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ORIGINAL ARTICLE

Carica Papaya Leaf Extract as a Neuroprotective Agent against Behavioral and Neurotransmitter Changes in Brain of the Rat Treated with Sodium Fluoride in Pre- and Post-Natal Periods

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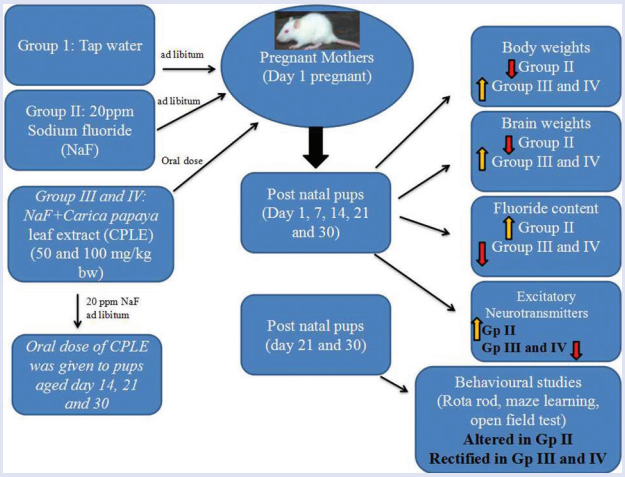
ABSTRACT

Background: Fluoride is an excitotoxin challenging the excitatory receptors and activate them continuously and also in proliferation of reactive oxygen and nitrogen species leading to neurodegeneration and its associated dysfunction. Chronic exposure to 20ppm sodium fluoride to pregnant rats is proven to be neurotoxic for the developing pups. Carica papaya is one of the medicinally important plants which exhibit anti-oxidative and anti-inflammatory responses. **Objective:** The present study aimed to evaluate the neuroprotective properties of Carica papaya leaf extract (CPLE) against fluoride induced behavioural change and neurotransmitter loss. **Materials and methods:** Timed pregnant Wistar rats (*n*=24) were chosen for the experiment and were divided into 4 groups (Group I: Control, Group II: 20 ppm Fluoride treated, Group III: 20 ppm Fluoride-50 mg CPLE, Group IV: 20 ppm Fluoride-100mg CPLE). The fluoride exposed groups received 20 ppm sodium fluoride (NaF) and two different concentrations of extract (50 and 100 mg/mL) through water for 52 days. The postnatal rat brains were collected after sacrificing them at regular time intervals (day 1, 7, 14, 21 and 30) and used to analyze the neurotransmitters alterations in cerebral cortex and hippocampus region of the brains. **Results:** Our results suggest that chronic exposure of NaF to pregnant rats and pups during embryonic and postnatal stages had detrimental effects on the brain. The concentration of fluoride increased in brain tissue and serum with respect to treatment duration in fluoride groups (groups 2, 3 and 4). The levels of glutamate and aspartate increased whereas the levels of acetylcholine, epinephrine, nor-epinephrine, dopamine and serotonin diminished on NaF exposure. The other neurotoxic effects of fluoride are behavioural changes including learning, motor control and other behavioural deficits in postnatal rats. **Conclusion:** The fluoride induced neurotransmitters and behavioural changes were ameliorated with CPLE administration, that were dose dependent i.e. 100 mg of CPLE showed better reversal efficiency than 50 mg extract.

Key words: Behavioral alterations, *Carica papaya* Linn., developing brain, fluoride toxicity, neurotransmitters

SUMMARY

The present study reports the neuroprotective properties of *Carica papaya* leaf extract (CPLE) against fluoride-induced behavioral and neurotransmitter loss in developing rats. Day 1 pregnant Wistar rats (*n* = 24) were chosen as a model of study and were divided into four groups (Group I: control, Group II: 20 ppm fluoride treated, Group III: 20 ppm fluoride-50 mg CPLE, and Group IV: 20 ppm fluoride-100 mg CPLE). The fluoride-exposed groups received 20 ppm sodium fluoride (NaF) along with CPLE 50 or 100 mg/mL orally for 52 days. The brains of postnatal rats were collected at regular time intervals (day 1, 7, 14, 21, and 30) and used to analyze the neurotransmitters alterations in cerebral cortex and hippocampus region of the brain. The chronic exposure of NaF during embryonic and postnatal stages had detrimental effects on the brain. The levels of glutamate, aspartate, and epinephrine increased, whereas the levels of acetylcholine (Ach), norepinephrine, dopamine, and serotonin diminished on NaF exposure. Chronic fluoride exposure induced behavioral deficits (learning and motor control) in postnatal rats. The administration of CPLE extract has successfully ameliorated the fluoride-induced alterations in a dose-dependent manner, i.e. 100 mg of leaf extract showed better reversal efficiency than 50 mg extract.



Abbreviations used: CPLE: *Carica papaya* leaf extract; NaF: Sodium fluoride; Ach: Acetylcholine; BSA: Bovine serum albumin; DA: Dopamine NE: Norepinephrine; EPN: Epinephrine; 5HT: Serotonin.

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INTRODUCTION

Chronic intake of compounds with 3–10 mg/L of fluorine content is known to cause fluorosis, resulting in dental and skeletal hypomineralization.[1-4] Recent reports indicate that most neurological defects due to fluorosis are found in children than in adults.[5,6] The chronic exposure of fluoride during pregnancy may cause deleterious effects in pre- and post-natal rats as the fluoride has the ability to

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cross placenta and blood–brain barrier (humans and animals) and it is also reported that fluoride can also pass to children through mother's milk.[5,7,8] Literature shows that fluorosis is also associated with central nervous system dysfunction.[9-11] Toxicity studies of fluoride were reported in both rats and mice, wherein their offspirngs showed altered brain functions and behavior.[5,12-15] Exposure to high levels of fluorated compounds through water, food, and environment leads to the accumulation of fluoride in all the soft and hard tissues,[5,6,16] leading to morphological,[3,9] physiological,[17,18] and biochemical changes.[19,20]

Being an excitotoxin,[2] the mechanism of action for fluoride starts with the activation of the glutamate receptors, resulting in the accumulation of calcium within the cytosol followed by triggering the activation of nitric oxide synthase and protein kinase C.[18,21,22] This can in turn induce free radical generation, lipid peroxidation, and activation of eicosanoids.[9] Fluoride also has the ability to suppress the cellular energy and mitochondrial electron transport enzymes. The suppression of neuronal and mitochondrial energy leads to the neurodegeneration.[2] Since fluoride has the ability to inhibit metabolic and functional enzymes, even at low concentrations, there is an increased possibility that excitotoxicity plays a significant role in this process.[2,20,22-25]

Since the last three decades, several research groups had put in their efforts to obviate the altering effects of fluoride by employing various natural compounds such as plant extracts and nutritional supplements (vitamins, fatty acids, and amino acids) in both *in vitro* and *in vivo* models.[26,27] Khandare *et al*.[22] and Manjula and Reddy[25] have reported to lessen the accumulation of fluoride content in bone tissue and soft tissues such as brain, liver, and kidney by administering the tamarind fruit pulp; it also eliminates the fluoride in urine.[22,25] The phytoconstituents collected from various medicinal and dietary plants have been used to assess their ability in ameliorating the neurological diseases and allied complications. Although the findings are encouraging, still it needs time before it can get into clinics.[23,28-31]

Carica papaya leaves are of medicinal importance, and they have been used to treat various ailments such as diabetes,[32] deworming,[32,33] dengue,[32] anticancer,[2,34] muscle relaxant,[21] and antimicrobial.[20] The fresh *C. papaya* leaves are consumed as an alternative for spinach in many parts of Asia. *C. papaya* leaf juice is served as an immune booster to dengue fever patients as it increases the white blood cells count and also detoxifies the vital organs.[20,33-35] The leaf juice is also known to work as an anticancer agent and boost the key signaling molecules levels, i.e., activation of Th1-type cytokines and the immune system.[34] The *C. papaya* leaf extract (CPLE) is also given to patients suffering from malaria and other plasmodial infections in the form of tea, though exact mechanism of action is still not clear. The *C. papaya* leaves are also known to increase the digestive function in humans due to the presence of chemical compounds such as carpains, papain, and chymopapain.[21] It also kills microorganisms which often interfere with the digestive function.[20] The nanoparticles synthesized using the CPLE have shown enhanced antimicrobial activity.[20] The CPLE was given as a medicine for controlling acne, anti-nausea, easing menstrual pain and also to increase appetite in olden days.[32,36]

The *C. papaya* leaves are an excellent source for antioxidants (Vitamin C and E), minerals (Ca, K, Mg, Zn, Mn, and Fe), and alkaloids (carpinine and carpaine), which work against various cardiovascular diseases and prevents the oxidation of cholesterol and other lipids.[32] The carpaine is believed to reduce blood pressure, relax uterine muscles, and act as calcium antagonistic agent.[34] Due to these extraordinary therapeutic properties, we have selected CPLE to study its effects on fluoride-induced alterations in neurotransmitters levels in the developing brains and its related neurobehavioral changes on developing rats.

MATERIALS AND METHODS

CPLE – *C. papaya* leaves were collected from neighboring districts of Telangana, India. The leaves were washed thoroughly with distilled water. The washed leaves were then minced and soaked in warm water (double-distilled water) followed by boiling at 60°C for 3 h. The resultant was cooled at room temperature and filtered using a fine sieve to obtain the aqueous extract which was then lyophilized into dry powder.[32]

Experiment design

Twenty-four female timed pregnant Wistar rats weighing 160–180 g were procured from the National Institute of Nutrition (NIN), Hyderabad, India. The rats were housed in individual polypropylene cages to acclimatize to the laboratory conditions and were given a standard pellet diet (NIN, Hyderabad) and water supplied *ad libitum* throughout the experiment.

The rats were divided into four groups with six animals per group. The first group (controls) received normal tap water, the second group was administered with sodium fluoride (NaF) (20 ppm NaF/kg bw) through oral gavage, and the third and fourth groups were treated with NaF (20 ppm NaF/kg bw) and powdered CPLE (50 and 100 mg/kg bw, respectively) concomitantly through gavage. The rats were maintained for 52 days right from timed pregnancy until the postnatal rat pups aging 30 days. The rat pups were sacrificed by cervical dislocation, and their brains were dissected out and immediately transferred to ice-cold conditions (initially at 4°C and then to −20°C) for further studies. The behavioral studies were done on pups aged 21 and 30 days.

Estimation of neurotransmitters

Protein estimation

Protein estimation was done using Lowry's method (1951).[37] Standard protein graph was prepared using bovine serum albumin (BSA; 1 mg/ml) as standard protein. BSA working solutions were prepared ranging from 0.05 to 1 mg/mL. A fixed volume of 0.2 ml of diluted protein solution from different test tubes was pipetted out and added to 2 ml of alkaline copper sulfate reagent (analytical reagent) and mixed well. The mix was then incubated at room temperature for 10 min. To the above mix, 0.2 ml of reagent Folin–Ciocalteu solution (reagent solutions) was added and incubated for 30 min. The optical densities (ODs) of the known and unknown protein solutions were measured at 660 nm.

Estimation of neurotransmitters

The brain tissue homogenates were prepared using 0.25 M sucrose solution and centrifuged for 10 min at 1000 rpm. Levels of dopamine (DA, excitation: 310 nm; emission: 410 nm), norepinephrine (NE, excitation: 387 nm; emission: 487 nm), epinephrine (EPN, excitation: 410 nm and emission: 500 nm), and serotonin (5HT, OD at 275 nm) were estimated in brain regions (cerebral cortex and hippocampus) of rats exposed to fluoride alone and simultaneous treatment of fluoride and CPLE using Jasco–FP750 fluorescence spectrometer.[19,38-43]

Estimation of excitatory neurotransmitters (glutamate and aspartate)

Glutamate was measured by employing the modified method of Ciarlose[19,38] and Margret *et al*.[43] Brains were homogenized in 3% perchloric acid and centrifuged at 1000 rpm for 10 min at 4°C. The supernatant was extracted and mixed with 1% ninhydrin and boiled for 10 min followed by cooling on ice; to the resulting mix, 0.4 ml of guanidine

carbonate, 1 ml of 100 mM lead acetate, 0.5 ml of 1 N NaOH, and 6 ml of dH_2O were added and vortexed. To the mix, 0.1% of 2,4 dinitrophenyl hydrazine dissolved in 0.01N HCl was added under ice-cold condition and incubated for 30 min. The color intensity was recorded at 420 nm in an ultraviolet-visible spectrophotometer (ThermoFisher). Color intensity was proportional to the concentration of substrate. The results were expressed as µg of monoamine/g wet weight of tissue.

Aspartate

Brains were homogenized in 1 ml of 3% perchloric acid and centrifuged at 1000 rpm for 10 min; supernatant was transferred to a fresh tube and to which 2 ml of citrate buffer; 1 ml of 1% ninhydrin solution, and 1 ml of 1% SnCl2 were added and the mix formed was then vortexed and heated for 15 min at 100°C, and cooled on ice thereafter for 10 min in a dark room. Later, 6 ml of isobutyl alcohol was added and vortexed. The alcohol layer was collected and OD was recorded at 570 nm.[39,44] The results obtained were expressed as µg of monoamine/g wet weight of tissue.

Extraction and estimation of acetylcholine

3% perchloric acid was used for homogenizing the brain tissues and centrifuged for 10 min at 1000 rpm. The levels of Ach were analyzed using the modified method of Kumar *et al*.[8,45]

Estimation of fluoride

Fluoride levels in the brain and serum of control and treated postnatal rats were analyzed using modified Birkel *et al*. method (1970), and fluoride content in the diluted samples was recorded on a fluorimeter (Orion R 94-09).[1] The results were expressed in µg of fluoride/g dry tissue.

Behavioral and locomotor activity studies

Maze learning test

Hampton Court maze was used as a research tool for studying animal learning and adaptability to surroundings and challenges on chronic exposure to fluoride [Figure 1].

The maze presents a complex branching passage through which the experimental animals were allowed to find a route to obtain reinforcements. This process is a popular experiment in behavioral laboratory, and it is the main method of studying spatial learning. The rats were starved for 12 h before the maze learning experimentation; the postnatal day pups aged 21 and 30 days from all experimental groups were allowed to spend time in maze training given before the recording the time to the reach the goal (i.e., food).[27,46]

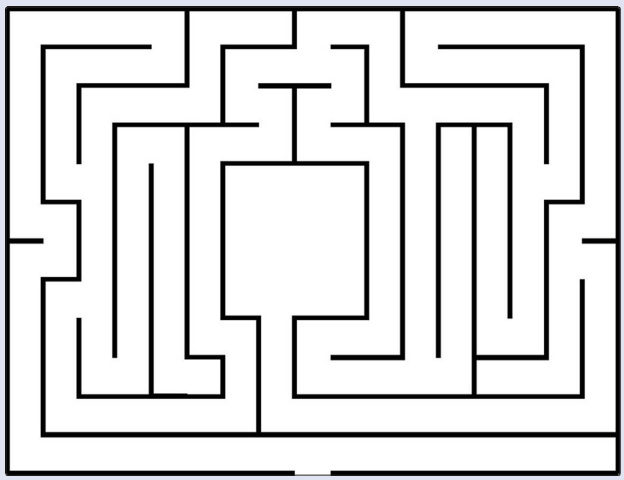

Figure 1: The Maze at Hampton Court Palace

Open-field test

The open-field test was designed to measure behavioral responses such as emotional reactivity/anxiety and locomotor activity.[45,47,48] This test was performed in 21- and 30-day old offsprings; Group II received 20 ppm of NaF in drinking water and Group III and Group IV received 20 ppm fluoride and CPLE (50 and 100 mg) simultaneously during pregnancy and lactation, respectively. Each rat (21 and 30 days old) was placed in an open area of 50 cm × 50 cm × 60 cm whose floor was divided into 12 cm × 12 cm squares by black lines.[6,34,49] The number of squares crossed by each rat with all four paws, rearings (occasions on which the animals stood on their hind legs), wall rearing, groomings (face washing, fore paw licking, and head stroking), sniffing and wall sniffing was scored every 5 min for 15 min. The number of squares crossed and the rearings of each rat was recorded as parameters of locomotor activity, whereas the number of groomings and sniffings was considered as parameters of behavior, i.e., emotionality. The open field was cleaned with a damp cloth after each animal was tested.[50-52]

Rotarod motor coordination activity test

The rotarod test is a performance test based on the rotating rod that forces the motor activity of animal. The rotarod performance test is used to measure the riding time (in seconds) or motor coordination endurance and evaluate the balance and coordination activity of the subjected drug effect on animals. The animals were placed horizontally oriented rotating cylindrical rod, the experimental animals usually make an effort to stay put on the rotating cylinder rod in order to avoid falling on the ground. Animal stay time on rotating rod shows the animal balance, coordination, physical condition, and motor activity. The speed of the rotarod is maintained with the help of motor and that is adjustable.[7]

RESULTS

The fluoride content in the brain and serum of the weanling rats was measured using fluorimeter. The concentration of fluoride in case of controls was minimal, whereas in the case of fluoride-induced rats, the concentration of fluoride increased with age and duration of dosing, based on the observed values its evident that the concentration keeps building up due to the lack of sources to reduce or control the accumulation of fluoride in soft tissue and serum. However, in case of rats which were given the treatment with CPLE simultaneously along with fluoride, it showed less accumulation of fluoride in brain and serum. The reduction of fluoride levels was significant with increasing age and dose of CPLE administration.

The chronic induction of fluoride has deteriorating effects on receiving rats, and when put in maze learning experiment, it showed that they were facing difficulty in navigating themselves through the maze and toward reaching the target (in this case its food pellets) [Table 1]. The time taken by fluoride-induced rats is much higher than the controls and rats receiving CPLE. The rats receiving extract showed improvement in maze learning ability and the fluoride effect of CPLE was ameliorating dose dependently.

The behavioral studies give the insights of stress and anxiety an animal is undergoing. By the open-field test, we made an observation that the rats treated with fluoride show decreased crossing, grooming, rearing, and sniffing in comparison to the control and the rats receiving CPLE. The crossing, grooming, rearing, and sniffing patterns increased in rats treated with extract dose dependently, and the patterns were very much similar to that of the controls; based on the results, it is clear that the CPLE works as a neuroprotectant against fluoride-induced toxicity.

The motor activity in the rats treated with fluoride gradually decreased, in comparison to that of controls and rats receiving CPLE. The rats' receiving the extract is certainly able to gain more and more activity

Table 1: Concentration of fluoride in whole brain and serum of weanling rats at 14, 21, and 30 days of control, fluoride, fluoride-*Carica papaya* leaf extract 50 mg, and fluoride-*Carica papaya* leaf extract 100 mg

Type of source	Groups	Age (days)		
		14	21	30
Brain (μg F/mL)	Control	0.223±0.102*	0.269±0.098*	0.352±0.052*
	Fluoride	2.65±0.122*	4.53±0.127*	5.37±0.043*
	Fluoride + CPLE 50 mg	2.55±0.122*	3.43±0.127*	4.37±0.043*
	Fluoride + CPLE 100 mg	2.35±0.122*	3.03±0.127*	3.87±0.043*
Serum (μg F/mL)	Control	0.023±0.012*	0.032±0.037*	0.039±0.058*
	Fluoride	0.068±0.013*	0.117±0.016*	0.165±0.019*
	Fluoride + CPLE 50 mg	0.048±0.013*	0.85±0.016*	097±0.019*
	Fluoride + CPLE 100 mg	0.038±0.013*	0.57±0.016*	0.85±0.019*

*$P<0.05$ statistically significant. CPLE: *Carica papaya* leaf extract

with age and dose; hence, it is evident that CPLE is a good source for controlling the deleterious effects of fluoride in developing rats.

The Ach is a major neurotransmitter in motor neurons controlling the activity in neuromuscular junctions, so Ach levels must be in abundance to keep the motor function intact. However, there was gradual loss of motor control in the rats as the levels of Ach decreased in both cerebral and hippocampus regions of developing rats when induced with fluoride. The motor activity and Ach levels were being restored in the rats receiving the CPLE and this increase was dose dependent. Although the activity in the groups receiving the CPLE was less in comparison to the controls, still its treatment was able to restrict the depletion of Ach and loss of motor control. The Ach levels were more in the cerebral cortex than in hippocampus, and the effect of fluoride on the levels is also more in cerebral region causing motor dysfunction and behavioral deficits.

The glutamate is an excitatory neurotransmitter which is most abundant in brain. The glutamate levels are gradually increased in the rats treated with fluoride, and the levels of glutamate are significantly high in both cerebral cortex and hippocampus regions of brains when compared to the controls and CPLE-receiving rats. The treatment of CPLE is efficiently decreasing the levels of glutamate with dose and duration; hence, the extract can be considered as an antiexcitatory source. The effect of fluoride on glutamate accumulation is high in cerebral cortex than in hippocampus, and the reversal effect of CPLE on glutamate levels is comparatively less in cortex region than in hippocampus region.

Aspartate is another excitatory neurotransmitter present in brain and its level increased gradually in the rats on fluoride exposure. The levels were significantly high in both cerebral cortex and hippocampus regions of brains, when compared to that the controls and CPLE-receiving rats. The CPLE treatment has efficiently decreased the levels of aspartate with dose and duration on simultaneous administration. Aspartate levels of CPLE-administered animals were in between the control and fluoride groups. The effect of fluoride on accumulation of excitatory neurotransmitter such as aspartate was found to be high in cerebral cortex than in hippocampus; reversal effect of CPLE in aspartate levels is less in the cortex region in comparison to hippocampus region.

EPN levels increased gradually on chronic fluoride exposure with levels significantly high in both cerebral cortex and hippocampus regions of brains when compared to that the controls and CPLE-receiving rats. The CPLE treatment significantly decreased the levels of EPN with dose and duration. The levels of CPLE-administered animals were in between the control and fluoride groups proving its protective efficiency.

The effect of fluoride on accumulation of excitatory neurotransmitter such as aspartate and stimulation of receptor is found to be high in the cerebral cortex than in hippocampus; reversal effect of CPLE in EPN levels is less in the cortex region in comparison to hippocampus regions.

The chronic exposure of fluoride has reduced the levels of NE in both cerebral cortex and hippocampus regions of developing brain. The levels were significantly less when compared to that in controls and CPLE receiving rats. The CPLE treatment significantly increased the levels of NE with dose and duration. The increase of NE levels is an indication of protective efficiency of CPLE and its ability in controlling neuroinflammation and its associated dysfunctions in developing brain of rats. The rats receiving CPLE showed that the levels which to be in between the control and fluoride groups.

The levels of DA decreased gradually on chronic exposure of fluoride in both cerebral cortex and hippocampus regions of brains. In comparison to the controls and CPLE-receiving rats, fluoride-induced rats have significantly less DA levels. The CPLE treatment significantly increased the levels of DA with dose and duration. The protective efficiency of CPLE can be examined from the above figure as it is able to restore the levels and the levels were in between the control and fluoride groups.

The prolonged exposure of fluoride in rats showed significant decrease in 5HT levels in both cerebral cortex and hippocampus regions of the brain, whereas in the controls and CPLE-receiving rats, the levels were normal. The CPLE treatment significantly increased and restored the 5HT levels to normal (i.e., similar to that of controls) with dose and duration. The 5HT works as an antistress and antidepressant neurotransmitter, and an increase of 5HT levels in CPLE-treated rats is an indication of CPLE efficiency as a neuroprotectant against fluoride-induced toxicity.

DISCUSSION

Literature study showed existing evidence on mechanism related to fluoride-induced toxicity in brain and other tissues functions in the postnatal rats. The mothers exposed to xenobiotics during gestation and lactation stages showed improper development of fetuses, the reason involving passage of xenobiotics through the placental and blood–brain barrier.[53] The latest research has indicated that the infants can get exposed to several environmental chemicals through breast milk (Human and Rodents).[6,15,16] The fetuses are vulnerable to toxins during gestation as there is only a partial protection is given by blood–brain barrier against them from entering the central nervous system.[45] New insights have been reported about the neurotoxicants and the neurodevelopmental consequences due to early exposure to these industrial chemicals.[11,17,52]

Fluoride (F) is considered as an essential trace element, excessive intake of which damages not only the skeletal system but also the nonskeletal organ systems.[4,15,54] It reveals a fairly consistent pattern of adverse effects on brain biochemistry,[52] neuropathology development behavior, and performance.[6,11,34] The ionic form passively passes through the intestinal mucosa and interferes with major metabolic pathways in the living system. Fluoride offers prophylactic influence on the dental system and inhibits dental caries at lower doses, but at high dose, it could lead to dental and skeletal fluorosis.[1,15,55] The cerebral cortex plays a key role in general movement, visceral functions, attention, memory and cognition, perceptual awareness, thought, language, and consciousness.[17,18,49] It

also integrates higher mental functions, behavioral reflexes, general movement, and visceral functions.[5,14,18,26] NaF exposure also has neurodegenerative changes in cerebral cortex which is evidenced by learning and memory deficits and impairment of motor activities and behavioral alterations.[5,7,13,23] The present study was conducted in pregnant rats to evaluate the protective efficiency of CPLE against fluoride-induced alterations in the levels of neurotransmitters and neurobehavioral changes in offsprings when mothers were intoxicated with fluoride during pregnancy and lactation stages.[1,26]

The principle findings in the present study are accumulation of fluoride in brain and increase concentration of fluoride in serum of the developing rats. The pharmacological observations showed behavioral impairment in the maze learning, open-field habituation, motor control, and neurotransmitter levels fluctuation on chronic NaF intake by adult rats. Our results are in concord with the literature.[14,43,47] The concentration of fluoride in serum and brain seems to increase with age and exposure time [Table 1].[56]

Decreased concentration of total proteins was observed in the brain lysates of fluoride group when compared to control and protectant groups. The results gained in our experiment are similar to that of literature showing the alterations in protein levels in hippocampus and neocortex of fluoride-induced brains.[19] Nonassociative behavioral habituation is considered as one of the most essential forms of learning and cognition in mammals. Wang *et al.*[49] illustrated the application of open-field test as a model for simultaneous assessment of anxiety and memory in experimental models (rodents). When the rats are introduced to open-field test, they display higher spatial exploration for gathering information and cognition[57] than in successive exposures. Exposure to a new environment also generates new recognition process that involves increased awareness and correlating with stored memories of formerly explored places with the new spatial information for evaluating its novelty.[6,36,49,50] Hence, the delayed response to successive exposures is taken as an index of memory of habituation.[50] The learning is an association between choice alternatives and their outcomes may be correlated to hippocampal dysfunction.[14]

This is the first time that CPLE was being used for evaluating its neuroprotective properties in developing rats against chronic fluoride exposure. To our knowledge, there are no reports published till date on CPLE as neuroprotectant in any of the neurological diseases. There are numerous reports about CPLE efficacy in increasing the platelets in case of dengue and hemorrhagic fever patients. The beneficial properties of CPLE reported to date are such as antitumor, immunomodulatory, antibacterial, antisickling, and antioxidative efficiencies, but not even a single report on neurobeneficiary effects.[20,33,36,35]

Fluoride exposure during developmental stages has led to altered motor coordination activity, emotional reactivity, maze learning goal oriented in experimental fluoride groups, whereas normal behavior patterns were observed in control and CPLE-fluoride-treated groups (aged 21st- and 30th-day postnatal rats).

The maze was designed [Figure 2] to observe the ability of rodents to learn and memorizes the location of food cognition.[10,27,49,50] Jett *et al.* described that the learning task is carefully chosen obtain a great deal of knowledge on the neurochemical, neuroanatomical, and neurophysiological basis-behavioral association.[36,41,51,52,58] The findings from the present study suggested that fluoride (F) exposure during development results in impaired performance in goal-oriented maze. The fluoride-treated rats were able to reach the goal, but due to impaired cognition and memory, the rats took significantly longer time than the control and CPLE-receiving animals to find the food.[46] Behavioral assessment based on maze learning task conducted in our study was similar to that of previous reports. Maze learning ability in relation to reference and working memory tasks in the fluoride-exposed rats differed and it was rectified by concomitant administration of CPLE. The decrease in latency in the finding the food on reference memory trials and working memory trials, suggesting a more convincing and significant amelioration of fluoride toxicity on short- and long-term memory in a dose-dependent manner.

The open-field [Figure 3] test has been regularly used to assess spatial memory and anxiety in rodents.[50,51] The emotional reactivity in the open-field test was assessed using parameters of motor activity, i.e., latency, grooming, and rearing. Spontaneous behavior in rats included monitoring of major body positions and movements such as sitting, standing, walking along with modified activities such as scratching, smelling as examples of motivated behavior. Prenatal and lactational exposure to fluoride causing cognitive impairment was shown by failure of habituation of experimental rats in an open-field task.[10,52] Based on crossing, rearing, and sniffing responses shown by the control and CPLE-receiving group, we reported

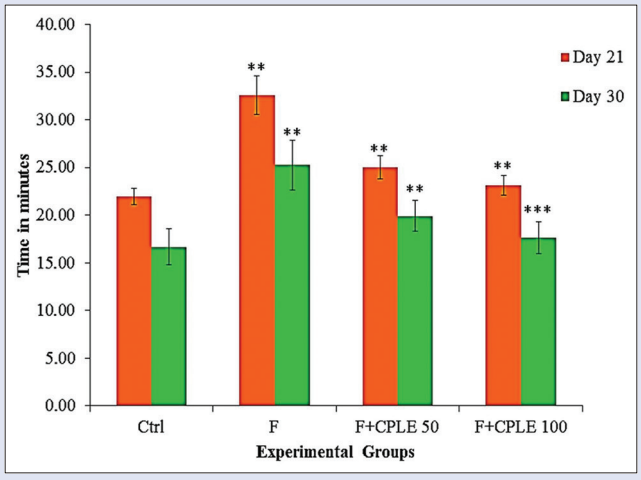

Figure 2: Maze learning ability in weanling rats of control, fluoride and fluoride-*Carica papaya* leaf extract treated groups. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, *P < 0.05, statistically significant

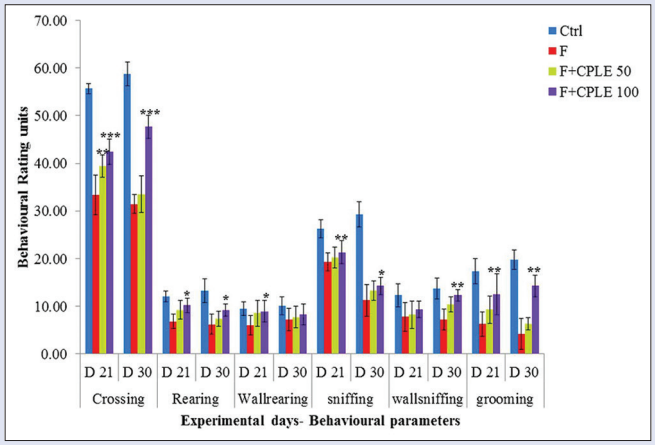

Figure 3: Behavioral observations in open field test in weanling rats of control (ctrl), fluoride (f), fluoride-*Carica papaya* leaf extract (fluoride + *Carica papaya* leaf extract) groups. Number of squares crossed and rearing's in each 5 min period and in the 15 min total test. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001, statistically significant

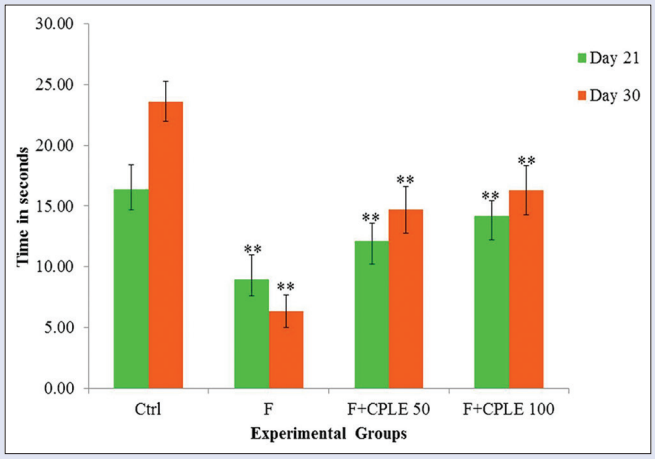

Figure 4: Effect of Fluoride on motor activity in 21st- and 30th-day old rats with and without *Carica papaya* leaf extract treatment. The values are mean ± standard deviation of 6 ($n = 6$) individual observations; one-way ANOVA, $^{**}P < 0.01$, $^{***}P < 0.001$, statistically significant

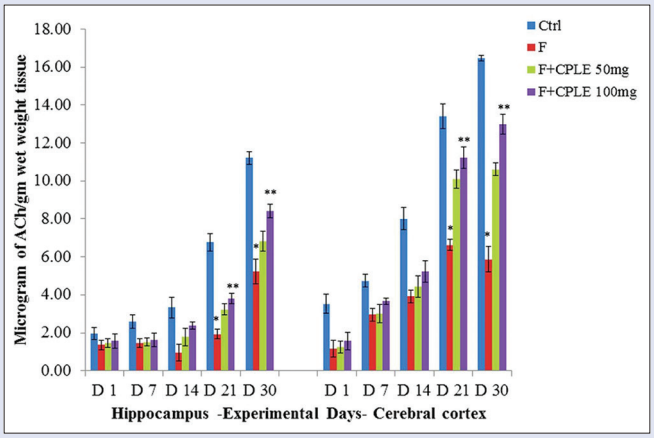

Figure 5: Effect of fluoride on the levels of acetylcholine in hippocampal and cerebral cortex of weanling rat brain with and without *Carica papaya* leaf extract treatment. The values are mean ± standard deviation of six ($n = 6$) individual observations; one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant. The acetylcholine content expressed as in mg ACh/g wet wt of tissue

that these rats had intact memory of habituation. The memory and habituation in the open-field test are processed by hippocampus, and hence, the exposure to fluoride causes loss of hippocampal neurons due to excitotoxicity.[27,58,59] Chioca *et al.*[60] demonstrate open-field habituation impairment and two-way active avoidance responses in adult rats when treated with high doses of NaF.[60] Similar, responses were observed in the experimental fluoride group, whereas the rats which were coadministered with CPLE showed better locomotion, exploration, and memory, which is an indication that the CPLE can act as antianxiety agent in a dose-dependent manner.

Rotarod is a test to find the ability of motor coordination endurance capacity in animal; to elicit this behavior, multiple factors involved are visual sense, skin sense, brain activity, and neuromuscular integration. The present study reports reduced motor coordination activity [Figure 4] in experimental pregnant rats when treated with NaF at 20 ppm/kg bw for 52 days (fluoride group), motor coordination analyses were done on rotarod apparatus, which is in agreement with the earlier reports showing lower the motor activity and coordination in female Wistar rats on NaF treatment.[36] Another study done by Ekambaram and Paul reported similar effects on motor activity when treated with NaF at 500 mg/L through drinking water.[18,36] Motor dysfunction was observed in the postnatal rats aged 14, 21 and 30 days on chronic exposure to NaF, whereas the controls and CPLE coadministered rats showed normal motor coordination when placed on rotarod apparatus. The discrepancies observed in the fluoride-treated postnatal rats were restricted on coadministration of CPLE in a dose-dependent manner.

Liu *et al.* reported about the latencies in pain reaction and longer conditioned reflexes in rats when intoxicated with high doses of fluoride in comparison to control animals.[11,45,47] Furthermore, chronic fluorosis induces disturbances in the development of brain in offsprings and also induces longer latency of the pain reaction and conditioned reflexes in rats receiving fluoride at higher concentrations have been observed as compared to control animals.[52]

An alteration in the Ach concentration was observed in rats intoxicated with fluoride in chronic fashion.[45,47] The Ach levels were significantly low in fluoride group in comparison to the controls and CPLE co-administered rats [Figure 5]. Ach is a neurotransmitter which plays a significant role in controlling the motor functions, and when the levels are diminishing, then it leads to loss of motor coordination, and this was the case with our fluoride-treated rats as they exhibited impaired balancing and motor function when they were put to rotarod test. The controls and CPLE coadministered rats showed better motor control than the rats which received only fluoride, indicating that the ameliorative property of CPLE against fluoride-induced toxicity and works as a neuroprotectant by controlling Ach levels in developing rats in a dose-dependent manner.

The fluoride being an excitotoxin, it can exert excitotoxicity in young and developing rats on chronic fluoride treatment. The increased levels of glutamate and aspartate in postnatal rats is associated with fluoride induced excitotoxicity.[6,52] The postnatal control rats showed normal levels of glutamate and aspartate during development stages, whereas CPLE coadministered postnatal rats reduced the levels of glutamate and aspartate gradually and in a dose-dependent manner, suggesting antiexcitotoxicity property of CPLE, and it could be used as alternative source for reducing excitotoxicity [Figures 6 and 7]. The results obtained in the present study are in accord to that of literature,[2,26,61] and in our attempt to ameliorate, the fluoride-induced excitotoxicity by coadministration of CPLE was successful as it was able to reduce the fluoride toxic effects by diminution of glutamate and aspartate levels in dose-dependent fashion.

The neurotransmitters such as EPN and NE are associated in maintenance of motor control, depression, memory, and cognition.[13,24,52] The present study reports that chronic fluoride treatment during the developing stages resulted in altering the levels of EPN (increased) and NE (decreased) significantly in postnatal rats, whereas in case of controls and CPLE coadministered rats, the EPN levels were less and the NE levels were higher in comparison fluoride-treated postnatal rats, suggesting that the CPLE has the ability to hinder the alterations in neurotransmitters levels induced by fluoride [Figures 8 and 9]. The results obtained in our experiments are in agreement to the literature, and our attempt to ameliorate the fluoride-induced toxicity by coadministration of CPLE was successful as it was able to decrease the levels of EPN and increase the levels of NE with time- and dose-dependent fashion in postnatal rats.[52,61]

In the present study, chronic fluoride intoxication has resulted in diminution of dopamine in the developing rats [Figure 10]. The levels of dopamine were found to be significantly low in fluoride-induced rats and whereas the controls and CPLE coadministered postnatal rats had normal dopamine.[19,26,13,18,39] The postnatal rats which were coadministered

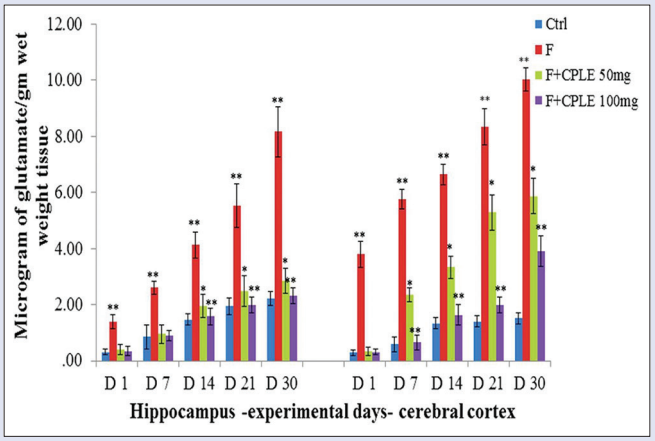

Figure 6: Alterations in glutamate levels in hippocampus and cerebral cortex of weanling rat brain on chronic fluoride and *Carica papaya* leaf extract treatment. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, * P < 0.05, ** P < 0.01, statistically significant. The glutamate level expressed as in µg amine/g wet wt of tissue

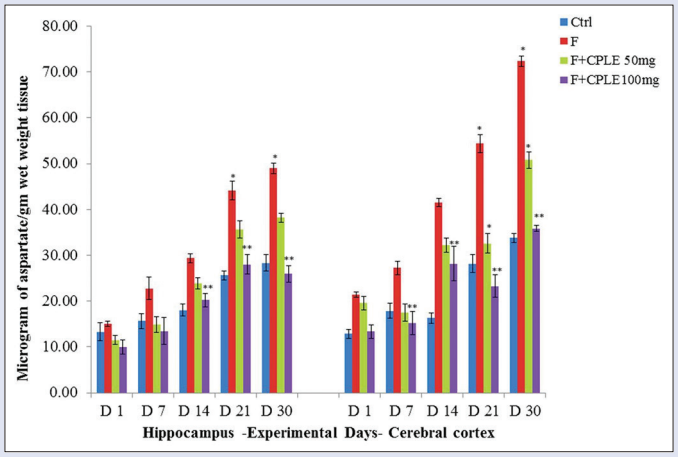

Figure 7: Effect of fluoride on levels of aspartate in cerebral cortex and hippocampus of the brain with and without *Carica papaya* leaf extract. The values are mean ± standard deviation of six (n = 6) individual observations; one-way ANOVA, * P < 0.05, ** P < 0.01, statistically significant. The Aspartate content expressed as in µg amine/g wet wt of tissue

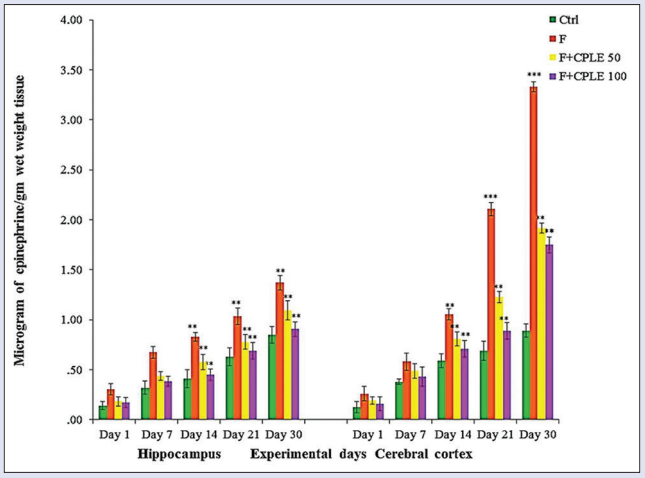

Figure 8: Epinephrine levels in different regions of the brain on chronic fluoride and *Carica papaya* leaf extract treatment. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, ** P < 0.01, *** P < 0.01, statistically significant. The epinephrine level expressed as in µg amine/g wet wt of tissue

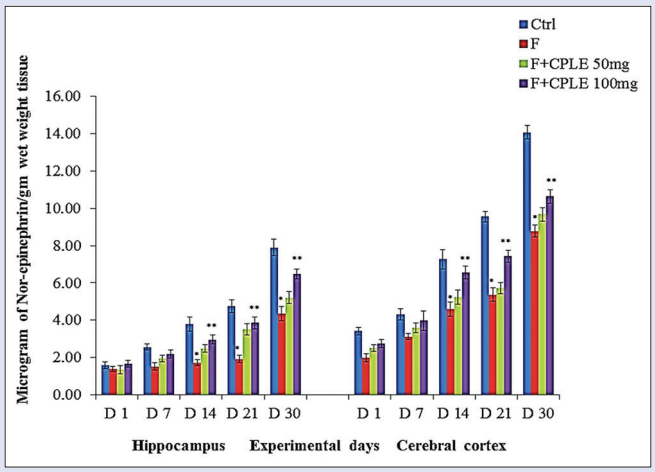

Figure 9: Effect of fluoride on norepinephrine levels in cerebral cortex and hippocampus of brain with and without *Carica papaya* leaf extract. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, * P < 0.05, ** P < 0.01, statistically significant. The norepinephrine level expressed as in µg amine/g wet wt of tissue

with CPLE had similar dopamine levels to that of controls, and it was significantly higher than the fluoride group. The reduced DA levels can lead to decrease in motor coordination, open-field rearing, and scratching behaviors in the rat. Literature review clearly indicates the association chronic fluoride intoxication and dopamine with complex stereotype behaviors intoxicated rats.[51,52] There is growing evidence about of fluoride on the molecular cascade of underlying neuronal plasticity, which allows the processing and storage of new information[16] these include neurotransmitter level, Ca^{2+} homeostasis, neural cell adhesion molecule polysialylation, long-term potentiation, long-term depression, NMDA receptors, and protein kinase C.[9,62] The study done by Madhusudhan *et al.* provided evidence on fluoride-induced effects on brain neurochemistry, which was more, pronounced in younger rats.[14] The mammals are very sensitive to fluoride exposure during their development stages and cause effects in relation to learning and behavior due to the intrinsic age-related differences at the molecular sites at which fluoride interacts.[4,6,45]

The 5HT is another neurotransmitter which controls the mood and depression in mammals.[19,18,39,24,60,48] Similarly to the other catecholamines, the levels of 5HT in fluoride-induced rats were significantly low, and the coadministration of CPLE has helped in recovering or maintaining the 5HT levels in the postnatal rats in dose-dependent fashion. 5HT levels protectant group were similar to that of postnatal control rats and significantly higher than fluoride group [Figure 11]. The behavioral studies (i.e., open-field test) done using postnatal rats showed that the anxiety, raring, habitual, and crossing activities were significantly less in rats which were induced with fluoride chronically, whereas the controls showed normal behavioral activities in open-field test and similar activity was observed in the rats which were receiving the CPLE concomitantly along with the fluoride.

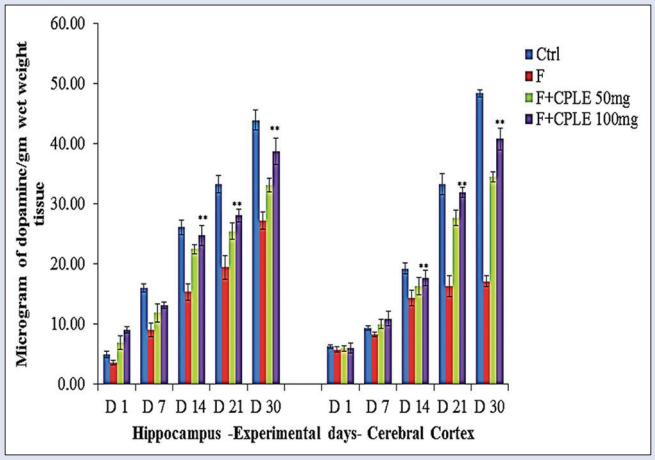

Figure 10: Effect on dopamine levels in hippocampus and cerebral cortex on fluoride and *Carica papaya* leaf extract treatment. The values are mean ± standard deviation of 6 ($n = 6$) individual observations; one-way ANOVA, $^{**}P < 0.01$, statistically significant. The dopamine level expressed as in µg amine/g wet wt of tissue

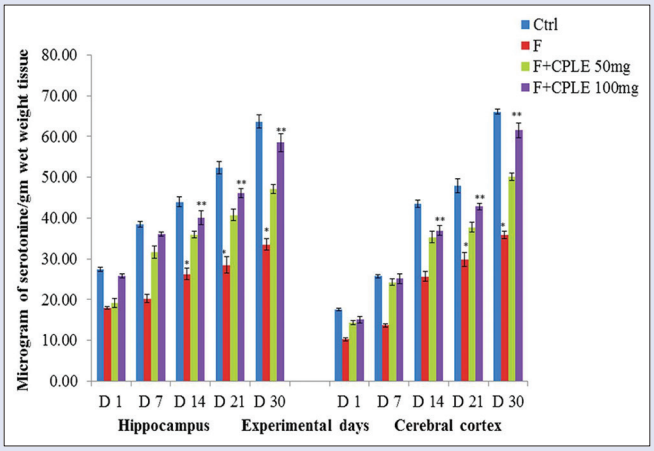

Figure 11: Chronic exposure of fluoride and *Carica papaya* leaf extract on serotonin levels in hippocampus and cerebral cortex of the brain. The values are mean ± standard deviation of six ($n = 6$) individual observations; one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant. The serotonin level expressed as in µg amine/g wet wt of tissue

This is the first occasion, wherein the CPLE was used to restrict the fluoride-induced neurobehavioral changes in developing rats. CPLE is good a source of micronutrients and its specific constituents such as Vitamin (C and E), minerals (Ca, K, Mg, Zn, Mn, Fe), and alkaloids (carpinine and carpaine) exhibited the properties for reversal of toxic effects of fluoride on neurotransmitters and behaviour, which are required for normal development and behavior in animals. Based on the results we can report that CPLE serves as a good neuroprotectant.

CONCLUSION

Chronic exposure of fluoride to pregnant rats caused alterations in total protein concentration in the brains of postnatal rats. The fluoride treatment caused behavioral deficits (memory, learning and cognition, anxiety, and emotional activity and motor control) and neurotransmitter discrepancies. The levels of brain bioamines namely EPN, NE, DA, and 5-HT in cerebral cortex and hippocampus of rat brain diminished significantly during chronic fluoride exposure and it was similar with Ach, whereas the other neurotransmitters such as glutamate and aspartate levels increased with chronic fluoride exposure in the postnatal rats is in corroboration of earlier studies on male and female rodents treated with NaF. The simultaneous administration of CPLE along with fluoride has successfully reduced the fluoride-induced toxic effects in developing rat brain, and it has successfully helped in rectifying the motor, habitual, and memory aspects in postnatal rats by improving the neurotransmitters levels to normal. The present study showed good neuroprotective responses of CPLE and further studies have to be performed to authenticate the mechanisms related to neuroprotective ability of CPLE.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Banala RR, Karnati PR. Vitamin A deficiency: An oxidative stress marker in Sodium fluoride (NaF) induced oxidative damage in developing rat brain. Int J Dev Neurosci 2015;47:298-303.
2. Blaylock RL. Excitotoxicity: A possible central mechanism in fluoride neurotoxicity. Fluoride 2007;37:301-14.
3. Sharma JD, Deepika S, Jain P. Prevalence of neurological manifestations in a human population exposed to fluoride in drinking water. Fluoride 2009;42:127-32.
4. Siddiqui AH. Neurological complications of skeletal fluorosis with special reference to lesions in the cervical regions. Fluoride 1970;3:91-6.
5. Choi AL, Sun G, Zhang Y, Grandjean P. Developmental fluoride neurotoxicity: A systematic review and meta-analysis. Environ Health Perspect 2012;120:1362-8.
6. Niu R, Sun Z, Cheng Z, Li Z, Wang J. Decreased learning ability and low hippocampus glutamate in offspring rats exposed to fluoride and lead. Environ Toxicol Pharmacol 2009;28:254-8.
7. Fassman DK. Prenatal fluoridation. A literature review. N Y State Dent J 1993;59:47-51.
8. Kumar T, Takalkar A. Study of the effects of drinking water naturally contaminated with fluorides on the health of children. Biomed Res 2010;21:423-7.
9. Sarri E, Claro E. Fluoride-induced depletion of polyphosphoinositides in rat brain cortical slices: A rationale for the inhibitory effects on phospholipase C. Int J Dev Neurosci 1999;17:357-67.
10. Williams DI, Russell PA. Open-field behaviour in rats: Effects of handling sex and repeated testing. Br J Psychol 1972;63:593-6.
11. Xiang Q, Liang Y, Chen L, Wang C, Chen B, Chen X. Effect of fluoride in drinking water on children's intelligence. Fluoride 2003;36:84-94.
12. Chinoy NJ, Memon MR. Beneficial effects of some vitamins and calcium on fluoride and aluminium toxicity on gastrocnemius muscle and liver of male mice. Fluoride 2001;34:21-33.
13. Gao Q, Liu YJ, Guan ZZ. Decreased learning and memory ability in rats with fluorosis: Increased oxidative stress and reduced cholinesterase activity in the brain. Fluoride 2009;42:277-85.
14. Madhusudhan N, Basha PM, Rai P, Ahmed F, Prasad GR. Effect of maternal fluoride exposure on developing CNS of rats: Protective role of *Aloe vera*, *Curcuma longa* and *Ocimum sanctum*. Indian J Exp Biol 2010;48:830-6.
15. Needham LL, Grandjean P, Heinzow B, Jørgensen PJ, Nielsen F, Patterson DG Jr., *et al.* Partition of environmental chemicals between maternal and fetal blood and tissues. Environ Sci Technol 2011;45:1121-6.
16. Mullenix PJ, Denbesten PK, Schunior A, Kernan WJ. Neurotoxicity of sodium fluoride in rats. Neurotoxicol Teratol 1995;17:169-77.
17. Sharma C, Suhalka P, Sukhwal P, Jaiswal N, Bhatnagar M. Curcumin attenuates neurotoxicity

induced by fluoride: An *in vivo* evidence. Pharmacogn Mag 2014;10:61-5.

18. Ekambaram P, Paul V. Calcium preventing locomotor behavioral and dental toxicities of fluoride by decreasing serum fluoride level in rats. Environ Toxicol Pharmacol 2001;9:141-6.
19. Chirumari K, Reddy KP. Dose dependent effects of fluoride on neurochemical milieu in hippocampus and neocortex of rat brain. Fluoride 2007;40:101-10.
20. Tiwari P, Kuldeep K, Rajnikant P, Pandey A, Pandey A, Sahu PK. Antimicrobial activity evaluation of the root of *Carica papaya* Linn. Int J PharmTech Res 2011;3:1641-8.
21. Keereevong K. Effect of Alkaloid from *Carica papaya L*. Leaves on Uterine Muscle Contraction in Rats. Master of Science Thesis in Pharmacology. Prince of Songkla University; 2002.
22. Khandare AL, Rao GS, Lakshmaiah N. Effect of tamarind ingestion on fluoride excretion in humans. Eur J Clin Nutr 2002;56:82-5.
23. Paun G, Neagu E, Albu C, Radu GL. Inhibitory potential of some Romanian medicinal plants against enzymes linked to neurodegenerative diseases and their antioxidant activity. Pharmacogn Mag 2015;11:S110-6.
24. Haelewyn B, Freret T, Pacary E, Schumann-Bard P, Boulouard M, Bernaudin M, *et al*. Long-term evaluation of sensorimotor and mnesic behaviour following striatal NMDA-induced unilateral excitotoxic lesion in the mouse. Behav Brain Res 2007;178:235-43.
25. Manjula MR, Reddy KP. Protective effects of aqueous extract of fruit pulp of *Tamarindus indica* on motor activity and metabolism of the gastrocnemius muscle of rats treated with fluoride. Int J Tox Pharm Res 2015;7:241-6.
26. Charles F, Reginald LD, Dorothy LW, Stephen KF, Bartus RT. Behavioural and neurochemical effects following neurotoxic lesions of a major cholinergic input to the cerebral cortex in the rat. Pharm Biochem Behav 1983;18:6973-81.
27. Small WS. An experimental study of the metal processes of the rat. Am J Psych 1900;11:133- 64.
28. Azza Z, Oudghiri M. *In vivo* anti-inflammatory and antiarthritic activities of aqueous extracts from *Thymelaea hirsuta*. Pharmacognosy Res 2015;7:213-6.
29. Gupta R, Kaur J. Evaluation of analgesic, antipyretic and anti-inflammatory activity on *Cordia dichotoma* G. Forst. Leaf. Pharmacognosy Res 2015;7:126-30.
30. Kumar M, Kaur D, Bansal N. Caffeic acid phenethyl ester (CAPE) prevents development of STZ-ICV induced dementia in rats. Pharmacogn Mag 2017;13:S10-5.
31. Nardi GM, Farias Januario AG, Freire CG, Megiolaro F, Schneider K, Perazzoli MR, *et al*. Anti-inflammatory activity of berry fruits in mice model of inflammation is based on oxidative stress modulation. Pharmacognosy Res 2016;8:S42-9.
32. Banala RR, Nagati VB, Karnati PR. Green synthesis and characterization of *Carica papaya* leaf extract coated silver nanoparticles through X-ray diffraction, electron microscopy and evaluation of bactericidal properties. Saudi J Biol Sci 2015;22:637-44.
33. Krishna KL, Paridhavi M, Patel JA. Review on nutritional, medicinal and pharmacological properties of papaya (*Carica papaya* Linn). Nat Prod Rad 2008;2008:364-73.
34. Otsuki N, Dang NH, Kumagai E, Kondo A, Iwata S, Morimoto C, *et al*. Aqueous extract of *Carica papaya* leaves exhibits anti-tumor activity and immunomodulatory effects. J Ethnopharmacol 2010;127:760-7.
35. Nguyen TT, Shaw PN, Parat MO, Hewavitharana AK. Anticancer activity of *Carica papaya*: A review. Mol Nutr Food Res 2013;57:153-64.
36. Paul V, Ekambaram P, Jayakumar AR. Effects of sodium fluoride on locomotor behavior and a few biochemical parameters in rats. Environ Toxicol Pharmacol 1998;6:187-91.
37. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
38. Ciarlose AF. Further modification of a fluorometric method for analyzing brain amines. Microchem J 1978;23:9-11.
39. Pal DK. Determination of brain biogenic amines in *Cynodon dactylon* Pers. and *Cyperus rotundus* L. Treated mice. Int J Pharm Pharm Sci 2009;1:190-7.
40. Jacobowitz DM, Richardson JS. Method for the rapid determination of norepinephrine, dopamine, and serotonin in the same brain region. Pharmacol Biochem Behav 1978;8:515-9.
41. Jett MF, McGuirk J, Waligora D, Hunter JC. The effects of mexiletine, desipramine and fluoxetine in rat models involving central sensitization. Pain 1997;69:161-9.
42. Kari HP, Davidson PP, Kohl HH, Kochhar MM. Effects of ketamine on brain monoamine levels in rats. Res Commun Chem Pathol Pharmacol 1978;20:475-88.
43. Margret S, Walter L, Heinrich L, Peter GW, Franz H. A fluorometric micro method for the simultaneous determination of serotonin, noradrenaline and dopamine in milligram amounts of brain tissue, Pergamon Press, Printed in Great Britain. Biochem Pharmacol 1974;23:2337-446.
44. Madhusudhan N, Basha PM, Rai P, Ahmed F, Prasad GR. Effect of maternal fluoride exposure on developing CNS of rats: Protective role of Aloe vera, Curcuma longa and Ocimum sanctum. Indian J Exp Biol 2010;48:830-6.
45. Long YG, Wang YN, Chen J, Jiang SF, Nordberg A, Guan ZZ, *et al*. Chronic fluoride toxicity decreases the number of nicotinic acetylcholine receptors in rat brain. Neurotoxicol Teratol 2002;24:751-7.
46. Kinjoh T. Effect of food deprivation on maze and discrimination learning in white rats. Ann Anim Psychol 1981;31:11-24.
47. Liu WX. Experimental study of behavior and cerebral morphology of rat pups generated by fluorotic female rat. Zhonghua Bing Li Xue Za Zhi 1989;18:290-2.
48. Mahut H, Zola-Morgan S, Moss M. Hippocampal resections impair associative learning and recognition memory in the monkey. J Neurosci 1982;2:1214-20.
49. Wang J, Yaming G, Hongmei N, Wang S. Effects of high fluoride and low iodine on biochemical indexes of the brain and learning-memory of offspring rats. Fluoride 2004;37:201-8.
50. Weiss, E., Greenberg, G. Open field procedures. In: Greenberg, G., and Haraway, M. (eds.), Comparative Psychology: A Handbook, Garland, New York: Routledge; 1996;603-62.
51. Winograd M, Viola H. Detection of novelty, but not memory of spatial habituation, is associated with an increase in phosphorylated cAMP response element-binding protein levels in the hippocampus. Hippocampus 2004;14:117-23.
52. Wu C, Xinli G, Yaming G, Jianhai Z, Wang J. Effects of high fluoride and arsenic on brain biochemical indexes and learning-memory in rats. Fluoride 2006:39:274-9.
53. Manna P, Sinha M, Sil PC. A 43 kD protein isolated from the herb *Cajanus indicus* L attenuates sodium fluoride-induced hepatic and renal disorders *in vivo*. J Biochem Mol Biol 2007;40:382-95.
54. Shashi A, Sharma N. Cerebral neurodegenration in experimental fluorosis. Int J Basic Appl Med 2015;5:146-51.
55. Den Besten PK. Dental fluorosis: Its use as a biomarker. Adv Dent Res 1994;8:105-10.
56. Vani LM, Reddy KP. Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscle of mice. Fluoride 2000;33:17-26.
57. Eichenbaum H. Is the rodent hippocampus just for 'place'? Curr Opin Neurobiol 1996;6:187-95.
58. Sun Z, Cheng Z, Hong J, Wang JD. Effects of fluoride and lead on the expression of N-methyl-D-aspartate receptor 1 in the hippocampus and learning – Memory of rats. Fluoride 2008;41:233-58.
59. Stewart J, Skvarenina A, Pottier J. Effects of neonatal androgens on open-field behavior and maze learning in the prepubescent and adult rat. Physiol Behav 1975;14:291-5.
60. Chioca LR, Raupp IM, Da Cunha C, Losso EM, Andreatini R. Subchronic fluoride intake induces impairment in habituation and active avoidance tasks in rats. Eur J Pharmacol 2008;579:196-201.
61. Pellegrini-Giampietro DE, Cherici G, Alesiani M, Carlà V, Moroni F. Excitatory amino acid release from rat hippocampal slices as a consequence of free-radical formation. J Neurochem 1988;51:1960-3.
62. Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, *et al*. Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 2001;4:382-90.