Research Article CODEN: IJPRNK Impact Factor: 5.567 ISSN: 2277-8713
B. Kadalmani, IJPRBS, 2017; Volume 6(6): 1-15 IJPRBS

INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

PROTECTIVE EFFECT OF *TAMARIND SEED COAT, SIDA ACUTA* ROOT AND *TRIDAX PROHUMBANS* ON FLUORIDE TOXICITY CAUSES ROS MEDIATED OXIDATIVE STRESS ALTERS ESTRUS CYCLICITY IN MICE

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Accepted Date: 13/10/2017; Published Date: 27/12/2017

Abstract: Fluoride is a toxic compound found in ground water has affected many countries of the world. It has been established that about 45% of drinking water in India are contaminated by fluoride. This toxic effect increases in every year by environmental hazarders. The adverse effect of fluoride at levels used for water fluoridation is dental fluorosis, skeletal and soft tissues. In the present study normal cyclic female mice weighing between 30-40g, were kept on fluoridated water 50, 100, 200 ppm for 30 days, conducted to find out the toxicity of fluoride in three month old female albino mice after *ip* of doses fluoride, and treated with three medicinal plant ethanol extract of *Tamarind seed coat, Sida acuta* root and *Tridax prohumbans* root daily dose of 15 days, in their distilled water per months and to reflect its impact on antioxidant status (from ovary and liver) and on female fertility is normal. The result revealed that the fluoride water exposure to female mice caused irregular estrus cycle, reduced fertility rate, and concentration of protein and enzyme activity of SOD, CAT, GPX, GSH, LPO, ALP, AST, and ALT, content of ovary and uterus and also liver, kidney decreased significantly as compare to control value. In this study chosen on fluoride induced reproductive toxicity with the ovarian dysfunction with the enzyme of antioxidants, after medicinal plants treatment (*Tamarind seed* coat, *Sida acuta* root and *Tridax prohumbans* root) to find out the fluoride decreased level of antioxidant level in reproductive organ, this is responsible for reproductive abnormalities.

Keywords: Fluoride, antioxidants, estrus cycle, fertility, ovary.

PAPER-QR CODE

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Access Online On:

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How to Cite This Article:

B. Kadalmani, IJPRBS, 2017; Volume 6(6): 1-15

INTRODUCTION

Fluorine is relatively rare element compared to other elements of nearby atomic weight. Fluoride is an inorganic, monatomic anion of fluorine that occurs mostly in metamorphic rocks, soils and water [1]. Fluoride levels in seawater ranges between 0.86 to 1.4 mg/l, although fluoride at low concentration was implicated to be beneficial in dental health to prevent tooth decay. Chronic exposure to high concentration of fluoride was proven to be detrimental causing health complications and can be toxic [2]. Medicinal plants are the great importance of medicinal properties due to their pharmacological properties. The importance of medicinal plants, extracts of plant parts are extensively explored for different bioactivities including antioxidants compared three medicinal plants using enzyme activities against the fluoride, shown in our report. Several studies indicate that follicular cell in mammalian species due to the accumulation of toxic metabolites often results from oxidative stress [3]. Increased cellular reactive oxygen species (ROS) levels of defensive antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxides (GPX) and catalyses (CAT), Reduced Glutathione (GSH), Lipid Per oxidation (LPO), Alkaline phosphate (ALP), Aspertate aminotransferase (AST), and Alanine aminotranferase (ALT). Previous works demonstrate that SOD protects against oxidative stress and important for ovarian functions [4-6]. To prevent damage due to ROS, cells possess a number of antioxidant enzymes, which are in the mitochondria and cytosol, respectively; they convert superoxide into hydrogen peroxide [7]. The fluoride effects of oxidative stress on irregular estrus cycle, premature ovarian follicles as well as oocyte due to infertility. Few reports have documented the presence of the antioxidant activity in reproductive organs in male and female mice [8].

In the present study, we have reported that *in vivo* exposure of 50 ppm fluoride for 30 days causes irregularity in estrus cycle and reduced the weights of reproductive organs in female albino mice (*Mus musculus)*. Furthermore exposure to 50 ppm fluoride *in vivo* modulates antioxidant enzyme profile in liver, kidney, uterus, and ovary suggesting ROS mediated oxidative damage. To confirm the oxidative damage at varying concentration of fluoride treatment (50, 100 and 200 ppm) with three medicinal plant ethanol extract of *T.india seed coat, S. acuta* root and *T.prohumbans* root daily dose of 15 days.

MATERIAL AND METHODS

Animals and Feeding Regimens:

Female albino mice (25 to 35 g) were used in our study and acclimatized to laboratory conditions for a week under standard environmental conditions. Animals were housed in a well-ventilated polycarbonate cage under enriched environmental conditions in groups of four animals per cage in a room under a 12-hour light/dark cycle under controlled temperature

(22°C ± 3°C) and humidity. The animals fed with standard chow diet (Sai Enterprises, Tamil Nadu, India) and water *ad libitum*. Animals were randomly divided into four groups (Control and Gr. I, II, III). A total of 30 mice (9 animals per group) were used. Fluroride toxicity was induced in experimental animals (Gr.I –III) by intra peritoneally (*ip*) injecting appropriate dose (50, 100, 200 ppm) of Sodium Fluoride (Loba Chemie, India) dissolved phosphate buffered saline (PBS, pH 7.4) vehicle for 30 days. All the experiments were performed strictly accordingly to the protocol approved by the Institutional Ethical Committee (IEC) and guidelines provided by Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) [BDU/IAEC/2016/NE/43/Dt. 17.03.2016], India.

Experimental design:

A fluoride toxicity study, Sodium fluoride 2.21 gram contains 1g of Fluoride. 1gram of fluoride dissolved in 1 liter of water (1000ppm). Experimental group of animals were divided into three groups I, II and III, exposed to fluoride (50,100,200ppm) for 30 days through intra peritoneally (*ip*). After induction of fluoride toxicity, the half of the animals from each group, *T. indica* seed coat*, S. acuta* root and *T. prohumbans* root ethanolic extracts were administrated orally at a daily dose of 100mg/kg. bw for 15 days. At the end of the experiment, the animals were sacrificed and used for further analysis.

Estrus cycle monitoring:

During experimental period, we performed vaginal smear cytology every morning between 9.00 to 10.00 hrs (GMT +5.30). Vaginal secretions were collected using cotton tipped swaps softened with a drop of saline (PBS, pH 7.4). Approximately 1-2 inches of the swap had been inserted into the vagina; the end was rotated through 2-3 revolutions, which will allow the cotton tip pick an adequate load of cells. The smears were wiping the glass slide, stained with methylene blue and observed under the light microscope.

Extraction Procedure:

Tamarind seed coat*, Sida acuta* root, *Tridax prohumbans* roots were collected and washed with tap water, and cut into the small pieces before drying. Cleaned plant material tried with under the shady condition in one week for drying. Then they were powdered by electric blender. Three plants material 25 g of powder was added to 150 ml ethanol and socked for 3d. Removal of the plant material from solvents was done by filtration through Maslin cloth, and the filtrate was concentrated using a rotary evaporator.

Organ Sample extraction:

After dissection the organs were washed with saline free blooded and weighted and the organs are homogenized with cold buffer, centrifuged at 10,000 rpm in 4˚C for 20 minutes. The supernatants were obtained and stored -80˚C for further analysis.

Estimation of Antioxidant Enzymes:

Superoxide dismutase (SOD) activity

The assay of superoxide dismutase was done according to the procedure described previously [9]. Briefly, The assay mixture for the enzyme contained 2ml of Tris- Hcl buffer (pH 8.2) 0.5ml of pyrogallol, 0.5 ml aliquots of the enzyme preparation and water to give a final volume of 4.5 ml. the blank is prepared simultaneously, which contains 2.0 ml of Tris- Hcl buffer (pH 8.2) and 2.5 ml of distilled water. The rate of inhibition of pyrogallol auto oxidation after the addition of the enzyme was noted with ELISA reader. The percentages inhibition in the auto oxidation of pyrogallol in the presence of tissue extract was converted to units of inhibition. The amount of enzyme required giving 50% inhibition of pyrogallol auto- oxidation is considered as 1unit of enzyme activity. The extract unit of enzyme activity was then calculated using the formula (560 nm). The enzyme activity was expressed as unit/mg protein (one unit is the amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation).

Catalase activity:

Catalase activity was measured described by [10]. To 0.9 ml of Phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of hydrogen peroxide were added. After 60 seconds, 2ml of dichromate acetic acid mixture was added. The tubes were then kept in a boiling water bath for 10 minutes and the color developed was read at 620 nm. Standards in the range of 2-10 µ M were taken and preceded as test with blank containing reagent alone. The activity was expressed as µ moles of H_20_2 consumed/ min/mg protein.

Glutathione Peroxides activity:

Glutathione Peroxides' (Gpx) was assayed by the method of [11]. To 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate/0.2 ml plasma were added to the mixture. 0.2 ml of glutathione followed by 0.1 ml of hydrogen peroxide was also added. The contents were mixed well and incubated at 37˚C for 10 minutes along with a tube containing all the reagents except the sample. After 10 minutes, the reaction was arrested by the addition of 0.5 ml of 10% TCA, centrifuged and the supernatant was estimated for glutathione by the method of [12]. The activity is expressed as µg of GSH consumed/min/mg protein.

Assay of Lipid Peroxidation:

LPO was estimated by measurement of thiobarbiaric acid reactive substances (TBARS) using the method of [13]. The pink chromogen produced by the reaction of thiobarbitaric acid with melondialdehyde,a secondary product of lipid peroxidation was estimated at 535nm.

Assay of Redused Glutothione:

The Reduced Gluototheone (GSH) content was determined by the method of [12], based on the development of a yellow color while adding 5,5-dithio (2-nitrobenzoic acid) (DTNB) to compounds containing sulphhydryl groups.

Serum biochemical parameters:

Total protein analysis with biuret method (Biosystem, Kit), Alkaline phosphate (ALP), Aspertateaminotransferase (AST), Alanine aminotranferase (ALT) were analyzed with commercial Kit (Biosystem).

Statistical analysis:

Values are expressed as Mean ± SE. Statistical analysis was done by one-way analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) *post hoc* at $P < 0.05$ level of significance by using SPSS software (17.0 version).

RESULT

Exposure level of fluoride induced intra peritoneal injection of 50, 100, 200 ppm concentration and control, three Exposure fluoride groups result revealed that reproductive organ An estrus cycle changes was observed in three treated groups. Three types of cell could be recognized: round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornfield cells; and the small round cells are leucocytes cells was observed by10x magnification. In this case estrus cycle is up normal when inducing high concentration of fluoride, each phase taking more hours to change the next phases. When we treat with medicinal plant estrus cycle change is slightly modified in all groups (Fig- 1).

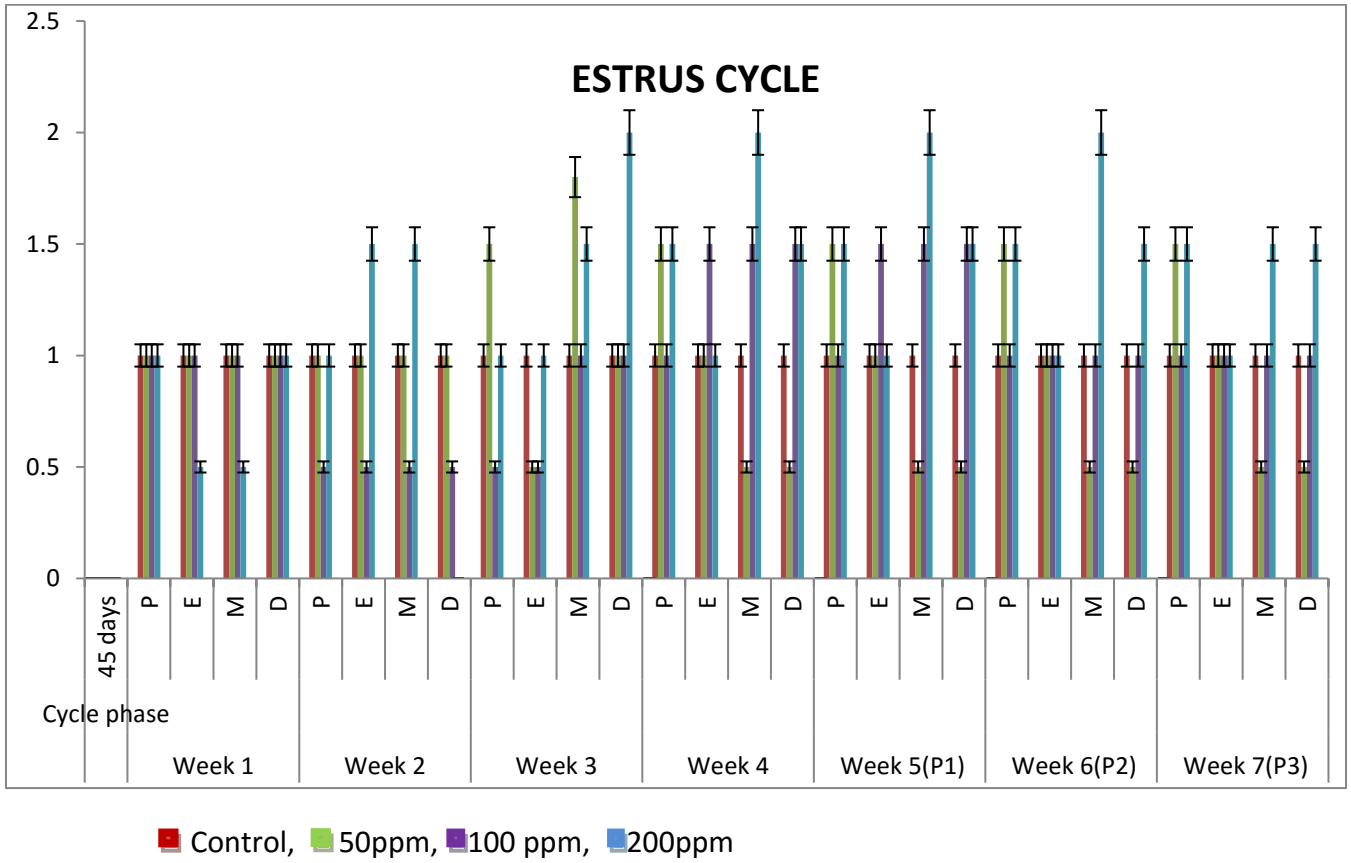

Fig. 1. Animals from each Fluoride groups treated with three medicinal plants ethanol extract of (P1) *Tridax procumbens* root, (P2) *Sida acuta* root and (P3) *Tamarind* seed coat daily dose beneficial effect in estrus cycle.

Result of Antioxidant Enzyme level:

The control organ extract of ovary, uterus, fallobian tube, liver, kidney showed given the enzymes are normal. Hense treated groups of 50, 100, 200 ppm fluoride induction suppressed the activity of the enzyme level is decrease when increased Fluoride concentration level. In this study focus on Antioxidant enzymes (SOD, CAT, GPX) very important in protecting organisms from reactive oxygen species (ROS).We are analyzed ALP, AST, AMP, SOD, Catalase and Gpx, GSH, LPO enzyme level in Serum, liver, kidney, ovary, uterus, and fallopian tube. The superoxide free radicals and per oxidation play an important role in fluorosis affected peoples [14]. SOD enzyme level of serum, liver, Kidney and reproductive organ level showed decrease compare with control (Fig-2), after medicinal plant treatment, Among the three groups the hightest activity of catalase was observed in control and lowest activity of catalase present in group 3 the activity was decreased highly concentraed with the reproductive organ (Fig-3). Glutothione peroxidase is a major role in regulating the concentration of H_2O_2 and a wide varity of organic peroxidase. Lpx was increased in all plant extracts treated groups compared with

control and fluoride groups (Fig-4). GPX and GSH also activity increased fluoride treated groups compare with control (Fig-5, 6). In our report ALT and ALP enzyme level decreased it may be affect the physiological changes in animals (Fig-7, 8). Decreased level of alkaline phosphates in mice after prolonged intake was reported by [15]. Aspartate amino transferase level significantly increased when highly intake of fluoride, it will affect hepatocellular damage in liver. Fluoride groups treated with three medicinal plant ethanol extract of, *T. procumbens* root, *S. acuta* root and *T.indica* seed coat ethanolic extract increased enzymatic and non enzymatic activity in all organs, particularly reduced glutathione level increased in fluoride treated groups compare with control, after treatment of plant extract significantly decreased (p<0.05) in all plants (Fig-6). Among these three plants possess antioxidant properties and protect the toxin induced oxidative stress in animals. These plants create the ability of the tissue to handle oxygen radicals decreased due to fluoride intoxication in reproductive organ of female mice.

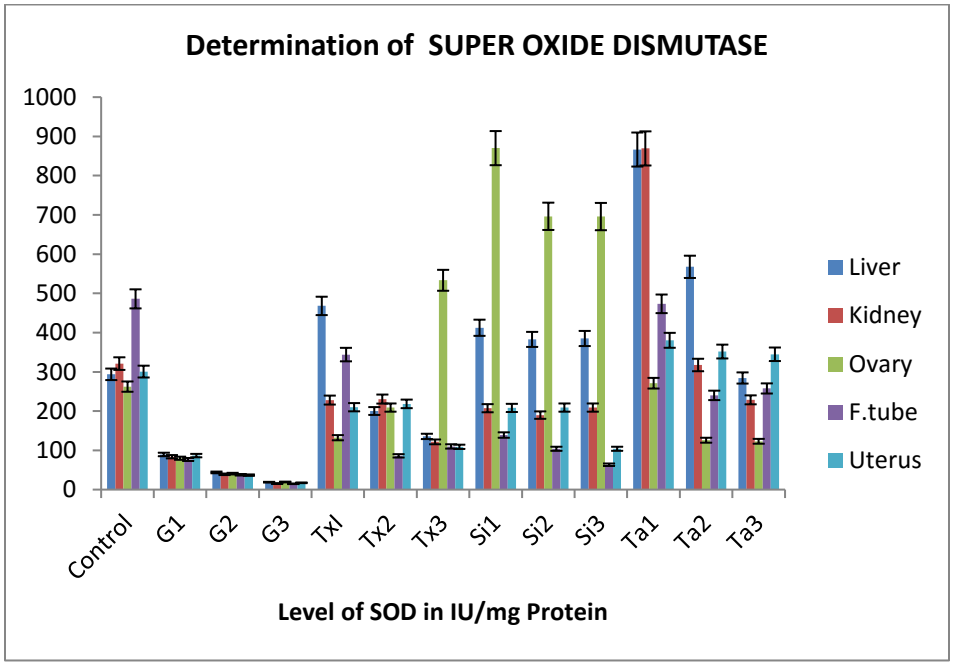

Fig. 2. Anti oxidant enzyme of SOD were significantly increased (p<0.05)

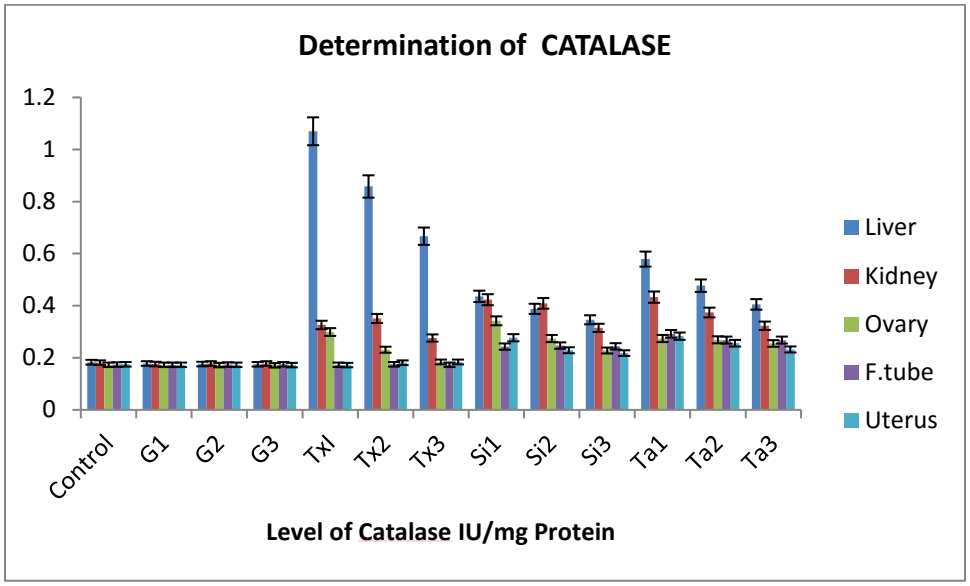

Fig. 3. Significantly increased p<0.05 treated plant extract

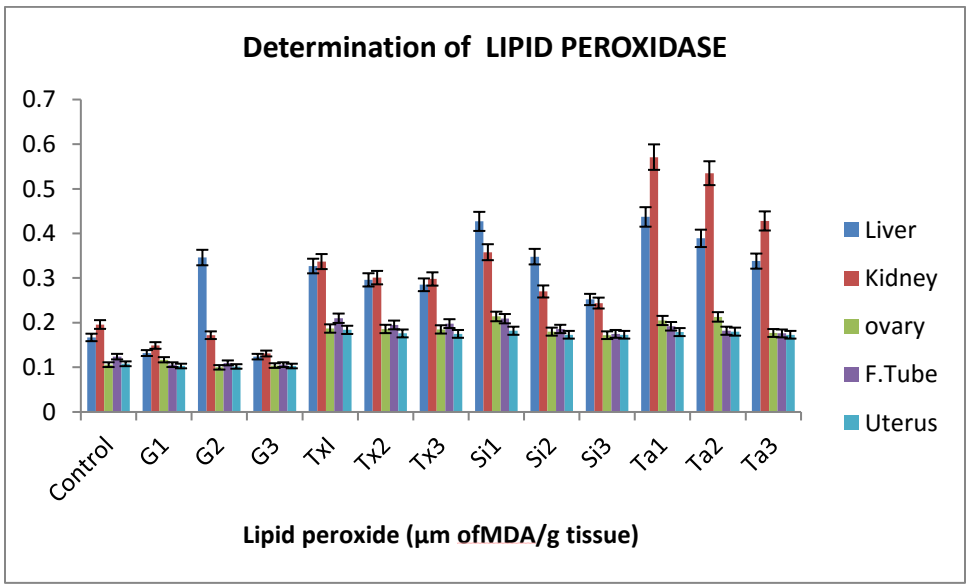

Fig. 4. Significantly increased in all plant extract groups *p*<0.05

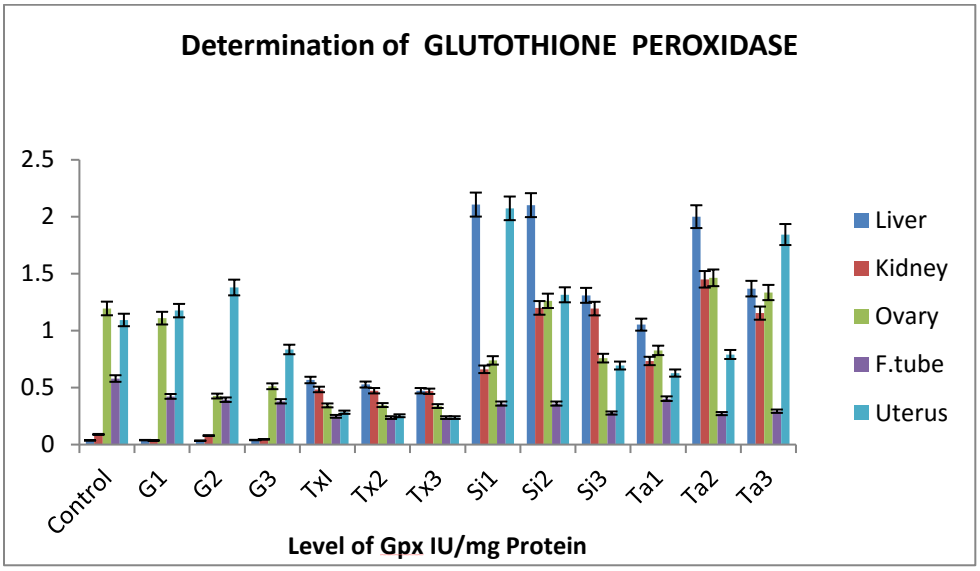

Fig. 5. Significantly increased in all plant extract groups $p<0.05$

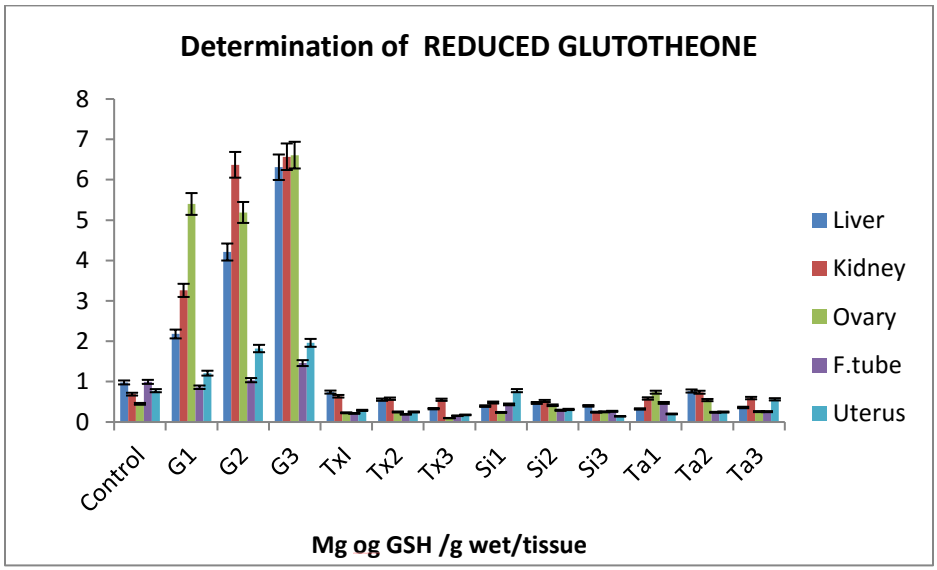

Fig. 6. Significantly decreased in all plant extract groups compare with control $p<0.05$

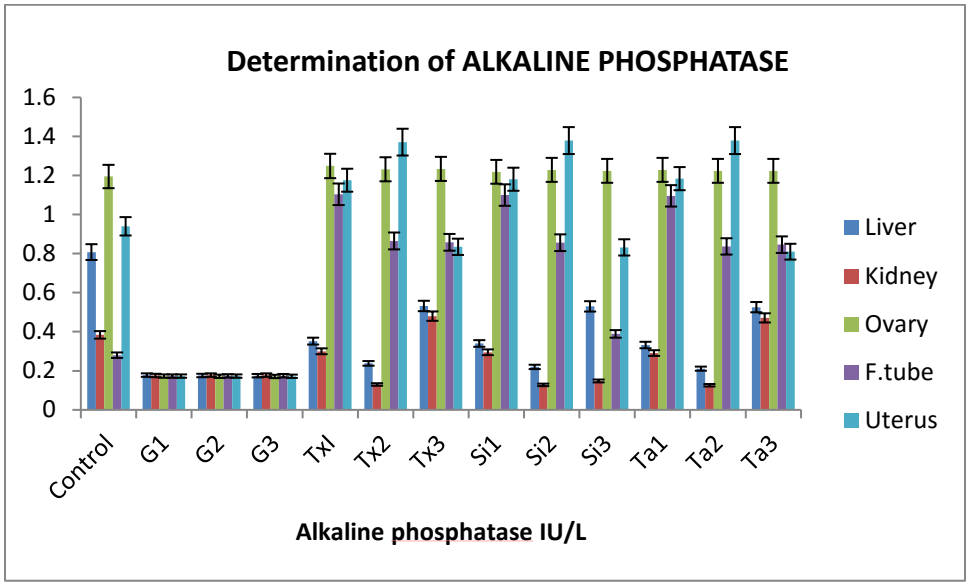

Fig. 7. Significantly increased in all plant extract groups $p<0.05$

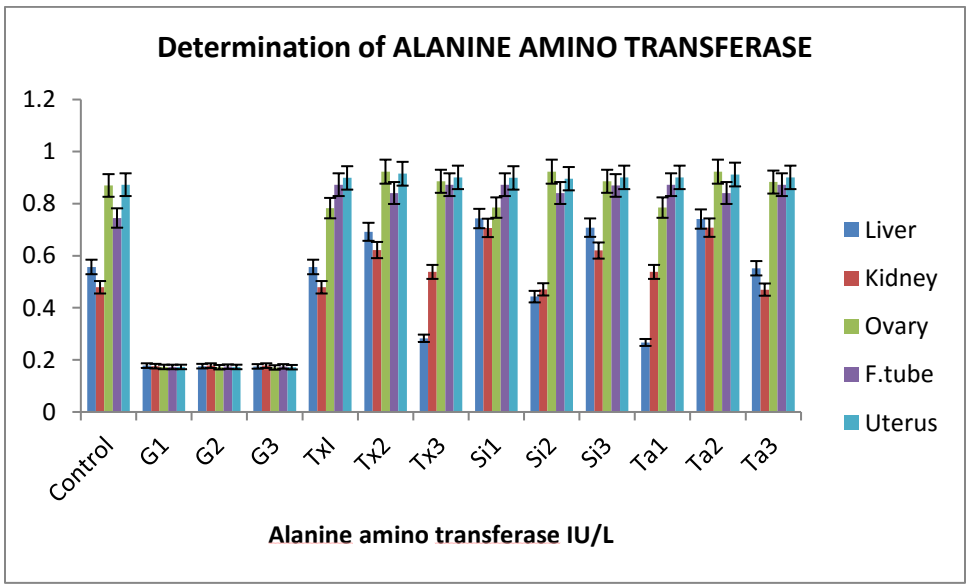

Fig. 8. Significantly increased in all plant extract groups $p<0.05$

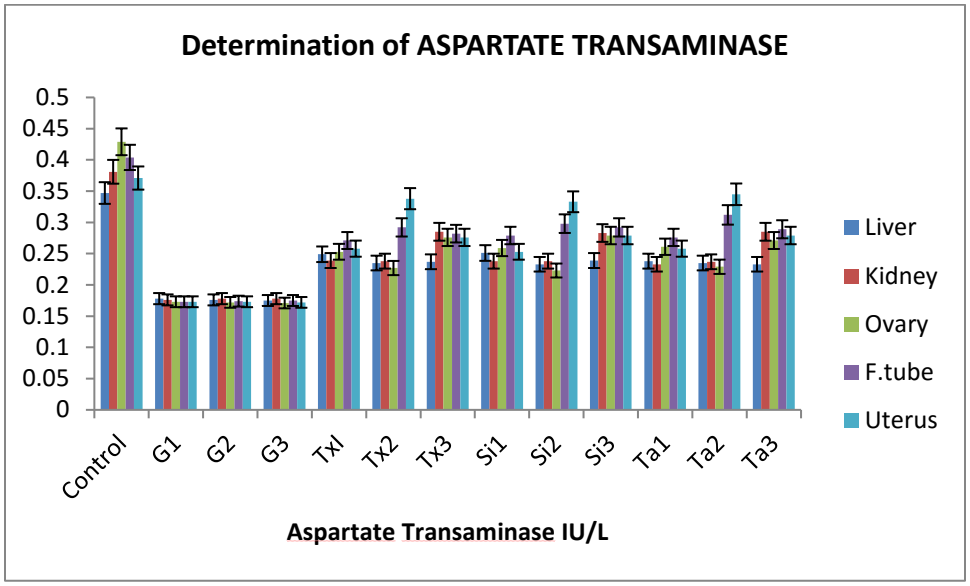

Fig. 9. Significantly increased in all plant extract groups $p<0.05$

DISCUSSION

Fluoride alters estrus cyclicity in our experimental mice, which can be particularly frustrating when trying to conceive. It's leading to infertility. Normally the enzyme and non-enzyme levels high in serum, liver and kidney, but in the case of treated groups similar amount present in reproductive organs, in our studies when increased concentration of fluoride inhibited enzyme level in serum, kidney, liver and reproductive organs [16, 17]. 5-25 ppm fluoride treated 12 weeks exposure induced kidney and liver damage leads to increased lipid peroxidation in rat [23]. Antioxidant enzyme level is decreased when increasing concentration of 200 ppm fluoride. Many reports on fluoride toxicity, affect several organs, like brain, bone marrow, kidney, and liver but few on steroids producing tissues and also the role of ascorbate as on antioxidant, particularly in mammals [18,19].We are choosing selected medicinal plants were used anti plasmodial, antimicrobial, antioxidant, cytotoxic activities, anticoagulant, antifungal and insect repellent, anti-inflammatory activity, Antidiabetic activity, Immunomodulatory Activity, and anti fungal activity, wound healing and many other properties in *T.indica seed* coat*, S. acuta* root, *T. prohumbans* roots [20,21]. All the three medicinal plant increasing antioxidant enzyme and reducing fluoride toxicity in ovary compare to control. *S. acuta* ethanolic root extract may reduce the fluoride toxicity and reverse effect of hormone level normal in estrus cycle, and regulate the ovarian function due to fertility in *S. acuta* plant extract, and also beneficial effect in *T. indica* and *T. procumbans* treatment.

Many studies indicate that high concentration of sodium fluoride in water can induced free radical toxicity in the Plants, animals and humans [14]. Fluoride has toxic effect on body organs

and reproductive system due to infertility. We are analyzed ALP, AST, AMP, SOD, CAT and GPX, GSH, LPO enzyme level in Serum, liver, kidney, ovary, uterus, and fallopian tube. AMP and ALP it is a marker enzyme of Fluoride toxicosis and bone pathology. The increasing level of this enzyme attributed to cell damage & disruptions of cellular organization, physical & chemical changes in liposomal membrane allowing release of hydrolytic enzyme and causing dislocation [22]. These changes were mainly caused by increased tissue burden of fluoride which resulted in increased lipid peroxidation, formation of oxygen free radicals and decrease in superoxide dismutase activity in the ovary–occupational exposure to organic Fluoride has been reported to [23]. While AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. AST may be elevated also in diseases affecting other organs, such as myocardial infarction, acute pancreatitis, acute hemolytic anemia, severe burns, acute renal disease, musculoskeletal diseases, and trauma. AST is similar to Alanine transaminase (ALT) in that both enzymes are associated with liver parenchyma cells.

The superoxide free radicals and per oxidation play an important role in fluorosis affected peoples .The medicinal plant of *T.indica* seed coat*, S. acuta* , *T. prohumbans* always used in traditional medicine, it rectify the liver metabolism and maintained the antioxidant enzymatic system, similar amount of antioxidant enzyme beneficial effect occur in estrus cycle present in reproductive organs. In the past, sodium fluoride and fluoride have been tested in several species of laboratory animals, revealed that ingestion of sodium fluoride by female mice caused alterations in reproductive organ and other organs also. These changes were mainly caused by increased tissue burden of fluoride which resulted in antioxidant enzyme level occupational exposure to organic fluoride has been reported to induce abnormal menstruation, increased in the frequency of miscarriages and pregnancy complication among the female workers in fluorine factories [24].

Taken together this study suggests that the administration of fluoride concentration increasing the free radical and reduce antioxidant enzyme via induced abnormal menstruation and infertility. Therefore, high concentration of fluoride induces infertility and reproductive disorder in women. Fluoride exposure effect is slightly decreased when inducing of *T. indica* seed coat*, S. acuta*, and *T. prohumbans* medicinal plant.

Conclusion:

We are concluding fluoride administered in drinking water to female mice for 30 days at 100 and 200 ppm /day doses intra peritoneal caused infertility. There was enzyme of antioxidant due to affect liver metabolism and altered ovulation. Finally, fluoride water exposure for 30 days caused infertility effect on reproductive organs abnormalities, leading to increased fertility reduced 15 days treatment of *T. indica* seed coat*, S. acuta*, and *T. prohumbans* medicinal plants.

ACKNOWLEDGMENT

We are grateful to the following funding agencies; BDU-URF, SAP-DRS-II, and DST- FIST II for providing financial support and instrumental facilities.

CONFLICTS OF INTERESTS

All authors have none to declare

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