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OXIDATIVE STRESS INDUCED ALTERATION OF PROTEIN AND NUCLEIC ACID METABOLISM IN FLUORIDE-INTOXICATED RAT BRAIN: PROTECTION BY 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID ISOLATED FROM *NEANOTIS WIGHTIANA*

Chaitali Sarkar¹, Niranjana Das², Sudipta Pal^{1*} and Biswanath Dinda³

Nutritional Biochemistry Laboratory, Department of Human Physiology¹, Tripura University, Suryamaninagar-799 022, West Tripura, India.

Department of Chemistry, Netaji Subhas Mahavidyalaya², Udaipur-799 114, Gomati Tripura, India

Department of Chemistry³, National Institute of Technology, Agartala, Jirania-799 055, India.

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Correspondence to Author:

Sudipta Pal

Assistant Professor
Department of Human Physiology
Tripura University
Suryamaninagar 799022
West Tripura Ttipura, India

E-mail: sudiptap12@yahoo.co.in

ABSTRACT: Increased production of reactive oxygen species plays important role in pathogenesis of chronic fluoride toxicity resulting from prolonged exposure to fluoride. Oral (NaF) fluoride administration to rats at 20 mg/kg b.w./day for 30 days resulted in elevation in MDA, free amino acid N₂, NO level and decreased activities of SOD, GPx, CAT, GR and reduced non enzymatic antioxidant like GSH. Significant alteration in protein and nucleic acid metabolism in discrete brain regions was observed associated with altered activities of proteolytic and transaminase enzymes in treated animals. *Neanotis wightiana* is traditionally used by local people of Tripura for brain and liver disorders. 3 α -hydroxy olean-12-en-27-oic acid isolated from the plant was administered to rats at a dose of 5 mg/kg b.w./day orally for the last 14 days of fluoride exposure in order to elucidate the antioxidative role of this triterpenoid compound in brain protein and nucleic acid metabolism during toxic insult. The present study indicates that the compound appreciably prevented NaF-induced changes in above mentioned parameters in rat brain by antagonizing the free radical generation and enhancing the antioxidant defence power of brain tissue.

INTRODUCTION: Fluorine is widely distributed in nature and is estimated to be the 13th most abundant element on our planet¹. It is the most electronegative of all chemical elements, and as a result, it never exists in elemental form, but rather combines with other elements; fluoride compounds represent about 0.06–0.09% of the content of the earth's crust². Fluoride is distributed universally throughout soil, plants and animals and is assumed to be an essential element in animals, including humans.

The distribution of fluoride in the environment is uneven and largely is believed to derive from geogenic causes. The natural sources of fluoride are fluorite, fluorapatite, and cryolite, whereas anthropogenic sources include coal burning, oil refining, steel production, brick-making industries and phosphatic fertilizer plants, among others.

Among the various sources of fluoride in the environment, those of anthropogenic origin have occasionally been considered to be major ones. Groundwater is more susceptible to fluoride accumulation and contamination than are other environmental media, primarily because of its contact with geological substrates underneath³. The impact of environmental fluoride on human health is well documented. When consumed in adequate quantity, fluoride prevents dental caries,

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assists in the formation of dental enamels and prevents deficiencies in bone mineralization³. At excessive exposure levels (more than 1 ppm); ingestion of fluoride causes dental fluorosis, skeletal fluorosis, and manifestations such as gastrointestinal, neurological and urinary problems. Reactive oxygen species is known to play important role in fluoride pathogenesis. Oxidative stress is implicated as one of the primary factors that contribute to the development of various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, dementia, etc.⁴.

Despite numerous biological defense systems, enhanced free radical generation has the potential to result in oxidative stress⁵ and promotes cellular injury. Since neural cells are highly susceptible to oxidative stress, over accumulation of free radicals in brain tissue cause subsequent damage to cells. Fluoride in combination with Ca⁺⁺ results in hypocalcemia⁶ and therefore affects the organism. Earlier studies revealed fluoride induced production of free radicals⁷ and disruption in activities of some antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)⁸. Nabavi et al.⁹ reported that fluoride may cross the blood brain barrier and induce neural cell degeneration mediated through oxidative stress. Fluoride toxicity causes increased lipid peroxidation in brain tissues¹⁰, leads to irregularity in brain cells' cellular mechanisms¹¹. Reactive oxygen species (ROS) is known to play crucial role in fluoride-induced oxidative stress in neural tissues¹².

Antioxidant defense mechanisms in brain are insufficient to prevent age-related increase in oxidative damage and exogenous intake of antioxidants might be beneficial for preserving brain function. Supplementation of antioxidants is thus needed to enhance the antioxidative power of cell¹³. A growing number of studies have been designed to test the antioxidant effects of some agents to prevent oxidative stress-induced neurodegenerative diseases and recent studies show mitigative action of natural compounds as antioxidative agents in ROS-induced neurological disorders⁹. Antioxidants that are well absorbed from the GI tract can effectively enter the nervous system and are ideal candidates for protection of the brain and nervous system from oxidative stress mediated damage¹⁴.

3 α -hydroxy olean-12-en-27-oic acid is assumed to have direct or indirect effect on antioxidant defense system as *in-vitro* superoxide radical scavenging activity of this pentacyclic triterpenoid was determined by nitroblue tetrazolium and NADH radical scavenging assay. However, literature regarding the biological activity of this derivative of oleanane-skeleton triterpenoid in oxidative stress is lacking. One report on the inhibitory activity of this triterpenoid on acyl CoA: cholesterol acyltransferase enzyme was obtained that could be related to anti-atherosclerotic activity of this compound¹⁵.

Therefore, to test this hypothesis, the present study was designed to investigate whether antioxidant level (enzymatic or non-enzymatic) would be changed in response to 3 α -hydroxy olean-12-en-27-oic acid administration in fluoride-treated rats' brain. To the best of our knowledge, this study is the first effort to determine whether 3 α -hydroxy olean-12-en-27-oic acid has any free radical scavenging action on fluoride-induced oxidative brain damage.

MATERIALS AND METHODS:

Chemicals and reagents

Sodium fluoride (NaF), purchased from Sigma Chemical Co. (St. Louis, MO, USA), other analytical laboratory chemicals and reagents, purchased from Merck (India), SRL (Mumbai, India) were used. Ultrapure water prepared by Millipore was used throughout the experiment to avoid metal contamination and for the preparation of reagents and buffers used for various biochemical assays in our study.

Plant material

The whole aerial parts of *Neanotis wightiana* were collected from Kalsi (Jolaibari), South Tripura in March 2008 and identified by Prof. B. K. Datta, taxonomist, Department of Botany, Tripura University. A Voucher specimen (#BD/02/08) has been deposited in the National Herbarium, Botanical Survey of India, Botanical Garden, Howrah 711 103.

Extraction and isolation

Fresh air-dried aerial parts of *N. wightiana* were crushed into coarse powder and 3 kg of the powder was extracted three times with MeOH (10 L \times 3) at room temperature for 1 week. The extract was then concentrated under reduced pressure in *vacuo* to a semi solid mass (400 g). The residue (350 g) was

suspended in 125 mL of triple distilled water and extracted three times with hexane, chloroform, ethyl acetate and *n*-BuOH (each 200 mL), successively.

The ethyl acetate soluble fraction (20.2 g) of the crude MeOH extract was column chromatographed through silica gel and eluted with stepwise gradient of CHCl₃/EtOAc (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, 10:90 each 500 ml). The fraction obtained from CHCl₃/EtOAc 90:10 gave a gummy residue, which after repeated column chromatography through silica gel gave 3 α -hydroxy olean-12-en-27-oic acid (45 mg).

Structure elucidation of compound 1

Compound **1** was isolated as amorphous solid (mp 248-250°C) from EtOAc fraction of MeOH extract of *Neanotis wightiana* aerial parts by repeated column chromatography. Its molecular formula was determined as C₃₀H₄₈O₃ from the quasi-molecular ion at *m/z* 457.3682 in HR-FAB-MS and analysis of ¹³C/DEPT NMR data. Its IR spectrum in KBr showed absorption bands for hydroxyl (3433 cm⁻¹), carbonyl (1697 cm⁻¹) and olefinic (1638 cm⁻¹) functions.

The ¹H-NMR spectrum in DMSO-*d*₆ (**Table-1**) showed an olefinic one-proton triplet at δ 5.13 and a broad singlet at δ 3.0 ($W_{1/2} = 7.0$ Hz), attributed to a CHOH group. The ¹H-NMR spectrum also showed seven tertiary methyl singlets at δ 0.71, 0.87, 0.89, 0.91, 1.04, 1.09, 1.28 (each 3H, s) and a carboxyl proton at δ 11.96 (1H, br s) indicating pentacyclic triterpene nature of the compound with a carboxylic group¹⁶. The ¹³C-NMR spectrum (**Table-1**) showed 30 carbon resonances, which on DEPT experiment revealed seven methyl, ten methylene, five methane and eight quaternary carbons. Two olefinic carbon resonance at δ 124.6 and 138.2 and a carboxyl carbon resonance at δ 179.2 suggested an olean-12-ene skeletal structure with a carboxyl group at C-14 position because of deshielding of C-12 carbon by ~ 3.8 and shielding of C-13 carbon by 5.4 ppm by the γ - and δ - effect of the carboxyl group¹⁷.

The carbinol carbon resonance at δ_C 76.9 coupled with broad singlet of carbinol methane proton at δ_H 3.0 suggested that C-3 hydroxyl group was alpha and axial. The proton signal at δ 2.73 due to H-18 was characteristic of olean-type triterpenes¹⁷. The spectral data of the compound were very similar to those of 3 α -hydroxy olean-12-en-27-oic acid¹⁸. On

the basis of the foregoing facts, the structure of the compound **1** was deduced as 3 α -hydroxy olean-12-en-27-oic acid. It is a known compound but is reported for the first time from this plant. Earlier it was reported from the leaves of tropical tree, *Cordia alliodora* (Boraginaceae)¹⁸.

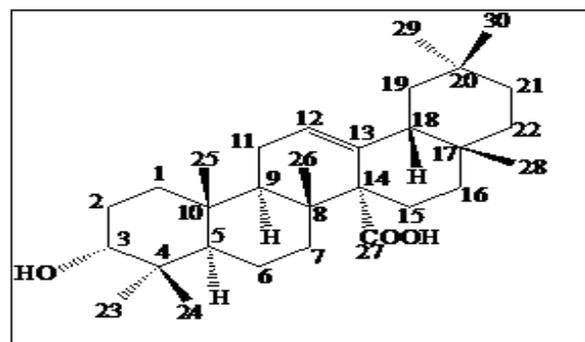


FIG. 1: STRUCTURE OF COMPOUND 1

TABLE 1: ¹H-(600 MHz) and ¹³C-(150 MHz) NMR SPECTRAL DATA OF COMPOUND 1 IN DMSO-*d*₆ (δ in ppm)

| No. (H/C) | δ_H^a | δ_C^b |
|-----------|------------------|-------------------------|
| 1 | | 36.6 (CH ₂) |
| 2 | | 27.5 (CH ₂) |
| 3 | 3.0 br m | 76.9 (CH) |
| 4 | — | 39.8 (C) |
| 5 | | 47.0 (CH) |
| 6 | | 18.0 (CH ₂) |
| 7 | | 32.7 (CH ₂) |
| 8 | — | 36.5 (C) |
| 9 | | 47.0 (CH) |
| 10 | — | 36.5 (C) |
| 11 | | 23.8 (CH ₂) |
| 12 | 5.13 t like | 124.6 (CH) |
| 13 | — | 138.2 (C) |
| 14 | — | 56.0 (C) |
| 15 | | 22.9 (CH ₂) |
| 16 | | 27.2 (CH ₂) |
| 17 | — | 32.7 (C) |
| 18 | 2.73 br d (11.5) | 47.0 (CH) |
| 19 | | 45.5 (CH ₂) |
| 20 | — | 31.0 (C) |
| 21 | | 32.7 (CH ₂) |
| 22 | | 36.3 (CH ₂) |
| 23 | 1.09 s | 28.3 (CH ₃) |
| 24 | 1.04 s | 22.6 (CH ₃) |
| 25 | 0.89 s | 15.2 (CH ₃) |
| 26 | 1.28 s | 18.0 (CH ₃) |
| 27 | — | 178.3 (C) |
| 28 | 0.87 s | 28.3 (CH ₃) |
| 29 | 0.71 s | 32.7 (CH ₃) |
| 30 | 0.91 s | 23.3 (CH ₃) |
| OH | 4.30 d (4.8) | |
| COOH | 11.96 br s | |

^a*J* values in Hz in parentheses. ^b Multiplicities were determined by DEPT.

Experimental animals

Healthy, adult male rats of Wistar strain, weighing approximately 130-140 g were obtained from

authorised animal supplier of CPCSEA. They received human care as per CPCSEA guidelines. Animal ethical committee of Tripura University approved the protocols of the experiments. Animals were kept in well ventilated cages at 22-25^oC, maintained under specific pathogen-free condition on a 12 h light dark cycle. Rats were supplied with standard 18% protein (casein) diet throughout the experiment and water *ad-libitum*. Food intake capacity of each group of animals was recorded periodically. Animals were treated according to the following schedule.

Experimental design

After 1 week of acclimatization, animals were divided into four groups, six rats in each group. Group 1 served as control and received the vehicle only. Group 2 was treated with NaF at dose of 20 mg/kg bw/day orally for 30 days. Group 3 was treated with NaF at 20 mg/kg bw orally for 30 days followed by administration of 3 α -hydroxy olean-12-en-27-oic acid (5 mg/kg b.w./day orally) for last 14 days of fluoride treatment. Group 4 was treated with NaF (20 mg/kg bw orally for 30 days) followed by supplementation with vitamin C (20 mg/kg b.w./day orally) for last 14 days of fluoride treatment. The last group served as positive control.

Biochemical analysis

Superoxide radical scavenging activity

Superoxide anion scavenging activity of methanolic extract of aerial part of *Neanotis wightiana* was measured according to the method of ¹⁹ with some modification. Samples were prepared in 100mM phosphate buffer (pH 7.4). 1ml of nitroblue tetrazolium (156 μ M), 1 ml of NADH (468 μ M) and 3 ml of MeOH extract of *Neanotis wightiana* (to produce final concentration of 1-200 μ g/ml) were mixed well. The reaction was started by adding 100 μ l of 60 mm phenazine methosulphate and the mixture was then incubated at 25^oC for 5 min. Absorbance was measured at 560 nm. The percentage of inhibition was calculated using the following formula

$$\text{Percentage of inhibition} = \frac{(\text{O.D. of control} - \text{O.D. of test})}{\text{O.D. of control}} \times 100$$

Body weight and organo-somatic index:

The body weight of each animal was taken onward the commencement of treatment and also noted periodically until sacrifice to observe the changes of body weight in different groups. The organ weight (whole brain) of respective group of

animals was also recorded after sacrifice. From these, the organo-somatic index (OSI) of brain was calculated ²⁰ using the following formula.

$$\text{Organo-somatic index} = \frac{\text{weight (g) of the organ}}{\text{Day 30 total body weight (g)}} \times 100$$

Estimation of RNA and DNA from rat brain

RNA and DNA were isolated from 5% tissue homogenates (in 0.1 M phosphate buffer, pH 7.4) of different brain regions (cerebrum, cerebellum, pons and medulla) of rats as per recommended method ²¹, except that DNA was extracted with 0.8 M PCA at 70^oC. The nucleotide contents were measured in the respective extracts by UV-absorption at 270 and 290 nm respectively on Dynamica double beam UV-VIS spectrophotometer (model Halo DB-20).

Tissue protein content

The acidic, basic, neutral, and total proteins were extracted separately by the methods of ^{22, 23}. The tissue was homogenized in ice-cold 10% trichloroacetic acid (TCA) to precipitate proteins. The homogenates were incubated at 70^o C for 20 min, then cooled and centrifuged. The residue left was washed with ethanol to remove lipids and nucleic acid and taken as the total protein.

The residue was mixed with known volume of 0.2 M HCl and incubated at 100^oC for 30 min and centrifuged. The resulting supernatant was taken as the extract of basic protein. The residue was treated with a known volume of 0.1 M NaOH and kept overnight at room temperature and centrifuged. The supernatant served as the aliquot of acidic protein. Neutral protein content was calculated by subtracting the sum of basic and acidic protein contents from total protein content. Determination of acidic, basic, neutral and total proteins was done spectrophotometrically by the method of ²⁴ using bovine serum albumin as standard.

Free amino acid nitrogen content

The amount of free amino acid nitrogen present in the brain tissue was determined by the method of ²⁵ using leucine standard curve. The 5% tissue homogenate was treated with 0.67 (N) H₂SO₄ and 10% Na-tungstate to get the protein free extract. After centrifugation the supernatant was treated with cyanide acetate buffer and ninhydrin solution and was heated at 100^oC in a water bath for 5 minutes. After cooling the diluent (methyl

cellosolve) was added to it. Readings were taken in a spectrophotometer at 570 nm wavelength.

Pronase activity

The pronase activity in rat brain (cerebrum, cerebellum, pons and medulla) was estimated by the method of ²⁶. The tissue homogenate was incubated at 40°C with casein substrate for 30 minutes and the reaction was stopped by addition of protein precipitating reagent. After centrifugation, the supernatant was collected and readings were taken in a spectrophotometer at 280 nm. Tissue pronase activity was expressed in terms of µg of tyrosine per minute per 100 mg tissue protein.

Trypsin activity

Tissue trypsin activity was measured by the method of ²⁷. In the sample tube, a definite volume of 5% tissue homogenate was added with 2.5 ml of Hb substrate and incubated at 25°C for 30 minutes. A buffer blank was prepared in which TCA was added before addition of tissue homogenate and substrate. After incubation, 5% TCA was added to the sample tube to precipitate the protein. The supernatant was collected after centrifugation and readings were taken in a spectrophotometer at 280 nm. Tissue trypsin activity was calculated from the tyrosine standard curve.

Cathepsin activity

Cathepsin activity of rat brain (cerebrum, cerebellum, pons and medulla) was measured by the method of ²⁸. The 5% tissue homogenate was added with 4% Hb substrate and incubated at 37°C for 60 minutes. Reaction was stopped by addition of 8% TCA. In a similar way, a buffer blank was also prepared in which TCA was added before addition of tissue homogenate and substrate. All the tubes were centrifuged to get the supernatant and O.D. of the samples was taken in a UV-vis spectrophotometer at 280 nm. Tissue cathepsin activity was expressed in terms of tyrosine per minute per mg protein.

Alanine aminotransferase (GOT) and Aspartate aminotransferase (GPT) activities

Tissue GOT and GPT activities were determined using standard kit (Coral clinical systems, Goa, India) followed by the method of ²⁹. Estimation of GOT activity was done using aspartate and α-ketoglutarate as substrates and the colour intensity was measured photometrically after specific period of incubation at 37°C temperature. In case of GPT,

the tissue homogenate was allowed to react with alanine and α-ketoglutarate, and the quantity of pyruvate formed was measured photometrically. The reaction does not obey Beer's law and hence a calibration curve was plotted using pyruvate standard. Both the enzyme activities were expressed as units per mg of tissue protein.

Lipid peroxidation

LPO was measured according to the method of ³⁰. The tissue homogenate was treated with 20% TCA and thiobarbituric acid. The reaction mixture was then added with 1 mM EDTA to chelate iron and reduce its interference in the peroxidation reaction of unsaturated fatty acids. It was then heated at 80°C for 5 minutes. The optical density was noted at 533 nm. The molar extinction coefficient, $1.56 \times 10^5 \text{ cm}^2/\text{mmol}$ of malondialdehyde was used to calculate the malondialdehyde production.

Reduced glutathione (GSH) content

Reduced glutathione content was assayed by the method of ³¹, modified by ³². The 5% tissue homogenate (in 0.1M PB) was treated with 20% TCA containing 1 mM EDTA to precipitate proteins. The supernatant was then mixed with Ellman's reagent and kept at room temperature for 20 minutes. The absorbance of the final reaction mixture was read at 412 nm. Tissue glutathione content was calculated from the standard curve of known GSH.

Nitric oxide (NO) content

Nitric oxide (NO) is synthesized in biological system by the enzyme nitric oxide synthase (NOS). The final products of NO are nitrite (NO₂⁻) and nitrate (NO₃⁻) *in vivo*. The nitrite concentration is the indicator of nitric oxide production. The method used depends on Griess reactions which convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance of the azo chromophore at 550 nm was measured according to the method of ³³. The 5% tissue homogenate was prepared in 0.25 M sucrose solution and centrifuged at 6000 rpm at 4°C for 10 minutes to obtain clear supernatant. Equal volume of 0.25 M sucrose solution, 1% sulfanilamide and 0.1% naphthylethylene diamine hydrochloride was then added to the supernatant and mixed well. After 20 minutes of incubation, the optical density was measured spectrophotometrically at 550 nm.

Superoxide dismutase (SOD) activity Superoxide dismutase (SOD) activity was assayed according to

³⁴. The assay was based on SOD-mediated increase in the rate of autooxidation of hematoxylin in aqueous alkaline solution, which yielded a chromophore with maximum absorbance at 560 nm. The enzyme activity was expressed as units per minute per mg of protein.

Catalase activity

Catalase activity was assayed following the procedure of ³⁵. A 5% tissue homogenate was prepared in 0.1M phosphate buffer (pH 7.4) containing Triton X-100. In a cuvette containing 200µl phosphate buffer (0.1M, pH 7.4) and 50µl of tissue extract, 250µl of 0.066M H₂O₂ (in phosphate buffer) was added and decrease in optical density was measured at 240 nm for 60 sec interval upto 5 mins. The molar extinction coefficient of H₂O₂ (43.6M cm⁻¹) was used to determine catalase activity. One unit of activity is equal to the moles of H₂O₂ degraded/min/mg protein.

Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured by the procedure of ³⁶. Supernatant obtained after centrifugation of 10% brain homogenate at 10,000g for 30 min at 4⁰C was used for GPx assay. One ml of reaction mixture was prepared with phosphate buffer (0.1 M, pH 7.4), GSH (10mM), glutathione reductase (0.2 unit) and 0.05 ml of the supernatant of respective brain tissues. After incubation at 37⁰C for 10 min, 1.5 mM NADPH was added in the reaction mixture and absorbance was recorded at 340 nm. The enzyme activity was expressed as nmoles of NADPH oxidized per min per mg protein.

Glutathione-S-transferase (GST) activity

GST activity was determined following the procedure of ³⁷. The assay system consisted of 0.01 M (pH 6.5) phosphate buffer containing 1 mM EDTA, 20 mM GSH, 20 mM CDNB and the supernatant. The increase in absorbance was noted at 340nm. The activity of GST was expressed as nmoles of GSH-CDNB conjugate formed per min per mg of protein.

Glutathione reductase (GR) activity

The GR activity was determined in the tissue by the method of ³⁸. The assay system consisted of phosphate buffer (0.2 M, pH 7.0, containing 2 mM EDTA), 20 mM GSSG, 2 mM NADPH and supernatant. The enzyme activity was quantitated at 25⁰C by measuring the disappearance of NADPH at 340 nm. The enzyme activity was expressed as

nmoles of NADPH oxidized per minute per mg of protein.

Tissue free hydroxyl radical (·OH) production

For free hydroxyl radical estimation, the animals of each group were treated with 30% dimethylsulfoxide at a dose of 0.4 ml per 100g body weight 2 hours before sacrifice. After sacrifice, a 5% tissue homogenate of different parts of the brain was prepared and used to determine the free hydroxyl radical formation according to the method of ³⁹. The homogenate was treated with 10N H₂SO₄ to allow precipitation of protein. The protein-free filtrate was then taken for extraction of methane sulfinic acid, which was produced from DMSO by the action of free hydroxyl radicals. Fast blue BB salt was used for production of yellow coloured product by reaction with MSA which was measured at 425 nm spectrophotometrically.

Protein assay

Protein contents in tissue homogenates and in the supernatant were determined by the method of ²⁴.

Histopathological examination

Brain tissues fixed in formalin (10%) were dehydrated through a graded ethanol series and embedded in paraffin wax (MP, 68°C). Brain sections at 9 µm were stained with Delafield's haematoxylin and eosin stain and analysed using compound microscope.

Statistical analysis

Significance of the results was statistically evaluated by using one way ANOVA (analysis of variance) using Statistica software (version 9) followed by multiple comparison t test to compare the difference between means of two different groups. The results were expressed as mean ± SD and values of P < 0.05 were considered statistically significant ⁴⁰.

RESULT:

Effect of fluoride on superoxide scavenging activity of MeOH extract of *Neanotis wightiana* in vitro

As shown in **Fig. 2**, the superoxide radical scavenging activities of this plant (aerial part) extract and the reference compound increased markedly with increasing concentration. Highest inhibition was found (52.32%) at concentration of 200µg/ml. Butylated hydroxyanisole, a good ROS scavenger (Festjens et al., 2006) showed maximum inhibition 42.77 % at 200 µg/ml concentration in this study. The plant extract showed higher

inhibition at lower concentration than the positive control.

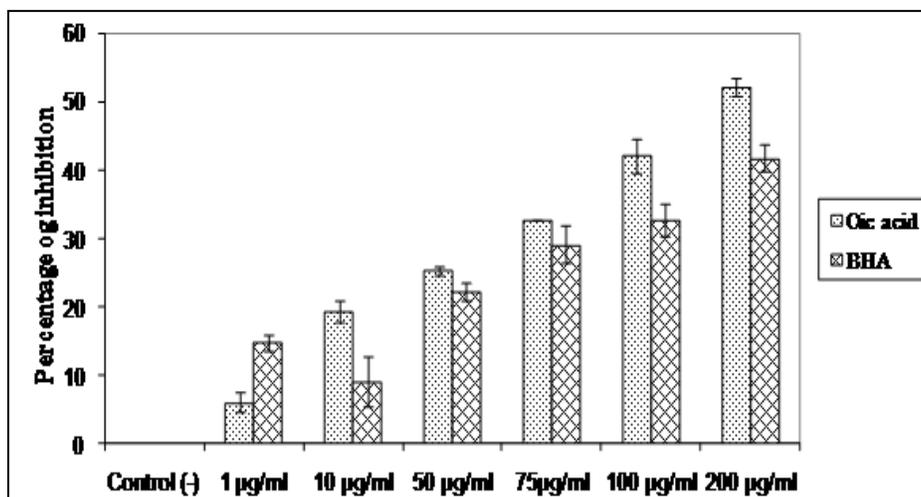


FIG: 2 EFFECT OF METHANOLIC EXTRACT OF NEANOTIS WIGHTIANA (AERIAL PARTS) TO ASSESS SUPEROXIDE RADICAL SCAVENGING ACTIVITY

Effect of sodium fluoride on body weight and organo-somatic index

Table 2 represents that fluoride treatment at the present dose and duration significantly ($p < 0.01$) decreased (17.36%) the body weight of rats as compared to the control group. The OSI (organo-

somatic index) of whole brain of fluoride-treated rats decreased by 18.27% ($p < 0.01$). 3α -hydroxy olean-12-en-27-oic acid and vitamin C supplementation at the test dose and duration exhibited significant counteractive effects against fluoride-induced changes in body weight as well as OSI.

TABLE 2. EFFECT OF NAF ON BODY WEIGHT AND ORGANO-SOMATIC INDEX OF BRAIN OF MALE RATS WITH OR WITHOUT 3α -HYDROXY OLEAN-12-EN-27-OIC ACID AND VITAMIN C SUPPLEMENTATION

| | Control (a) | NaF-treated (b) | NaF treated + 3α -hydroxy olean-12-en-27-oic acid (c) | NaF treated + Vit.C (d) | % change |
|--|--------------------|-------------------------------------|--|---------------------------------------|--|
| Body weight (g) | 160.2±2.5 (N=6) | 132.7±1.09 (N=6) $p^a < 0.01$ | 135.5±2.15 (N=6) $p^b < 0.05$ | 138.33 ±1.65 (N=6) $p^b < 0.05$ | 17.36 (a & b) 3(b&c) 5.15 (b & d) |
| Organo-Somatic Index (OSI) of brain | 1.04±0.02 (N=6) | 0.85±0.019 (N=6) $p^a < 0.01$ | 0.95±0.025 (N=6) $p^b < 0.05$ | 0.96±0.008 (N=6) $p^b < 0.05$ | 18.27 (a & b) 11.76(b&c) 12.94 (b & d) |

Values are Means±S.D. p^a compared with control group and p^b compared with fluoride treated group, N is the number of animals in each group

Effect of fluoride on brain proteolytic enzymes

Moreover, treatment with NaF caused significant decrease in proteolytic enzyme (like cathepsin, trypsin and pronase) activities. **Table 3** represents that the cathepsin activity decreased by 23.08% ($p < 0.01$), 46.15% ($p < 0.001$), 53.85% ($p < 0.001$) and 54.55% ($p < 0.001$) in cerebrum, cerebellum, pons and medulla, respectively after fluoride treatment. The pronase activity also decreased significantly in all studied regions of rat brain (**Table 3**). The decrease was found to be 17.39% ($P < 0.01$), 12.5% ($p < 0.05$), 35.71% ($p < 0.001$) and 50% ($p < 0.001$) of the control value in cerebrum,

cerebellum, pons and medulla, respectively. It is further revealed from **Table 3** that the trypsin activity decreased (37.93%, 50%, 54.17%, and 60.87% in cerebrum, cerebellum, pons and medulla) significantly in studied regions of brain due to fluoride. Supplementation of 3α -hydroxy olean-12-en-27-oic acid with NaF could alleviate the depressing effects of NaF on brain proteolytic enzyme activities and restored their respective control values. Vitamin C also restored all enzyme activities in the studied regions of brain of fluoride-treated rats.

TABLE 3. EFFECT OF FLUORIDE ON PROTEOLYTIC ENZYME ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA OF RAT'S BRAIN WITH OR WITHOUT 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID SUPPLEMENTATION

| Parameters | Control | | | | NaF treated | | | | NaF treated + 3 α -hydroxy olean-12-en-27-oic acid | | | | NaF treated + Vit.C | | | |
|---|-------------------|-------------------|-------------------|-------------------|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla |
| Cathepsin e Activity (μ moles of tyrosine/min/mg of protein) | 0.013 \pm 0.001 | 0.013 \pm 0.005 | 0.013 \pm 0.001 | 0.011 \pm 0.001 | 0.011 \pm 0.003, p ^{a***} | 0.007 \pm 0.001, p ^{a***} | 0.006 \pm 0.001, p ^{a***} | 0.005 \pm 0.001, p ^{a***} | 0.016 \pm 0.004, p ^{b***} | 0.014 \pm 0.002, p ^{b***} | 0.009 \pm 0.002, p ^{b***} | 0.008 \pm 0.001, p ^{b***} | 0.015 \pm 0.002, p ^{b***} | 0.013 \pm 0.002, p ^{b***} | 0.009 \pm 0.001, p ^{b***} | 0.009 \pm 0.002, p ^{b***} |
| Trypsin e Activity (μ moles of tyrosine/min/mg of protein) | 0.029 \pm 0.003 | 0.030 \pm 0.001 | 0.024 \pm 0.001 | 0.023 \pm 0.001 | 0.018 \pm 0.002, p ^{a***} | 0.015 \pm 0.002, p ^{a***} | 0.011 \pm 0.001, p ^{a***} | 0.009 \pm 0.001, p ^{a***} | 0.031 \pm 0.003, p ^{b***} | 0.027 \pm 0.002, p ^{b***} | 0.014 \pm 0.001, p ^{b***} | 0.013 \pm 0.002, p ^{b***} | 0.033 \pm 0.004, p ^{b***} | 0.026 \pm 0.003, p ^{b***} | 0.022 \pm 0.001, p ^{b***} | 0.019 \pm 0.001, p ^{b***} |
| Pronase Activity (μ moles of tyrosine/min/mg of protein) | 0.023 \pm 0.001 | 0.024 \pm 0.003 | 0.014 \pm 0.001 | 0.020 \pm 0.001 | 0.019 \pm 0.002, p ^{a**} | 0.021 \pm 0.001, p ^{a**} | 0.009 \pm 0.001, p ^{a***} | 0.01 \pm 0.001, p ^{a***} | 0.027 \pm 0.003, p ^{b***} | 0.027 \pm 0.004, p ^{b**} | 0.014 \pm 0.002, p ^{b***} | 0.019 \pm 0.001, p ^{b***} | 0.024 \pm 0.003, p ^{b***} | 0.027 \pm 0.006, p ^{b**} | 0.015 \pm 0.002, p ^{b***} | 0.020 \pm 0.001, p ^{b***} |

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates p<0.001, ** indicates p<0.01, * indicates p<0.05, # indicates insignificant difference

Effect of fluoride on DNA and RNA contents and free amino acid nitrogen level in rat brain

Table 4 represents that tissue DNA and RNA contents decreased whereas amino acid nitrogen concentration increased in different regions of rat brain (cerebrum, cerebellum, pons and medulla). The study reveals that DNA content decreased by 25.68% (p<0.001), 28.95% (p<0.001), 34.62% (p<0.001) and 34.15% (p<0.001) in cerebrum, cerebellum, pons and medulla, respectively following exposure to fluoride. 3 α -hydroxy olean-12-en-27-oic acid supplementation in fluoride-treated rats restored the reduced DNA content of proposed areas of brain by 30.91% (p<0.001), 25.19% (p<0.001), 29.41% (p<0.001) and 18.52% (p<0.001).

Similarly, vitamin C exhibited partial protective effects against fluoride-induced alteration of DNA content in the mentioned areas by 23.64 % (p<0.001), 33.33% (p<0.001), 41.18% (p<0.001) and 40.74% (p<0.001) in cerebrum, cerebellum, pons and medulla, respectively. RNA content of fluoride-treated animals also decreased by 28.35% (p<0.001), 9.2% (p<0.001), 31.03% (p<0.001) and

32.18% (p<0.001) in cerebrum, cerebellum, pons & medulla respectively. Administration of 3 α -hydroxy olean-12-en-27-oic acid restored the reduced RNA content in the above mentioned areas of rat brain by 26.37% (p<0.001), 4.38% (p<0.05), 23.33% (p<0.001) and 11.86% (p<0.05), respectively.

Vitamin C also exhibited partial ameliorative effects in restoration of RNA content in those specific brain regions. The present study further reveals that free amino acid N₂ level increased (75.2%, 59.05%, 41.25% and 50.32%) appreciably in the studied brain regions of fluoride-treated rats. 3 α -hydroxy olean-12-en-27-oic acid has the ability to restore free amino acid nitrogen content to the respective control level.

The restoration was more effective in cerebellum (34.05% restoration) in comparison to cerebrum (27.30% restoration), pons (26.21% restoration) and medulla (25.58% restoration). Similarly, vitamin C also exhibited partial protective effects against fluoride-induced alteration of free amino acid N₂ level. The restoration was found to be

20.07%, 31.99%, 17.41% and 17.62%, respectively in the above mentioned areas of rat brain.

TABLE 4. EFFECT OF FLUORIDE ON RNA, DNA AND FREE AMINO ACID NITROGEN CONTENTS IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA OF RAT'S BRAIN WITH OR WITHOUT 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID SUPPLEMENTATION

| Parameters | Control | NaF treated | NaF treated + 3 α -hydroxy olean-12-en-27-oic acid | NaF treated + Vit.C |
|--|---------------------------------------|---------------------------------------|---|---------------------------------------|
| | Cerebrum | Cerebellum | Pons | Medulla |
| RNA (mg/100 mg of tissue) | 0.127 \pm 0.009 | 0.176 \pm 0.007 | 0.087 \pm 0.003 | 0.087 \pm 0.001 |
| | Cerebrum | Cerebellum | Pons | Medulla |
| DNA (mg/100 mg of tissue) | 0.074 \pm 0.005 | 0.19 \pm 0.013 | 0.078 \pm 0.005 | 0.082 \pm 0.003 |
| | Cerebrum | Cerebellum | Pons | Medulla |
| Free amino acid N content (μ g of leucine/mg of tissue protein) | 199.89 \pm 3.08 | 235.9 \pm 1.32 | 248.3 \pm 3.32 | 314.4 \pm 3.15 |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 0.160 \pm 0.005, p ^{a****} | 0.06 \pm 0.004, p ^{a****} | 0.059 \pm 0.002, p ^{a****} | 0.160 \pm 0.005, p ^{a****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 0.115 \pm 0.006, p ^{b****} | 0.167 \pm 0.007, p ^{b****} | 0.074 \pm 0.004, p ^{b****} | 0.066 \pm 0.004, p ^{b****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 0.120 \pm 0.003, p ^{b****} | 0.170 \pm 0.005, p ^{b****} | 0.076 \pm 0.002, p ^{b****} | 0.070 \pm 0.003, p ^{b****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 0.072 \pm 0.006, p ^{b****} | 0.169 \pm 0.006, p ^{b****} | 0.066 \pm 0.006, p ^{b****} | 0.064 \pm 0.006, p ^{b****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 0.180 \pm 0.010, p ^{b****} | 0.255 \pm 2.59, p ^{b****} | 0.072 \pm 0.002, p ^{b****} | 0.076 \pm 0.003, p ^{b****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 254.58 \pm 2.54, p ^{b****} | 247.46 \pm 1.62, p ^{b****} | 258.78 \pm 2.42, p ^{b****} | 351.71 \pm 1.26, p ^{b****} |
| | Cerebrum | Cerebellum | Pons | Medulla |
| | 279.91 \pm 1.90, p ^{b****} | 255.16 \pm 2.59, p ^{b****} | 289.66 \pm 1.23, p ^{b****} | 389.08 \pm 1.49, p ^{b****} |

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, **** indicates p<0.001, ** indicates p<0.01, * indicates p<0.05, # indicates insignificant difference.

Effect of fluoride on acidic, basic, neutral, total protein contents and brain transaminase activity

The treatment with NaF caused significant decrease in different protein levels (acidic, basic, total and neutral) of rat's brain (Table 5). The decrease in acidic protein was found to be 77.74% (p<0.001), 71.09% (p<0.001), 70.86% (p<0.001) and 71.54% (p<0.001), respectively in cerebrum, cerebellum, pons and medulla regions of brain whereas the basic protein level diminished by 65.53% (p<0.001), 72.63% (p<0.001), 79.22% (p<0.001) and 83.51% (p<0.001), respectively in those brain regions.

The inhibition level of neutral protein content in above mentioned brain areas was found to be 58.72% (p<0.01), 73.55% (p<0.001), 65.22% (p<0.001) and 70.86% (p<0.001) as compared with control group. Finally, the total protein level was also inhibited by 71.91% (p<0.001), 72.71% (p<0.001), 72.89% (p<0.001) and 776.04%

(p<0.001). Supplementation of 3 α -hydroxy olean-12-en-27-oic acid can ameliorate NaF-induced changes in protein contents.

Vitamin C showed appreciable beneficial effects in restoration of depleted protein contents in different parts of brain.

The activities of AST and ALT were significantly increased in brain tissue of rats treated with NaF (Table 5). GPT activity in the experimental group was increased by 20.66% (p<0.001), 63.44% (p<0.001), 22.40% (p<0.001) and 41.70% (p<0.001) in cerebrum, cerebellum, pons and medulla, respectively. 3 α -hydroxy olean-12-en-27-oic acid supplementation restored the increased GPT activity of proposed areas of brain by 8.08% (p<0.001), 10.60% (p<0.001), 9.60% (p<0.01) and 14.05% (p<0.001). Similarly, vitamin C exhibited partial protective effects against fluoride-induced alteration in the increased GPT activity in the observed areas of brain. Tissue GOT activities also

increased in fluoride-exposed animals by 61.26% (p<0.001), 44.74% (p<0.001), 35.47% (p<0.001) and 41.67% (p<0.001) in those above mentioned regions of brain. 3 α -hydroxy olean-12-en-27-oic acid supplementation restored the increased GOT activity in proposed areas of brain by 21.23%

(p<0.001), 28.32% (p<0.001), 16.81% (p<0.001) and 14.05% (p<0.001), respectively. Vitamin C also exhibited similar protective effects as 3 α -hydroxy olean-12-en-27-oic acid against fluoride-induced alteration of the increased GOT activity in the studied brain regions.

TABLE 5. EFFECT OF 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID ON FLUORIDE-INDUCED ALTERATION OF PROTEIN CONTENTS (MG%) AND TISSUE TRANSAMINASE ACTIVITIES IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA OF RAT'S BRAIN

| | Control | | | | NaF treated | | | | NaF treated + 3 α -hydroxy olean-12-en-27-oic acid | | | | NaF treated + Vit.C | | | |
|-----------------------|------------|------------|------------|------------|-------------------------------|------------------------------|------------------------------|-------------------------------|---|-------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | Cerebrum | Cerebellu | Pons | Medulla | Cerebrum | Cerebellu | Pons | Medulla | Cerebrum | Cerebellu | Pons | Medulla | Cerebrum | Cerebellu | Pons | Medulla |
| GPT (U/mg of tissue) | 38.77±0.95 | 29.43±2.45 | 49.1±1.81 | 45.71±1.02 | 46.78±1.61, p ^{a***} | 48.1±1.1, p ^{a***} | 60.1±2.1, p ^{a***} | 64.77±1.52, p ^{a***} | 43±1.10, p ^{b***} | 43±1.10, p ^{b***} | 54.33±1.97, p ^{b**} | 55.67±1.51, p ^{b***} | 38.67±1.03, p ^{b***} | 38.33±1.97, p ^{b***} | 50.33±1.97, p ^{b***} | 52±2.19, p ^{b***} |
| GOT (U/mg of tissue) | 26.9±1.31 | 26.91±1.33 | 42.15±1.38 | 35.23±2.07 | 43.38±2.09, p ^{a***} | 38.95±1.9, p ^{a***} | 57.1±2.71, p ^{a***} | 49.91±2.6, p ^{a***} | 34.17±2.04, p ^{b***} | 27.92±1.02, p ^{b***} | 47.5±1.58, p ^{b***} | 44.58±1.88, p ^{b***} | 30.42±2.92, p ^{b***} | 25.83±1.29, p ^{b***} | 42.92±1.88, p ^{b***} | 38.33±1.29, p ^{b***} |
| Total protein (mg%) | 16.16±0.81 | 17.48±0.58 | 14.79±0.20 | 14.65±0.36 | 4.54±0.7, p ^{a***} | 4.77±0.74, p ^{a***} | 4.01±0.3, p ^{a***} | 3.51±0.13, p ^{a***} | 7.79±0.44, p ^{b***} | 6.13±0.29, p ^{b***} | 5.37±0.34, p ^{b***} | 4.84±0.36, p ^{b***} | 10.65±1.08, p ^{b***} | 12.29±0.64, p ^{b***} | 8.40±0.42, p ^{b***} | 7.71±0.14, p ^{b***} |
| Neutral protein (mg%) | 1.72±0.67 | 1.21±0.24 | 1.61±0.31 | 1.51±0.13 | 0.71±0.44, p ^{a**} | 0.32±0.12, p ^{a***} | 0.56±0.12, p ^{a***} | 0.44±0.13, p ^{a***} | 0.91±0.25, p [#] | 0.42±0.08, p [#] | 0.73±0.11, p ^{b*} | 0.56±0.14, p ^{b**} | 0.98±0.40, p [#] | 0.72±0.13, p ^{b***} | 0.89±0.17, p ^{b**} | 0.70±0.15, p ^{b***} |
| Basic protein (mg%) | 4.41±0.77 | 3.91±0.31 | 3.32±0.13 | 2.91±0.11 | 1.52±0.56, p ^{a***} | 1.07±0.31, p ^{a***} | 0.69±0.24, p ^{a***} | 0.48±0.21, p ^{a***} | 1.99±0.58, p ^{b***} | 1.69±0.21, p ^{b***} | 1.29±0.24, p ^{b***} | 0.97±0.15, p ^{b***} | 3.08±0.84, p ^{b***} | 2.22±0.47, p ^{b***} | 1.72±0.17, p ^{b***} | 1.30±0.24, p ^{b***} |
| Acidic protein (mg%) | 11.32±0.88 | 11.69±0.64 | 9.54±0.31 | 9.31±0.09 | 2.52±0.61, p ^{a***} | 3.38±0.51, p ^{a***} | 2.78±0.34, p ^{a***} | 2.65±0.15, p ^{a***} | 4.99±0.26, p ^{b***} | 4.02±0.11, p ^{b***} | 3.67±0.17, p ^{b***} | 3.09±0.18, p ^{b***} | 6.86±0.89, p ^{b***} | 9.34±0.43, p ^{b***} | 5.93±0.32, p ^{b***} | 5.60±0.14, p ^{b***} |

Values are Means±S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates p<0.001, ** indicates p<0.01, * indicates p<0.05, # indicates insignificant difference

Effect of fluoride on brain variables suggestive of oxidative stress, glutathione metabolism and tissue free hydroxyl radical (OH) production

Changes in GSH content, LPO level and free hydroxyl radical were represented in **Table 6**. A significant decrease in GSH level in the cerebrum (47.48%), cerebellum (49.33 %), pons (46.52%) and medulla (46.48%) was found in the fluoride

treated group. 3 α -hydroxy olean-12-en-27-oic acid supplementation almost completely restored the depleted glutathione level in the observed areas of rat's brain. Vitamin C also exhibited appreciable beneficial effects in restoration of decreased glutathione content in all regions of brain. Thiobarbituric acid reactive substance (TBRAS) was used to measure the extent of lipid

peroxidation induced by NaF in different regions of brain (cerebrum, cerebellum, pons and medulla) of rats.

Results indicated that the level of TBARS increased significantly in fluoride-exposed animals. The increase was found to be 45.74% ($p < 0.001$), 48.33% ($p < 0.001$), 37.47% ($p < 0.001$) and 40.80% ($p < 0.001$) in cerebrum, cerebellum, pons and medulla, respectively as compared to their respective control values. A remarkable protection against overproduction of lipid peroxides in all regions of brain was found in 3 α -hydroxy olean-12-en-27-oic acid supplemented group. Enhanced lipid

peroxidation in fluoride-treated animals was also checked by vitamin C supplementation.

Similarly, free \cdot OH radical production significantly increased in brain (79.11% in cerebrum, 70.90% in cerebellum, 88.37% in pons and 113.50% in medulla) tissue at the present dose of fluoride, which was appreciably counteracted by 3 α -hydroxy olean-12-en-27-oic acid supplementation and the amelioration was found to be 26.54%, 29%, 20.29% and 19.52%. Supplementation of vitamin C appreciably prevented increased production of free \cdot OH radical in the above regions of brain.

TABLE 6. EFFECT OF SODIUM FLUORIDE ON GSH, LPO AND FREE HYDROXYL RADICAL FORMATION IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA OF RAT'S BRAIN WITH OR WITHOUT 3 α -HYDROXY-OLEAN-EN-27 OIC ACID SUPPLEMENTATION

| | Control | | | | NaF treated | | | | NaF treated + 3 α -hydroxy olean-12-en-27-oic acid | | | | NaF treated + Vit.C | | | |
|--|------------------|------------------|------------------|------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|---|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla | Cerebrum | Cerebellum | Pons | Medulla |
| Free hydroxyl radical (μ moles/g of tissue) | 12.54 \pm 0.6 | 14.71 \pm 0.54 | 9.89 \pm 1.28 | 8.52 \pm 0.91 | 22.46 \pm 1.12, p ^{a***} | 25.14 \pm 0.89, p ^{a***} | 18.63 \pm 1.21, p ^{a***} | 18.19 \pm 1.19, p ^{a***} | 16.50 \pm 3.05, p ^{b***} | 17.85 \pm 2.82, p ^{b***} | 14.85 \pm 2.71, p ^{b**} | 14.64 \pm 2.20, p ^{b**} | 15.74 \pm 0.86, p ^{b***} | 16.07 \pm 1.05, p ^{b***} | 13.6 \pm 0.74, p ^{b**} | 13.48 \pm 1.27, p ^{b***} |
| LPO (nmoles of MDA/g of tissue) | 18.19 \pm 0.35 | 17.32 \pm 0.77 | 11.45 \pm 0.94 | 11.2 \pm 0.91 | 26.51 \pm 0.72, p ^{a***} | 25.69 \pm 0.57, p ^{a***} | 15.74 \pm 0.72, p ^{a***} | 15.77 \pm 0.71, p ^{a***} | 17.79 \pm 0.92, p ^{b***} | 18.88 \pm 1.34, p ^{b***} | 11.81 \pm 1.01, p ^{b***} | 10.94 \pm 0.60, p ^{b***} | 19.19 \pm 0.26, p ^{b***} | 20.07 \pm 0.87, p ^{b***} | 12.90 \pm 0.57, p ^{b***} | 12.58 \pm 0.78, p ^{b***} |
| GSH (μ M/mg of protein) | 56.07 \pm 0.55 | 54.21 \pm 0.97 | 48.04 \pm 1.04 | 45.61 \pm 1.06 | 29.45 \pm 0.67, p ^{a***} | 27.47 \pm 1.32, p ^{a***} | 25.69 \pm 1.13, p ^{a***} | 24.41 \pm 1.34, p ^{a***} | 49.39 \pm 1.15, p ^{b***} | 51.76 \pm 1.56, p ^{b***} | 43.81 \pm 1.76, p ^{b***} | 40.00 \pm 1.66, p ^{b***} | 53.35 \pm 0.79, p ^{b***} | 54.49 \pm 1.51, p ^{b***} | 46.26 \pm 1.39, p ^{b***} | 42.49 \pm 1.56, p ^{b***} |

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

The alterations in oxidative enzyme activities in brain of rat during administration of NaF with or without 3 α -hydroxy olean-12-en-27-oic acid/Vitamin C were shown in Fig. 3, 4, 5, 6, 7. The NaF treatment resulted in significant decrease in activities of catalase, SOD, GST, GPx and GR. The SOD activity decreased significantly in cerebrum (18.32%), cerebellum (19.16%), pons (34.05%) and medulla (35.41%) of fluoride-exposed animals. Catalase activity also decreased

significantly in those specific brain regions of fluoride-treated animals by 37.9%, 39.04%, 43.29% and 44.36%, respectively. Supplementation of 3 α -hydroxy olean-12-en-27-oic acid along with NaF exposure reversed the enzymatic antioxidant systems towards control. Vitamin C supplemented group also exhibited almost complete restoration of the decreased SOD and catalase activities after fluoride exposure.

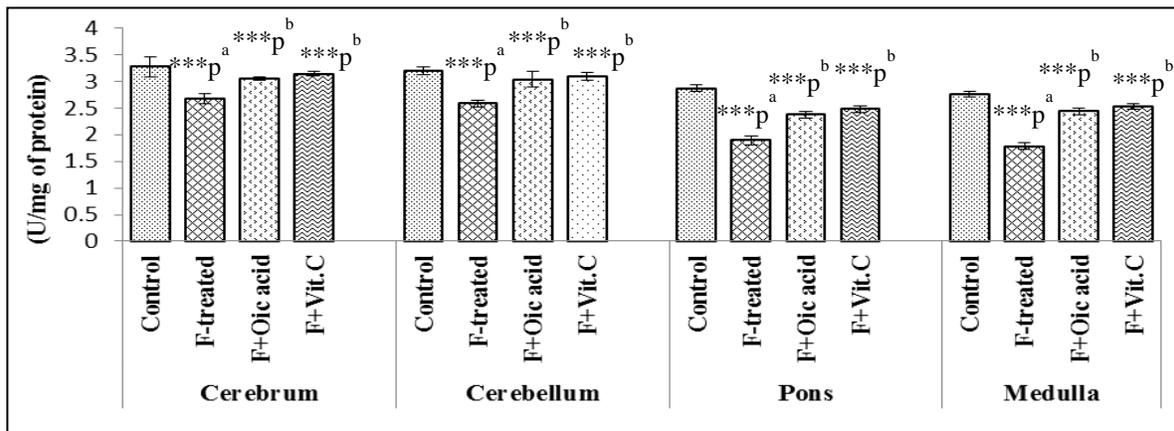


FIGURE 3: CHANGES IN SUPEROXIDE DISMUTASE (SOD) ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA FOLLOWING FLUORIDE EXPOSURE WITH OR WITHOUT 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID AND VITAMIN C SUPPLEMENTATION

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

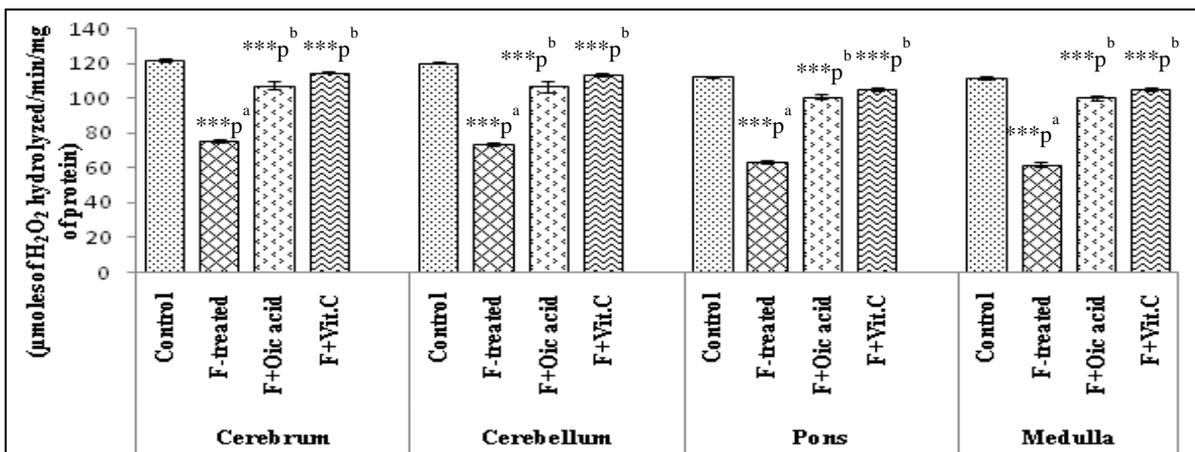


FIGURE 4: CHANGES IN CATALASE (CAT) ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA FOLLOWING FLUORIDE EXPOSURE WITH OR WITHOUT 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID AND VITAMIN C SUPPLEMENTATION

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

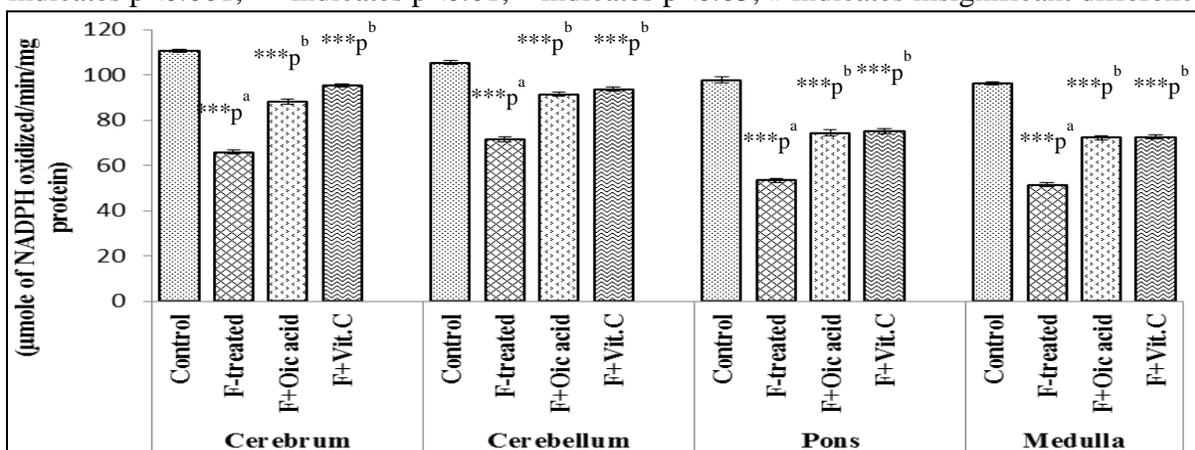


FIGURE 5: EFFECT OF 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID ON FLUORIDE-INDUCED CHANGES IN GLUTATHIONE PEROXIDASE (GPX) ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

As seen in **Fig. 5**, GPx activity was inhibited by 40.43% ($p < 0.001$), 32.12% ($p < 0.001$), 45.37% ($p < 0.001$) and 46.5% ($p < 0.001$), respectively in cerebrum, cerebellum, pons and medulla of rat brain after fluoride treatment. The administration of 3 α -hydroxy olean-12-en-27-oic acid in fluoride-treated rats resulted in reversal of the GPx activity in those studied brain regions by 33.92%, 27.82%, 39.26% and 40.58%. Fluoride treatment also

decreased the activities of GST (23.33%, 18.08%, 19.63% and 16.93%) and GR (57.78%, 58.44%, 41.14% and 36.35%) markedly in the studied brain regions. 3 α -hydroxy olean-12-en-27-oic acid supplementation also exhibited appreciable counteractive effects in restoration of both GST and GR activities in cerebrum, cerebellum, pons and medulla

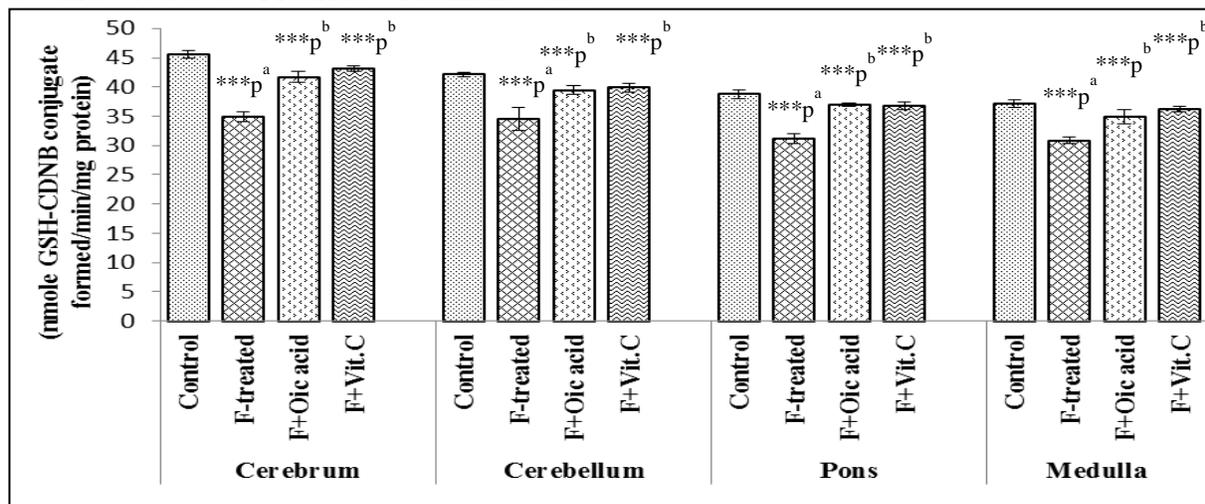


FIGURE 6: EFFECT OF 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID ON FLUORIDE-INDUCED ALTERATION IN GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA

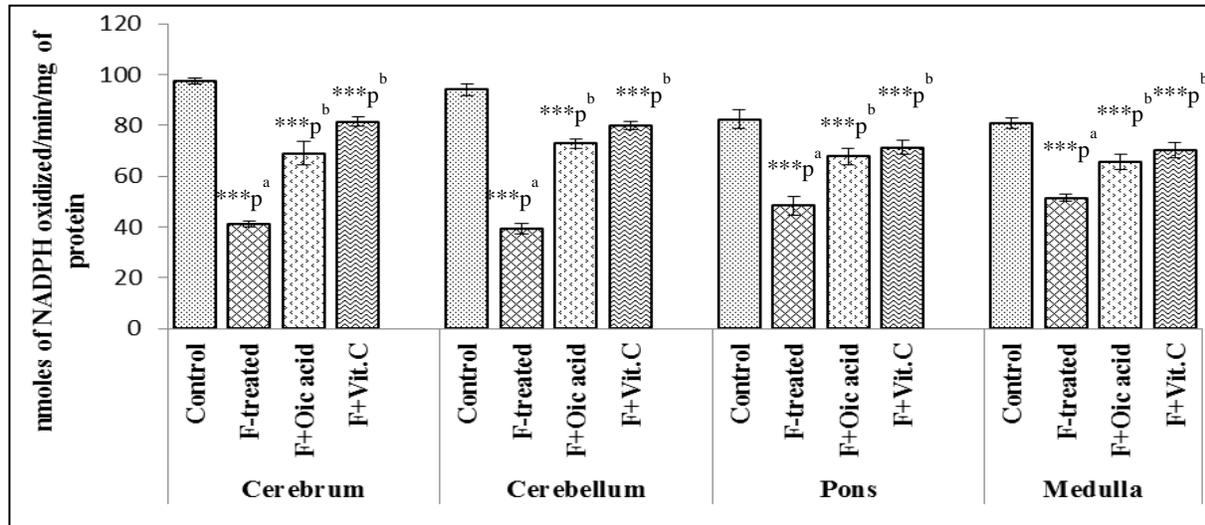


FIGURE 7: CHANGES IN GLUTATHIONE REDUCTASE (GR) ACTIVITY IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA FOLLOWING FLUORIDE EXPOSURE WITH OR WITHOUT 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID AND VITAMIN C SUPPLEMENTATION

Values are Means \pm S.D. p^a compared with control group and p^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

Fluoride exposure caused a significant elevation in NO level (**Fig. 8**) in cerebrum, cerebellum, pons and medulla of rat brain. The increase was found to be 92.72% ($p < 0.001$), 116.08% ($p < 0.001$), 97.72% ($p < 0.001$) and 40.24% ($p < 0.001$), respectively. 3 α -hydroxy olean-12-en-27-oic acid supplementation in

fluoride exposed animals caused partial restoration of NO level in the above mentioned areas. In presence of Vitamin C, elevated NO level was restored more efficiently than 3 α -hydroxy olean-12-en-27-oic acid in cerebrum and cerebellum, pons and medulla towards the control value.

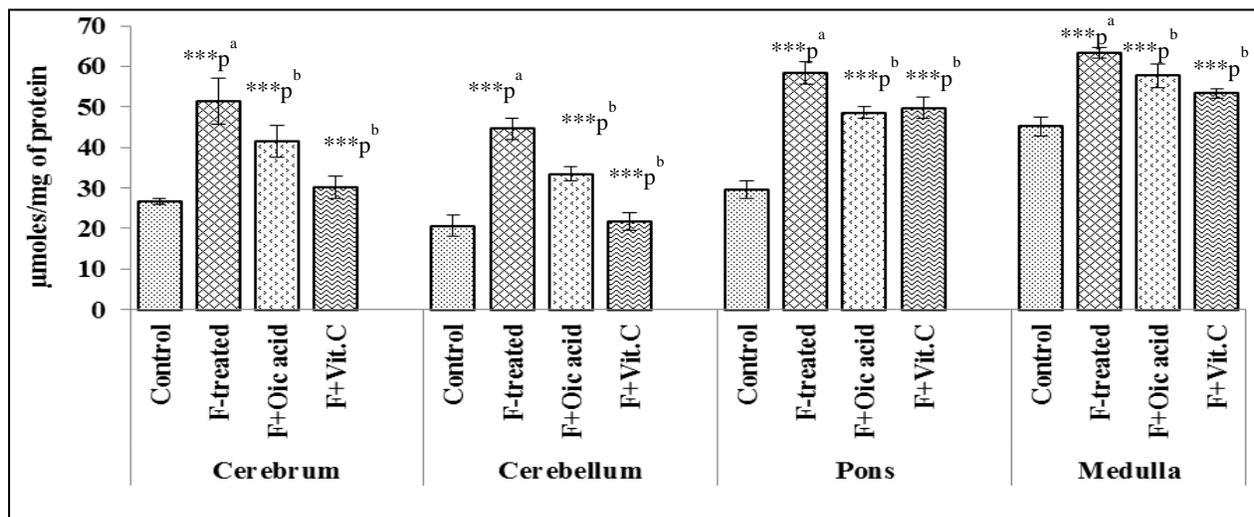


FIGURE 8: EFFECT OF 3 α -HYDROXY OLEAN-12-EN-27-OIC ACID ON FLUORIDE-INDUCED CHANGES IN NITRIC OXIDE (NO) "LEVEL" IN CEREBRUM, CEREBELLUM, PONS AND MEDULLA

Values are Means \pm S.D. ^a compared with control group and ^b compared with fluoride treated group, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, # indicates insignificant difference

Histological studies

Histological analysis of whole brain after fluoride exposure is depicted in **Figure 9**. Control animals show normal cellular compositions in all parts with intact neuron and cytoplasm (**Fig. 9A**). The ultrastructure shows normal oligodendrocytes and few astrocytes. Fluoride exposure (**Fig 9B**) showed chromatolysis of nuclear material, shrinkage of some Purkinje neurons and mild necrosis which is

depicted by hyperchromasia and disintegrated cytoplasm. Cytoplasm showed edema depicted by vacuoles at many instances. Many neurons were shrunken, pyknotic, and darkly stained with small nuclei, and there was a decrease in their overall cell number. Supplementation of 3 α -hydroxy olean-12-en-27-oic acid (9C) and Vitamin C (9D) shows partial protection in the brain of rat.

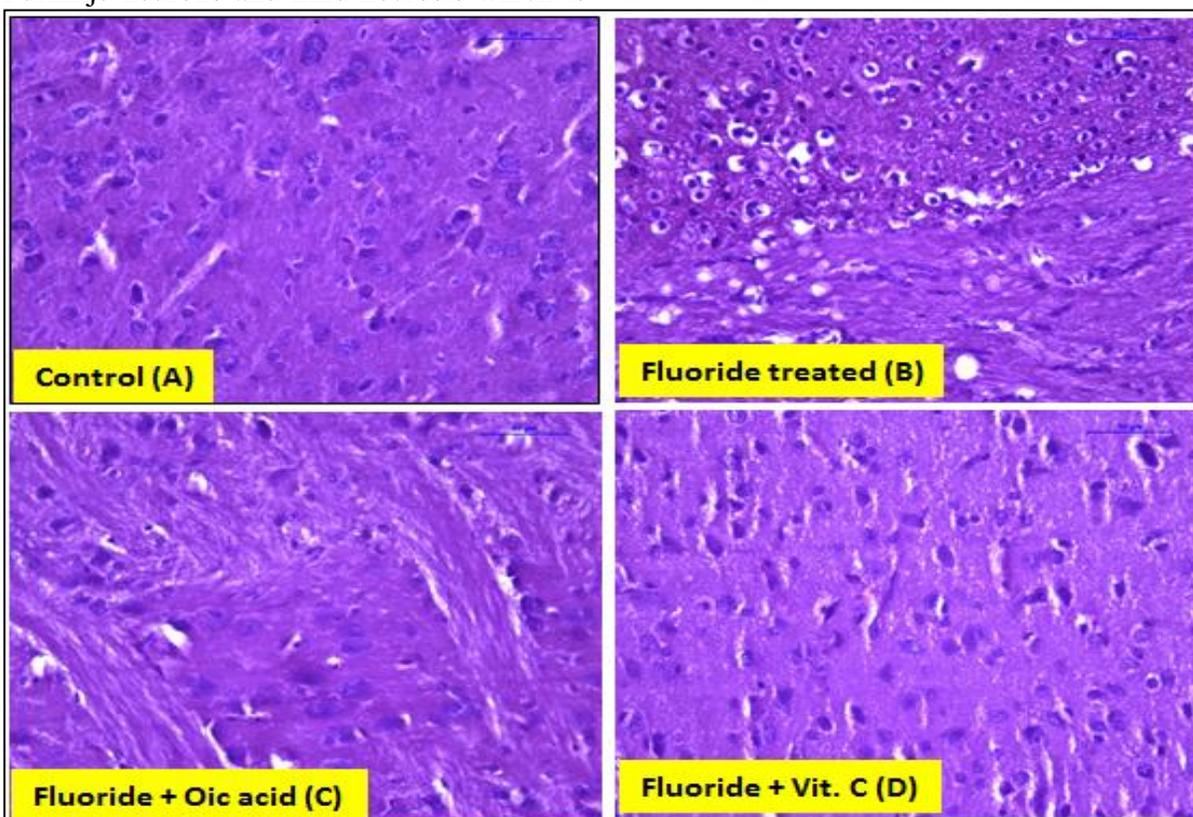


FIG 9: HISTOPATHOLOGICAL CHANGES IN RAT BRAIN AFTER FLUORIDE TREATMENT WITH OR WITHOUT OIC ACID SUPPLEMENTATION

DISCUSSION: To evaluate the role of 3 α -hydroxy olean-12-en-27-oic acid isolated from *Neanotis wightiana* in mitigating fluoride-induced oxidative stress associated with alteration of brain protein and nucleic acid metabolism, effects of oral exposure of fluoride on certain biochemical parameters of brain tissue were studied extensively. Though regarded as an essential trace element, excess intake of fluoride causes adverse health effects in animals. Over-accumulation of fluoride leads to a large number of haematological, hepatic, renal and neurological disorders⁴¹. High fluoride exposure can result in damage to brain cells as the soft tissues like brain are susceptible to oxidative stress. Although the blood-brain barrier is relatively impermeable to fluoride, it does not pose an absolute barrier and fluoride has the ability to enter the brain⁴².

Increased free radical generation is reported to be a causative factor for fluoride-induced oxidative damage⁴³. Due to high electronegative nature, fluoride forms strong hydrogen bonds with –OH and –NH moieties causing oxidative stress resulting in neurodegeneration⁴⁴. Oxidative stress is thought to be involved in DNA damage⁴⁵, which elicits a wide variety of cellular events, such as cell-cycle arrest, apoptosis and necrosis^{46, 47}. Reactive oxygen species (ROS) are implicated as important pathologic mediators in disorders such as inflammation, aging, mutagenicity and carcinogenicity¹⁹. ROS, capable of damaging DNA, proteins, carbohydrates and lipids are generated in aerobic organisms. These ROS include superoxide anion radical (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]) and singlet molecular oxygen¹⁹.

Various phytochemicals have been reported to have antioxidant activity against fluoride-induced oxidative stress. Thymoquinone⁴⁸, quercetin⁴⁹ and lycopene⁵⁰ etc. have been found to be beneficial against sodium-fluoride induced toxicity in liver, brain, red blood cells and cardiac tissue of rats. To assess *in vitro* antioxidant activity of 3 α -hydroxyolean-12-en-27-oic acid, free radical scavenging activity (**Fig. 2**) was measured by calculating percentage of inhibition of the extract on superoxide radical generation and it was found that the compound possesses appreciable inhibitory effects against free radical generation *in vitro*. It has been reported that flavonoids are effective

antioxidants mainly because of their scavenging action against superoxide anions⁵¹. Superoxides are produced from molecular oxygen due to over-activation of oxidative enzymes⁵² or *via* non-enzymatic reaction such as auto-oxidation by catecholamines⁵³. The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of 3 α -hydroxyolean-12-en-27-oic acid towards the generation of superoxides in *in vitro* reaction mixture.

Fluoride treatment at the present dose and duration altered organ weight in relation to body weight (**Table 2**). Significant decrease in body weight of NaF-treated rats was also reported earlier⁵⁴. Decrease in organo-somatic index after fluoride exposure may be due to breakdown of tissue proteins or depressed protein synthetic machinery due to fluoride treatment⁵⁵.

Proteolytic enzymes' (pronase, cathepsin and trypsin) activity and different protein level (acidic, basic, neutral and total) significantly (p<0.001) decreased after NaF treatment (**Table 3**). The decrease in proteolytic enzyme activity might be due to less availability of the substrates in brain tissues after fluoride exposure. Changes in the activity of proteolytic enzymes depend on regional level of tissue proteins and differential distribution pattern of proteins in observed brain regions. Another suggestive mechanism of decreased proteolytic activity after fluoride treatment may be the direct inhibitory effect of fluoride on the enzyme activity. Adverse effects of fluoride on brain metabolic activity may result from inhibitory effects of fluoride on the activity of certain enzymes like oxidoreductase, transferase, hydrolase, TCA cycle enzymes and other enzymes which synthesize protein and DNA^{56, 57, 58, 59}. This may be due to high binding affinity of fluoride with the coenzymes like Ca²⁺ and Mg²⁺⁶⁰ that hampers the activities of these enzymes.

Fluoride treatment appreciably suppressed the activity of cathepsin, pronase and trypsin in all observed regions of rat brain. This is supported by the studies of⁶⁰, where fluoride in Elmex green fluid preparation used for maintaining oral hygiene inhibited salivary cathepsin C activity. *In vitro* studies also revealed that oral hygiene preparation containing fluoride has inhibitory effect on proteolytic enzyme like cathepsin D in the saliva⁶¹.

Disturbed protein metabolism due to fluoride in different brain regions may alter learning, memory, posture and equilibrium maintenance and other cognitive functions.

Transaminases are enzymes involved in the catabolism of amino acids and are involved in the catalytic process of interconversion of a pair of amino acids and a pair of α -keto acids. The enzymes GOT and GPT are known to act as a link between carbohydrate and protein metabolism. An elevation of the activities of the brain transaminases is clear indication of cellular leakage and loss of functional integrity of cell membrane and impaired brain function due to fluoride exposure. It is evidently indicated that fluoride can impair the activities of a series of enzymes, such as transaminases (GOT and GPT) as well as those which lead to ATP production and synthesize protein and DNA^{62, 58}. The present study further reveals that both DNA and RNA contents were significantly reduced after fluoride treatment in all observed areas of rat brain (**Table 4**). Fluoride can interfere with the metabolism of carbohydrate, lipid and nucleic acids (DNA and RNA), injure immune system and damage different vital organs of the body^{63, 64}.

Fluoride treatment also disturbs protein metabolism in brain due to either increased breakdown of proteins or decreased synthesis. Proteolysis, an important metabolic stress after fluoride exposure was suggested by many investigators^{23, 65, 55}. Studies of Trivedi et al.⁶⁶ also confirm our present findings that acidic, basic and neutral protein contents in cerebrum, cerebellum, pons and medulla oblongata were significantly reduced after oral administration of fluoride for 30 days. Protein breakdown in fluorosis may be attributed to inhibition of oxidative decarboxylation of branched chain amino acids as suggested by⁶⁷. Increased proteolysis after fluoride treatment may be due to oxidative stress mediated alteration of cellular proteins.

Fluoride toxicity caused oxidative damage in different tissues which may be ascribed to cellular dysfunctions. Another suggestive mechanism in favour of fluoride-induced decreased brain protein contents may be due to decreased ability of brain tissues to synthesize amino acids as a result of suppressed activity of certain metabolic enzymes like glutamine synthetase and methionine

activating enzymes⁶⁸. So less availability of amino acids may be a causative factor for decreased protein synthesis. In spite of increased amino acid level (**Table 4**) in brain tissues, it is not properly utilized to synthesize proteins, as amino acids are rapidly mobilized by the catalytic action of transaminases from the studied regions of the brain to other areas, causing less availability of substrates for synthesis of desired proteins in those specific brain regions.

Additionally, free hydroxyl radical (**Table 6**) production also increased in fluoride-treated animals. Earlier studies revealed the increase in free radicals in the erythrocytes of fluorotic humans⁶⁹ and in liver, kidney, brain and ovary of experimental animals^{70, 71, 72}. This would not only increase free radical injury but would also enhance excitotoxicity, since reactive oxygen species as well as nitrogen species and lipid peroxidation products can trigger the excitotoxic process. Antioxidant enzyme inhibition would necessarily enhance the toxicity of other neurotoxic elements, pesticides, herbicides, and environmental pollutants including fluoride⁷³.

Moreover, fluoride can alter cellular reduced glutathione level^{74, 75} resulting in excessive production of ROS at the mitochondrial level, leading to damage of cellular components. GSH, an endogenous free radical scavenger protects tissues against fluoride-induced oxidative damage, thus depletion of this cellular antioxidant aggravates the chance of imbalance between antioxidant and pro-oxidant level in cells, inducing oxidative stress. Fluoride induced decreased GSH level was found in all observed brain regions of rats, which indicates oxidative threat to brain tissues.

It is assumed that reactive intermediates can react with GSH either by glutathione transferase mediated reaction and converts GSH to GSSG, as a result GSH content decreased in fluoride intoxicated rat brain. Fluoride also depleted the cellular antioxidant level in liver, kidney, testis and erythrocytes in mammalian model^{76, 77, 78}. Depleted GSH content after fluoride exposure is associated with increased lipid peroxidation level in brain tissue as evidenced from our study and even in other tissues^{79, 80}. Increased LPO level may also be due to decreased activity of antioxidant enzymes especially SOD and catalase. The lipid peroxide production was more pronounced in cerebrum and

cerebellum as compared to that of pons and medulla in fluoride-intoxicated rats. This may be due to tissue specific metabolic efficacy to eliminate free radicals. Enhanced LPO level may be one of the important causative factors of fluoride-induced brain damage, as because brain is rich in polyunsaturated fatty acids, which are highly susceptible to oxidative stress^{69, 81}. Shao et al.⁸² also revealed that fluorosis is associated with increased transformation of polyunsaturated fatty acids to saturated fatty acids due to oxidative metabolic conversion. Excessive ROS production leads to macro-molecular oxidation, resulting in free radical attack on membrane phospholipids causing membrane damage via induction of lipid peroxidation, mitochondrial membrane depolarization and apoptosis⁸².

Fluoride treatment resulted in decreased activities of antioxidant enzymes like catalase, SOD, GST, GPx, GR. Fluoride has been reported to alter oxidative stress markers in various biological systems in different animal models^{41, 72}. Oxidative stress is a recognized mode of action of fluoride exposure that has been observed *in vitro* in several types of cells and also in soft tissues of animals and humans^{83, 84, 78, 80}. Fluoride is thought to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR)^{85, 86}. SOD activity decreased by direct or indirect action of fluoride, the direct effect may be due to competitive inhibition of that enzyme by fluoride⁸⁷.

These observations are in conformity with our present investigation where sub-acute exposure to fluoride is associated with alteration of certain important antioxidant enzymes like SOD, catalase, GPX, GR, GST. Inhibition of antioxidant enzymes by fluoride accelerates overaccumulation of free radicals in the observed tissues resulting in oxidative stress. The supplementation of 3 α -hydroxy olean-12-en-27-oic acid along with NaF exposure reversed pro and antioxidant systems towards control.

A report from China establishes that sodium fluoride significantly increased nitric oxide synthase (NOS) activity⁸⁸ (**Fig. 8**). Interestingly, excitotoxins stimulated NOS activity, which increased intracellular nitric oxide (NO) content. This is of particular importance because NO

combines readily with superoxide radicals forming very powerful toxic peroxynitrite radical, which plays a major role in all neurodegenerative diseases, primarily by damaging mitochondrial energy production, inhibiting glutamate re-uptake and stimulating lipid peroxidation^{89, 90}.

Fluoride has also been shown to inhibit superoxide dismutase, which would increase intracellular levels of the superoxide radical, the substrate for peroxynitrite formation⁹¹. Protective effects of 3 α -hydroxyolean-12-en-27-oic acid on fluoride-induced alteration in free radical scavenging as well as metabolic enzymes were found in brain tissues of rat. Information regarding the biological activity of this triterpenoid is little. Previous studies indicate this triperpenoid has anti-atherosclerotic activity due to its inhibitory effects on acylCoA:cholesterol acyltransferase which helps in cholesterol absorption and esterification¹⁵. Protective effects of 3 α -hydroxyolean-12-en-27-oic acid have been established for the first time against oxidative stress-induced alteration of metabolic activities in fluoride-intoxicated rat brain.

Changes in histological architecture (**Fig. 9**) followed by fluoride treatment were partially counteracted by 3 α -hydroxy olean-12-en-27-oic acid and Vit.C supplementation. It is thus suggested that this pentacyclic triterpenoid compound may be used as prospective protective agent against fluoride-induced metabolic toxicity in brain tissue. However, further studies are needed to fully characterize the mechanisms of neuroprotective action of this plant triterpenoid.

CONCLUSION: Fluoride causes neurotoxicity by altering oxidant/antioxidant homeostasis and protein and nucleic acid metabolism. Adverse effects of fluoride toxicity *via* oxidative stress mediated damage can be mitigated by supplementation of 3 α -hydroxy olean-12-en-27-oic acid that appreciably eliminates the harmful effects of fluoride on rat brain tissue probably due to its antioxidant property. Thus this pentacyclic triterpenoid compound may be used as a prospective protective antioxidative agent that can mitigate fluoride induced brain toxicity. Further studies are still needed to fully characterize the mechanisms of neuroprotective action of this plant triterpenoid.

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