334 ± 6.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.2 ± 2.47&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF + Spirulina (500 mg/kg)</td>
<td>3.72 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>258 ± 2.95&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4.54 ± 0.16&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>17.1 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>391 ± 6.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>44.9 ± 2.53&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
<sup>a</sup> p < 0.05 compared with the control.
<sup>b</sup> p < 0.05 compared with the fluoride-treated group. NaF: Sodium fluoride.
due to deficient iodine uptake or interference in the synthesis of thyroid hormones (Kendall-Taylor, 1972; Yu, 1985). We perceive that the structural semblance between fluorine and iodine might enhance the uptake of fluorides, thereby affecting iodide uptake and ultimately affecting the synthesis of thyroid hormones (Obel, 1982). Treatment with Spirulina elevated the levels of FT3, FT4 and TSH, indicating that it either interfered with the uptake of fluoride or displaced fluoride from the gland or catalysed the organification of trapped iodides; thereby, preventing fluoride-induced depletion of thyroid hormones.

Deficiency of thyroid hormones can affect the genesis of cerebellar Purkinje cells and interneuron connections with the granule cells (Oppenheimer & Schwartz, 1997; Potter, Facchinetti, Beaudoin, & Thompson, 2001). Degeneration of Purkinje neurons was also observed on fluoride exposure. Co-supplementation with higher dose of Spirulina restored the structural features of the cerebellar neurons suggesting its ability to rescue the region from fluoride-induced toxicity. By obstructing cytoarchitectural changes in the cerebellum, it could be presumed that Spirulina enhanced the availability of thyroid hormones, which in turn might be responsible for improved behaviour.

Several studies have reported that consumption of fruits and vegetables can reduce the risk of developing cerebrovascular disorders, cardiovascular diseases (Rimm et al., 1996), and cancer (Steinmetz & Potter, 1996). On exposure to toxicants, a drastic increase in the formation of free radicals occurs, compromising the body's ability to counteract them. In times such as these, fortification with antioxidants can provide protection to the intracellular and cellular machinery. Radical-scavenging ability of Spirulina was evident from the DPPH scavenging (Amarowicz, Pegg, Rahimi-Moghadam, Barl, & Weil, 2004) and β-carotene linoleic acid assay. Spirulina counteracted the effects of fluoride on the cerebellum and the thyroid gland by its powerful antioxidant nature, and by facilitating the displacement of fluorides, thereby reducing their accumulation in the body. The ability of Spirulina to prevent peroxidation of lipids could be linked to the armamentarium of antioxidants encompassing phycocyanin, β-carotene, vitamin C, E, and chlorophyll. The brilliant blue polypeptide phycocyanin and allophycocyanin are important constituents of phycobilisomes (Bhat & Madyastha, 2001; Riss et al., 2007) capable of exerting strong antioxidant action. Chlorophyll and its derivatives scavenge free radicals (Ferruzzi, Böhm, Courtney, & Schwartz, 2002); hence, the presence of chlorophyll in Spirulina can contribute to the antioxidant action. S. platensis also contains x-lipoic acid, riboflavin, xanthophyll phytopigments, SOD enzyme, selenium, magnesium and manganese, which can contribute to the antioxidant effect (Berm-ejo, Pinero, & Villar, 2008; Gong, Ding, Liu, Chen, & Liu, 2005; Upasani & Balaraman, 2003).

In conclusion, fluoride can cause irreparable damage to the developing foetus leading to behavioural impairment and poor cognitive performance. Advocating nutritional supplements can avoid the deleterious impact of fluoride on the developing brain and thyroid gland. Therefore, it is worthwhile to use Spirulina as a nutritional supplement during gestation in geographical areas beset with high fluoride in ground water, thus minimising the risk of neuroendocrinal alterations and neurodevelopmental disorders.

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References


