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Curative Effect of Ethanolic Extract of Medicinal Plants on Fluoride Induced Female Infertility Using Mice Model

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Abstract

Female infertility, problems are increased gradually due to several reasons, especially environmental toxicity and water contaminations. Fluoride is the poisonous element, alters the hormonal regulation, folliculogenesis, ovulation and surface epithelial cell damage, finally, it creates infertility and risk of developing ovarian cancer. Sodium fluoride was administrated to female mice at 50, 100, 200ppm for 30d (days) through IP injection. After the fluoride treatment, three animals from each group were sacrificed and analyzed for biochemical parameters, hormone level and histopathological study. The remaining animals treated with ethanol extract of *Tamarindus indica* (*T. indica*) seed coat, *Sida acuta* root (*S. acuta*) and *Tridax procumbens* root (*T. procumbens*) at a daily dose of 100mg/kg bw for 15 days. The plant extracts treated animals were sacrificed to analyze the same process. In addition, histopathology of an ovarian surface are the irregular shape, follicle cell size and cells accumulations increased. Ethanolic extract of three medicinal plants reduced the fluoride toxicity *S. acuta* root extract was found to be more effective than other plant extracts. The GC-MS report also had pharmacological properties that reduced the fluoride toxic. These plant extracts also revealed beneficial effect in the ovary, follicle cell count, hormone levels, and ovarian epithelial cell damage. The fluoride adverse effects on fertility are reversible when the extract treatment is observed in all three plants.

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Keywords

Female mice
Fluoride
Infertility
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Ovary

Introduction

Fluoride is one of the toxic compounds. It is a major inorganic pollutant which naturally originates in ground water in many geographical areas rich in fluoride-containing rocks (Agalakova and Gusev, 2012). One ppm of fluoride is essential for growth and bone development increase amount of fluoride prove to toxic plant, animal and human (Chinoy and Narayana, 1994). Fluoride drinking water is easily absorbed by the intestine and distributed throughout the body. It affects the reproductive organs *via* blood, it altered surface epithelial tissue hardening irregular function of ovulation in ovary leading to infertility. Epithelial cells which play an important role in signal transduction during ovulation. Ovarian surface epithelial cells (OSE) cells involved in ovulation release proteolysis enzymes that degrade the basement membrane and the underlying apical follicular wall, weakening the ovarian surface to the point of rupture by environmental toxicants (Vanderhyden et al., 2003).

The OSE cells directly over the point of rupture undergo apoptotic cell death before ovulation and the wound created at the adulatory site surface is repaired by the rapid proliferation of OSE cells from the perimeter of the ruptured follicle (Auersperg et al., 2001). Infertility is one of the most complex issues in all over the world. Mostly early stage of infertility is curable. We had chosen three traditional plants, which have have always been a source of efficient natural medicines for people (Bahmani et al., 2015).

The present study was conducted to find out the toxicity of fluoride in three-month-old female albino mice weighing 30-40g after intraperitoneal injection of certain doses fluoride in their distilled water for 30days and to reflect its impact female fertility. In this study, fluoride-induced infertility *via* ovarian surface epithelial cell damage it may affect ovarian functions. Nowadays, several medicinal plants are used in different manners. We have used three medicinal plants used to remove the fluoride content from body organs and treated for fertility and inflammation of ovarian surface and epithelial cell alteration (risk in cancer) in an ovary. In the present study, ethanolic extracts of *Tamarindus indica* (seed coat), *Sida acuta* (root) and *Tridax procumbens* (root) were used to treat infertility; ovarian surface epithelial cell alteration may risk in ovarian cancer.

Materials and methods

Healthy adult female albino mice (*Mus musculus*) weighing between 30-40g were used for experiments. The animals were maintained under standard husbandry conditions on a standard diet and water *ad libitum*. Further, the animals were kept under the air cooled condition and exposed to 12 h light /dark cycle. Preparation of fluoride water a stock of 1000ppm fluoride solution was prepared by dissolving 2.21g of sodium fluoride (equal to 1g of fluoride) dissolved in 1L of distilled water. Fluoride water concentration at 50, 100, 200 (ppm) were prepared and administered to animals for 30d. After fluoride treatment the animals were cured with the following plant ethanolic extracts of *Tamarindus indica* seed coat, *Sida acuta* root and *Tridax procumbens* root for 15d. All procedures performed were in accordance with the Institutional Animal Ethical Committee [IAEC] approval [BDU/IAEC/2016/NE/43/ dt. 17.03.2016].

Experimental design

No of Groups	Description
Group-1 (n=3 Female)	Control
Group-2 50 ppm Fluoride (30d) (n=9)	*T.indica* seed coat, *S. acuta* root and *T. procumbens* root ethanolic extract (100mg/kg. b.wt) for 15d (Each group with 3 animals).
Group-3 100ppm Fluoride(30d) (n=9)	*T.indica* seed coat, *S. acuta* root and *T. procumbens* root ethanolic extract (100mg/kg. b.wt) for 15d (Each group with 3 animals).
Group-4 200ppm Fluoride(30d) (n=9)	*T.indica* seed coat, *S. acuta* root and *T. procumbens* root ethanolic extract (100mg/kg. b.wt) for 15d (Each group with 3 animals).

Plant samples

Three medicinal plants were selected for the study. The seed coat of *Tamarindus indica* L. (Leguminosae), roots of *Tridax procumbens* Linn. (Asteraceae), and roots of *Sida acuta* Burm *f.* (Malvaceae) were collected from in and around Bharathidasan University, Tiruchirappalli, Tamilnadu were used for the study.

Extraction procedure

In the laboratory, *T. indica* seed coat, *S. acuta* roots, *T. procumbens* roots were washed with fresh water and cut into the small pieces before drying. Cleaned plant materials were dried under the shady condition in one week for drying. Then they were powered by an electric blender. Three plants material 25g of powder was added to 150ml 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.

GC-MS analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed with chromatogram D:\GCMS\GC-MS (30m/0.25mm/0.5µm) capillary column. 6µ of 1mg/ml plant extract dissolved in methanol was injected under the following conditions: Injector temperature, 280°C; carrier gas, helium; pressure150kpa; ion voltage, 60eV; temperature gradient, 20°C per minute from 100 to 315°C. The compounds were identified on the basis of their spectral data.

Estrus cycle and fertility study

The vaginal smears were checked daily, and estrus stage analyses every week in the fluoride groups and plant extracts treated groups. Estrus stage is morphologically changes were noted and fertility study was performed in this study.

Water consumption analysis

Water consumption level was monitored and recorded every day in fluoride groups and plant extracts treated groups.

Hormone level analysis of blood serum

After the administration of fluoride for 30d and plant extracts 15d, the mice were sacrificed. Blood samples were then collected from the hearts of each group and centrifuged with 300 rpm for 10 minutes to collect the serum. The serum samples were used for further analysis of Estrogen, Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) levels (Zhou et al., 2013).

Estimation of urine sample analysis

The administration of fluoride induction 30days and plant extracts treatment 15days. The urine samples were analyzed Urea and creatinine.

Follicle cell counting

Follicles were counted in ovaries stained with H&E. Every 20th section the number of oocytes classified into primordial follicles contained single layer of squamous granulose cells, single layer of cuboidal granulosa cell is primary follicle, two layers of granulose cell is secondary follicle cell stage, multilayer of granulosa cells and fluid-filled antral space (Graafian follicle stage) (or) antral follicle (Hoyer et al., 2009).

Histological analysis

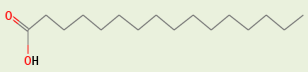
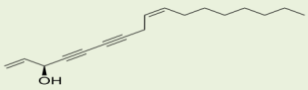
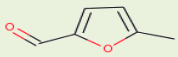
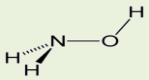
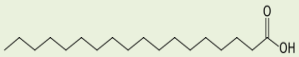
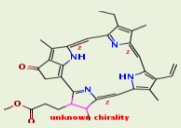
The ovaries were fixed in 4% formalin solution 24 hours, transferred to ethanol dehydration embedded in paraffin blocks, sectioned 5um, and every 20th section was mounted and stained with Hematoxylin and Eosin (H&E).

Results

GC-MS analysis of three plant extracts

The GC-MS analyzed the plant extract compounds are Falcarinol, Ethyl palmitate (Hexadeconoicacid), 5-Hydroxymethyl furfural, Ethyl-9-12-Octadecadienoate, Octadecanoic acid, ethyl ester. Hydroxylamine, 3-Phorbinepropanic acid, these compounds are highly present in medicinal plants (Table 1).

Table 1. GC-MS analysis of plant extracts.

S. No	Structure	Compounds	% Area	Medicinal plant
1.		Hexadecanoic acid	11.22% 3.29%	*S. acuta* *T. procumbens*
2.		Falcarinol	0.64% 63.63%	*S. acuta* *T. procumbens*
3.		Furfural	31.50% 7.83%	*S. acuta* *T. procumbens*
4.		Ethyl(9z,12z)-9-12-	21.92% 1.05%	*S. acuta* *T. procumbens*
5.		Octa decadienoate Hydroxylamine	26.037% 6.640%	*T. indica* *T. indica*
6.		Octa decanoic acid	2.16% 1.05%	*S. acuta* *T. procumbens*
7.		3-Phorbinepropanic acid	10.762% 25.605%	*T. indica* *T. indica*

In these GCMS result commonly analysis the 9, 12 Octadecanoic acids found in three plants. The result of the present study support the traditional medicinal use of *S. acuta, T. procumbens* and *T. indica* suggest that a great attention should be compensated to these plants, which is found to have many pharmacological properties used to cure the several diseases (Figs. 1-3).

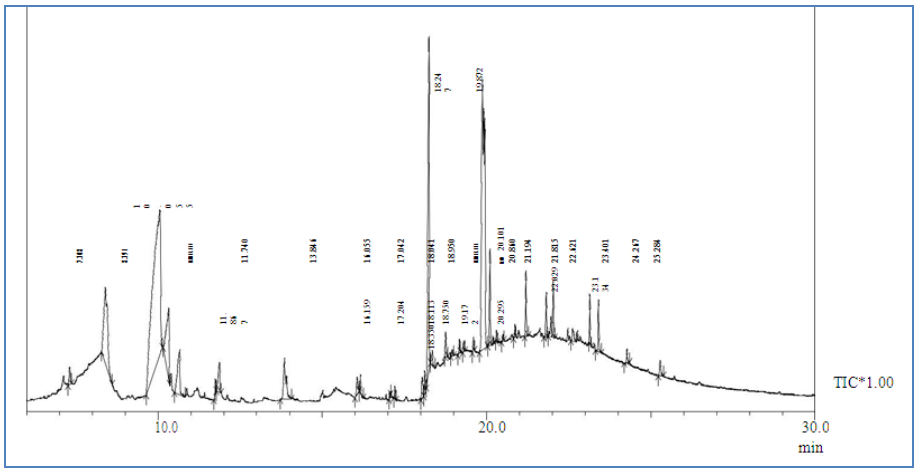


Fig. 1: Ethanolic root extract of *Sida acuta.*

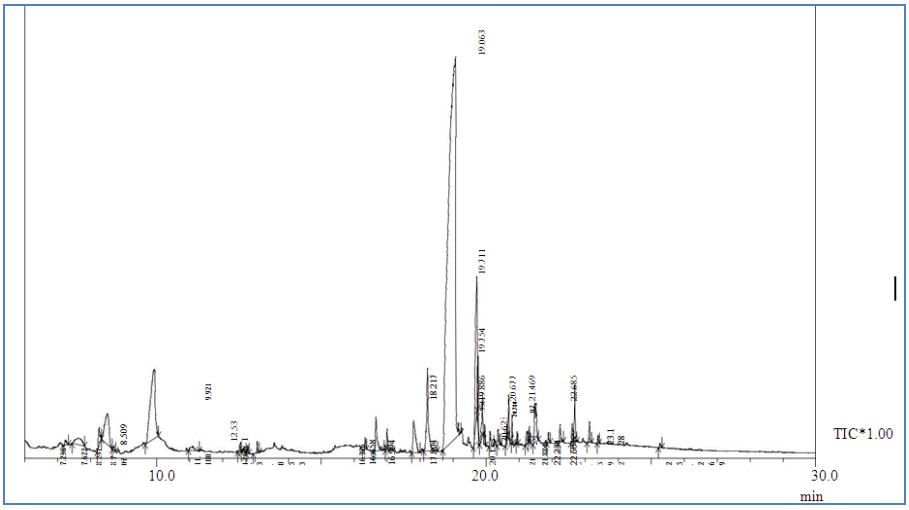
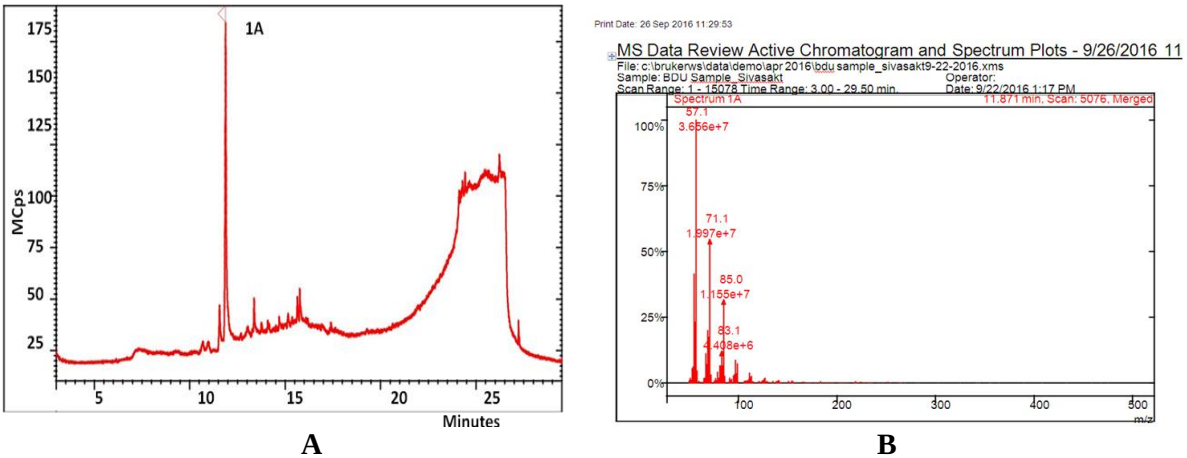


Fig. 2: Ethanolic root extract of *Tridax procumbens.*



A **B**

Fig. 3(A, B): Ethanolic seed coat extract of *Tamarind indica.*

In female mice, the estrus cycles were checked by vaginal smear. The female mice treated with fluoride for 30 days, revealed that the irregular estrus cycle and disturb the ovarian cyclicity. Fluoride-treated groups mating were decreased when compared with medicinal plants treated animals (Table 2).

Table 2. Infertility of female mice.

Group	Dose of Fluoride	F	P1	F	P2	F	P3	No to Animals/Pregnancy %
Control	Distilled water	-	-	-	-	-	-	- (100%)
G1	50ppm	2	5	1	5	1	3	18/17 (94%)
G2	100ppm	1	4	1	4	1	3	18/14 (77%)
G3	200ppm	1	1	1	2	0	2	18/7 (38%)

P1- Plant 1 (*T. procumbens*), P2-Plant 2 (*S. acuta*), P3-Plant 3 (*T. indica*).

The result indicates that the rate of pregnancy following fluoride exposure declined after treatment rate of successful pregnancy is increased compared with control. Everyday water consumption levels were monitored in fluoride exposure and treated groups. The water consumption level was significantly decreased ($P<0.05$) in 200ppm fluoride exposure groups compared with control (Fig. 4). In the plant extracts, treated groups water intake levels were significantly increased (mean±SD).

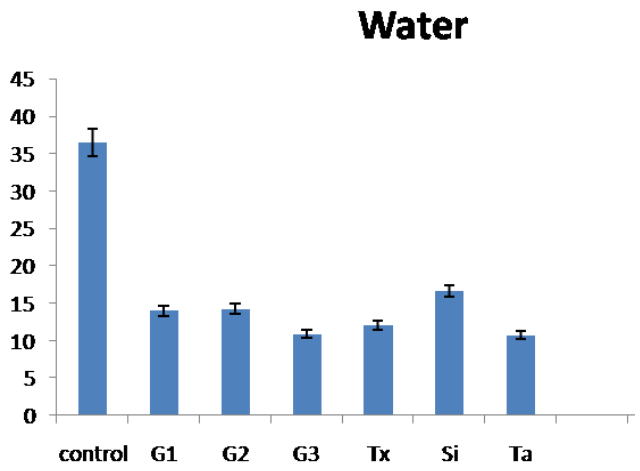


Fig. 4: Water consumption level analysis (ml/L).

Hormone level analysis of blood Serum concentration of FSH and LH hormone decreased in a dose-dependent manner after fluoride exposure for 30d compare with control. The medicinal plant treated with female mice FSH and LH serum concentrations slightly increased (Figs. 5, 6). The mean FSH concentrations were the lowest of the three fluorides treated groups and were significantly ($P < 0.05$) decreased compared to those in the control. Significantly increased in *S. acuta* and *T. procumbens* ($P < 0.05$) plants extract treated groups than *T. indica* (mean±SD).

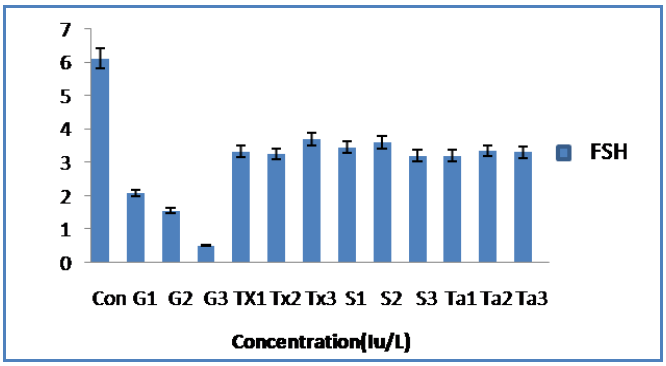


Fig. 5: FSH levels in the serum of female mice $P < 0.005$ significantly increased in medicinal plant treatment.

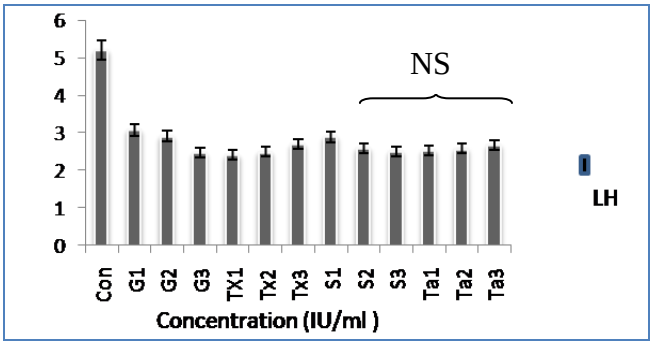


Fig. 6: LH levels in the serum of female mice LH concentrations in the serum were no significant difference. NS (non- significance).

The gonadotropin hormone promotes the secretion of estrogen (E2) and progestogen stimulate the follicular cell development and responsible for secondary sexual characteristics and reproductive cyclicity. Estrogen concentration decreased in a dose-dependent manner after fluoride treatment. However, no statistically significant differences were found between the plant extracts treated groups. The concentrations of estrogen in the fluoride-treated groups were statistically decreased when compared to the medicinal plant treated (Fig. 7).

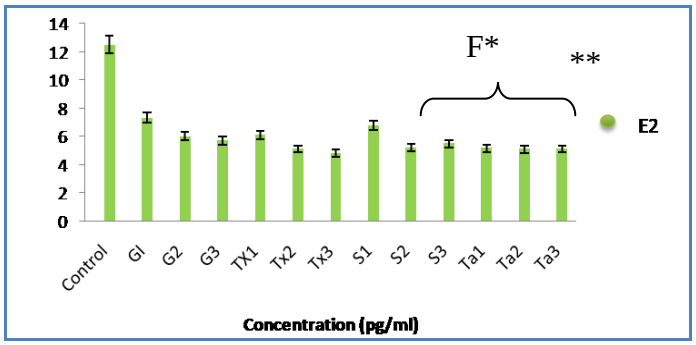


Fig 7a: Serum estrogen level in female mice. Estrogen level was significantly increased after *S. acuta and T. indica* ethanol extract ($P < 0.05$). F* fluoride groups estrogen level is significantly decreased compare with control. **($P < 0.005$) *S. acuta* plant.

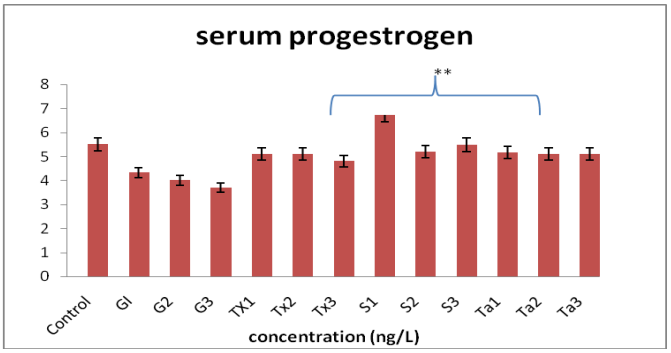


Fig. 7b: Progestrogen level was significantly increased after *S. acuta* and *T. indica* ethanol extract ($P < 0.05$). F* fluoride groups progestrogen level is significantly decreased compare with control.

The effect of urea and creatinine level in urine sample

The urea and creatinine levels were found to be significantly ($P < 0.05$) increased and decreased, respectively in both fluoride exposed and plant extract treated. *S. acuta* plant root extract gradually decreased in urea level, effective with *T. procumbens* and *T. indica* (Figs. 8, 9) (mean±SD).

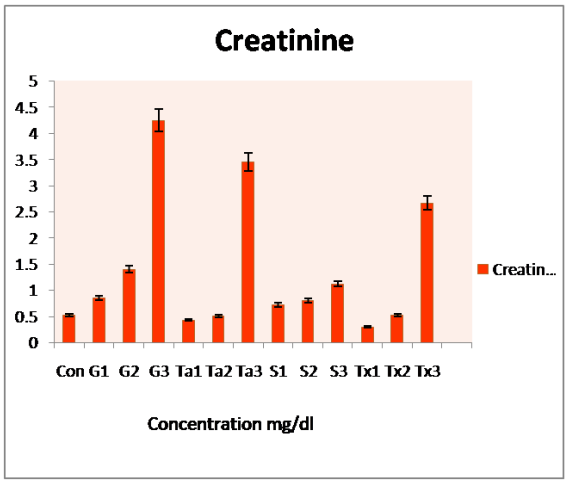


Fig. 8: Urea in urine of female mice.

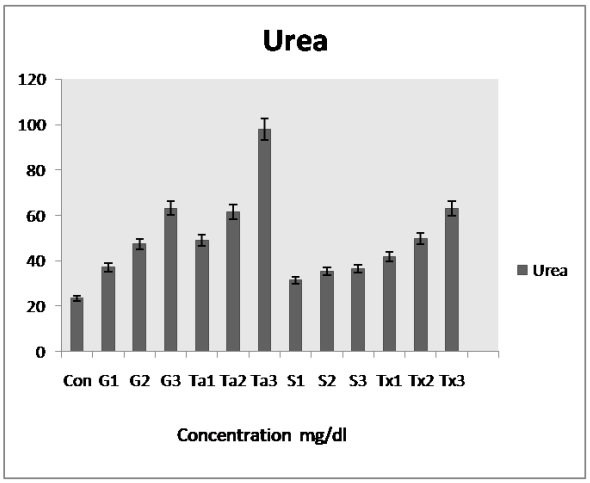


Fig. 9: Creatinine in urine of female mice.

The urine urea and creatinine levels were found to be increased in fluoride groups ($P < 0.05$). Plant extract treated group's decreased urea and creatinine level reducing fluoride in body organs.

Follicle cell count in ovary

The total number of follicles (primary, secondary, antral or Graafian follicles) measured in adult female mice ovaries were counted by a light microscope with digital camera. Secondary and antral follicle numbers calculated. The higher concentration of fluoride inhibits the follicular development which leads infertility. The primary follicles per ovary determined fluoride exposed groups were decreased. In the *S. acuta*, *T. procumbens*, and *T. indica* extract treated groups the primary follicle cells increased and antral follicles numbers increased only in *S. acuta* plant extract treated animals (mean±SD) ($P < 0.05$) (Fig. 10).

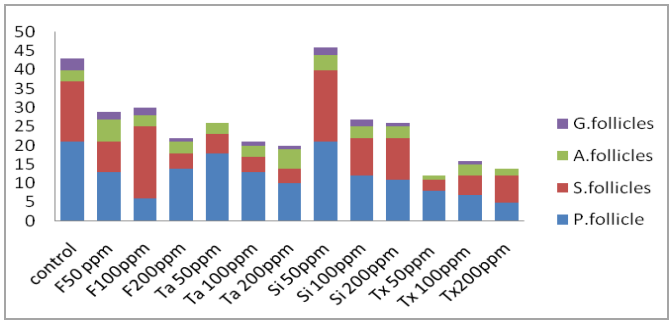


Fig. 10: Follicle cell count in fluoride and plant treated groups.

Histological analysis

In the light microscopic observation, the control mice showed normal histo-architecture in the ovary (Fig. 11a). In fluoride treated animal's ovaries, the follicles were found undergoing degenerative changes and they had lost their normal shape and arrangement of granulosa cells (Fig. 11b-d). In *T. procumbens* plant extracts treated group there are no changes in the ovarian morphology (Fig. 11e-g). *S. acuta* and *T. indica* plant extract treated animal's ovary had the normal nucleus in ovum (Fig. 11h-m).

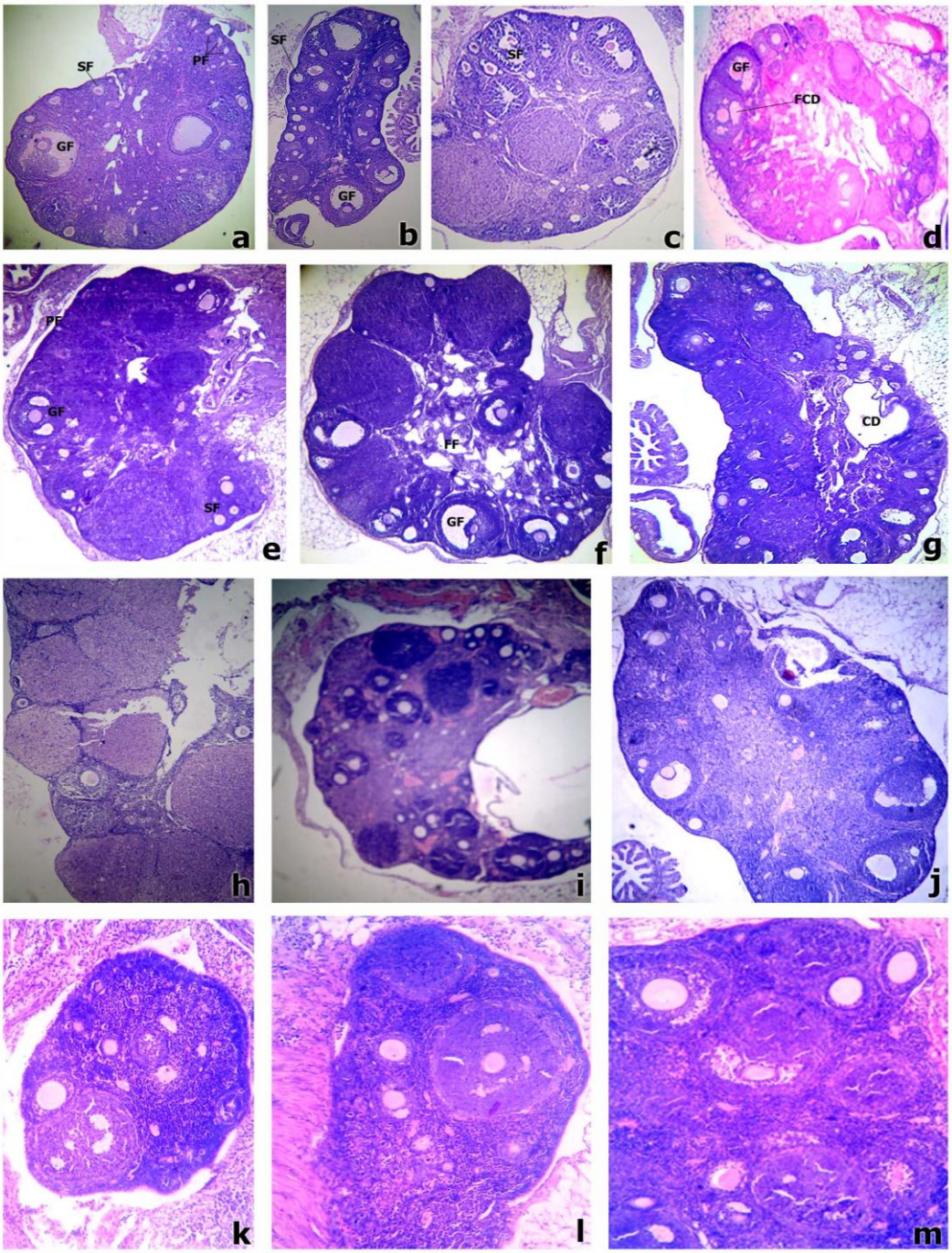


Fig 11. H & E stained section of mice ovary in control, fluoride and plant ethanolic extracts treatment showed histo-architecture. PF- Primary Follicle, SF-Secondary Follicle, GF-Graafian Follicle. FCD-Follicular Cell Death, CD- Cell Death. (**a**-Control; **b-d** Fluoride treatment; **e-g** *Sida acuta*; **h-j** *Tridax procumbens*; **k-m** *Tamarindus indica*).

The surface epithelium of ovary in control group was showing normal epithelia (Fig. 12a). The fluoride exposure group's epithelium of ovary was observed disorganized and depletion of epithelium in the ovary (Fig. 12 b-d). The epithelial cells of *S. acuta* and *T. indica* plant extract treated animals showed normalcy (Fig. 12h-m) than *T. procumbens* plant extract treated group (Fig. 12e-g).

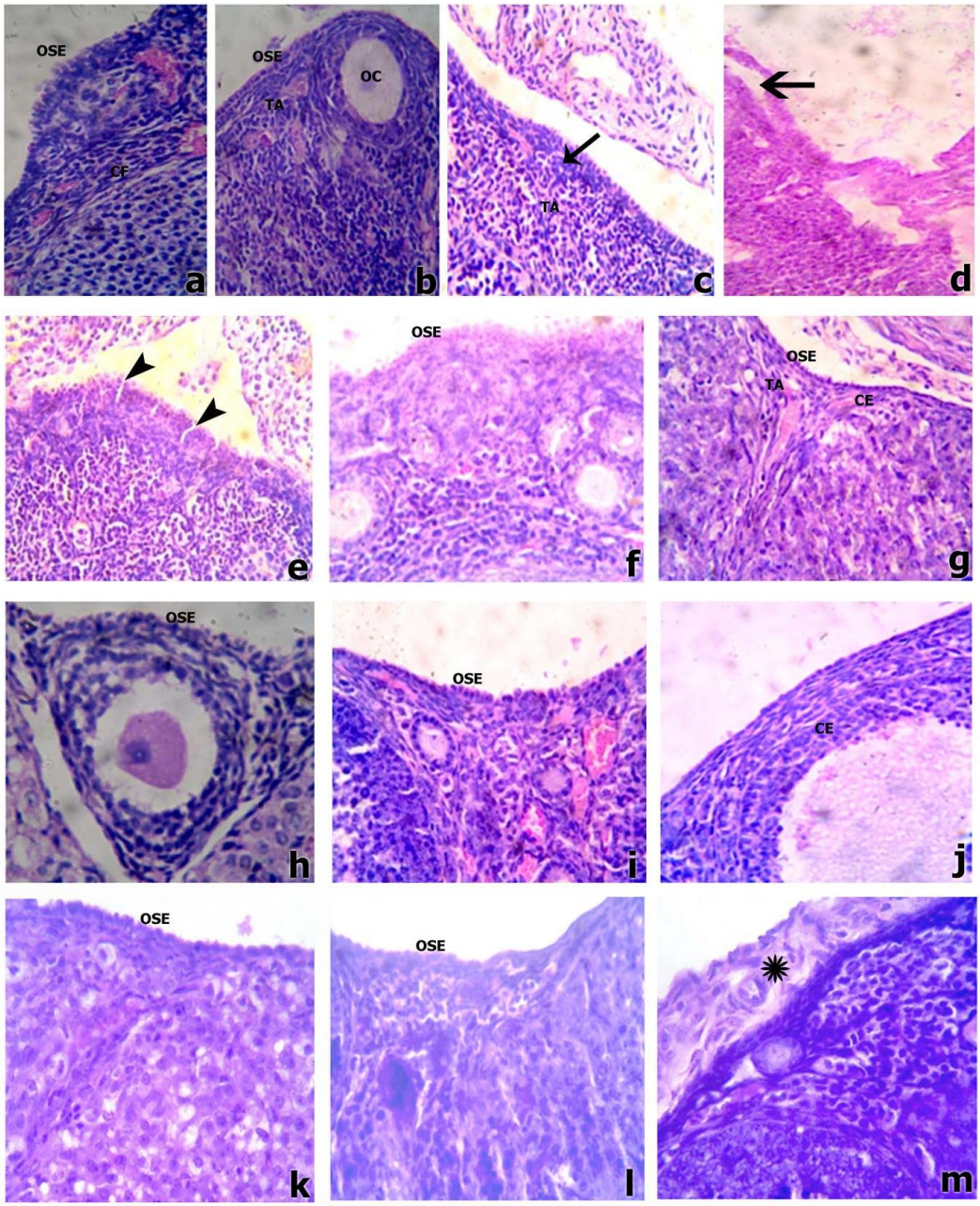


Fig 12. H & E stained section of mice ovarian epithelium in control, fluoride and plant ethanolic extracts treatment showed histoarchitecture. OC-Oocyte, OSE-Ovarian Surface epithelium, CE- Columnar Epithelium, TA- Tunica Albuginea. Arrow indicates a thick layer of an epithelium; solid arrow-depletion of the epithelium; arrowheads- damaged epithelium (**a**-Control; **b-d** Fluoride treatment; **e-g** *Sida acuta*; **h-j** *Tridax procumbens*; **k-m** *Tamarindus indica*).

In the ovarian oocyte control, mice were showed fully mature Graafian follicle with oocyte which surrounded by follicular the corona radiata (Fig. 13a). Fluoride high concentration treated group mice showed Graafian follicles with oocyte that surrounded by a mass of dead follicular cells (Fig. 13b-d). The *S. acuta* root extract

and *T. indica* seed coat ethanolic extracts treatment of ovarian Graafian follicle cell with oocyte showed in normal (Fig. 13h-m). Among the three plant extracts, the *S. acuta* root extract was found to be more effective than *T. indica* seed coat and *T. procumbens* root extracts for fluoride toxicity.

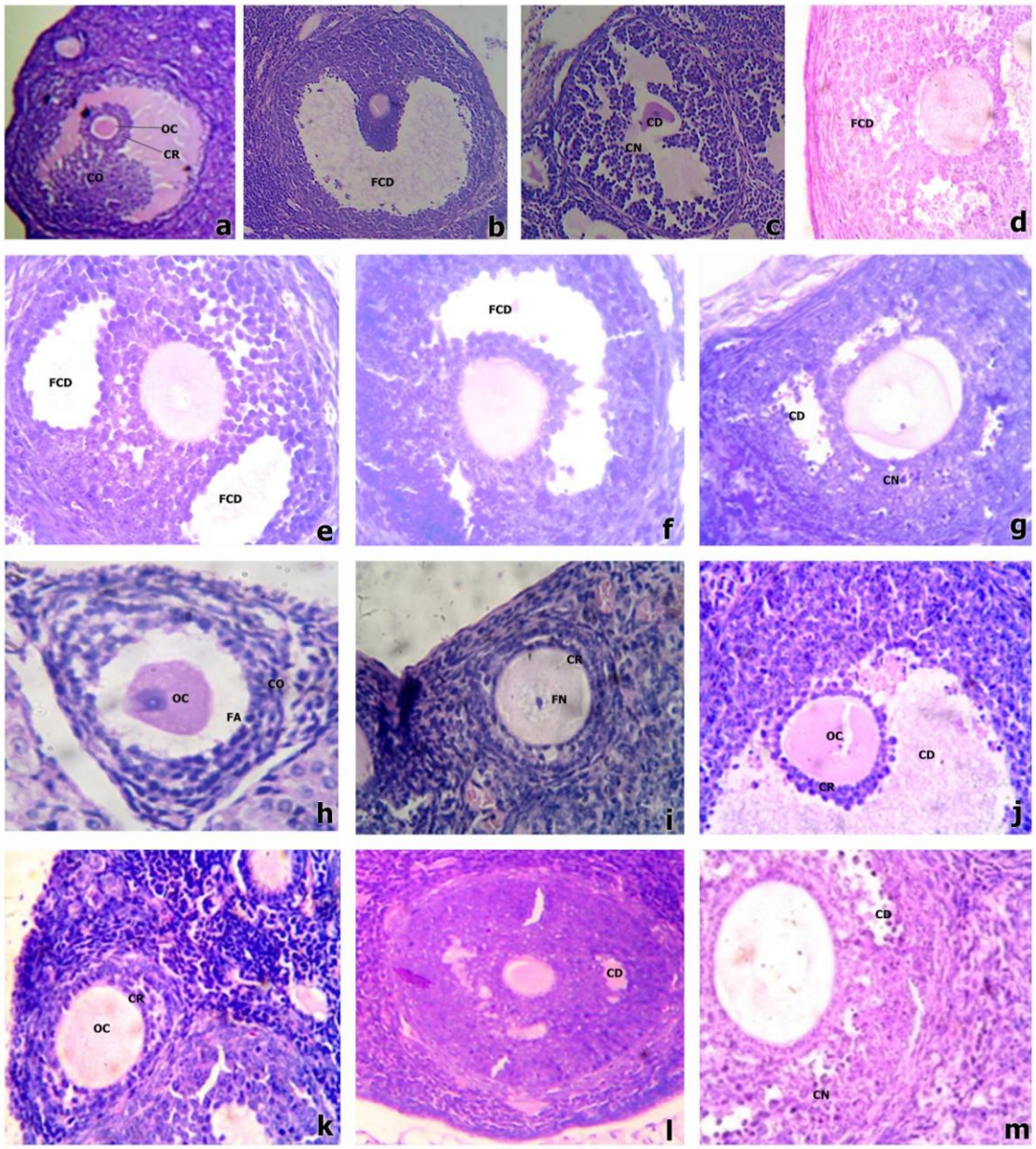


Fig. 13: H & E stained section of mice oocyte in control, fluoride and plant ethanolic extracts treatment showed histo-architecture. FCD-Follicular Cell Death, CD- Cell Death. CR- corona radiate, CN-Cell Necrosis, CD- Cell Death. OC-Oocyte, CO- Cumulus Oophorus, FA- Follicular Antrum, CE- Columnar Epithelium (**a**-Control; **b-d** Fluoride treatment; **e-g** *Sida acuta*; **h-j** *Tridax procumbens*; **k-m** *Tamarindus indica*).

Discussion

The fluorides are poisonous element occur in groundwater sources (Zhou et al., 2013). Uptaking groundwater used for agriculture purposes and directly used for food process and drinking water via fluoride can cause the reproductive function. Reproductive toxicity assessment of female animals, few reports only in mice model research, at the same time remedy of the fluoride is very few reports only available. Several medicinal plants have been used for thousands of years in folk medicines in Asian and African populations and

many plants are consumed for their health benefits in developed nations. An overview of selected medicinal plants, of *S. acuta*, *T. procumbens*, and *T. indica* derived compounds that have anticancer therapeutic properties and their developments in this field.

At present, the animal model used in the study has been previously employed in several reports and evaluating the adverse effect of different compounds on male and female rat and mice reproductive function. Fluoride is a toxic compound in our study used medicinal plant against the fluoride toxicity. We choose the female albino mice as a suitable animal model due to their strong resistance to disease. We also detected everyday estrus cycle and consumption of water level were increased in control groups slightly decreased in treated groups in 30d fluoride exposure after plant extracts treated animals water consumption level was slightly increased.

The result of the GC-MS analysis showed Falcarinol (carotatoxin) 0.64% present in *S. acuta*, 63.63% in *T. procumbens*. Falcarinol is a natural pesticide and fatty alcohol found in carrots. As a toxin, it protects from fungal diseases, and also it against in cancer. Hexa decanoic acid or palmitic acid 3.29% in *T. procumbens*, (11.22% *S. acuta*) is one of the saturated fatty acids found in animals and plants. It is a highly antioxidant compound and a source of Vitamin A, excess amount of palmitic acid alterations in central nervous system, control of insulin secretion, suppression of plants appetite (Benoit et al., 2009). Hydroxylamine is an inorganic compound with the formula NH_2OH. It is also an intermediate in biological nitrification. In biological nitrification, the oxidation of NH_3 to hydroxylamine is mediated by the enzyme ammonia monooxygenase (AMO) (Lawton et al., 2014). Hydroxylamine oxidoreductase (HAO) further oxidizes hydroxylamine to nitrite. Do deconic acid (2.55% in *S. acuta*) (1% of *T. indica*) is a fatty acid, quickly metabolized via coenzyme-A intermediates through β-oxidation and the citric acid cycle to produce carbon dioxide, acetate and ketone bodies (Chang et al., 2013). 9,12-Octa decanoic acid (26.037% in *T. indica* seed coat extract), (1.05% in *T. procumbens*) (21.92% *S. acuta*) is a saturated fatty acid it otherwise called Stearic acid used in cosmetics and soaps. In these GC-MS result commonly analysis the 9, 12 Octadecanoic acids found in three plants. The result of the present study support the traditional medicinal use of *S. acuta* and *T. indica* suggest that the attention should be compensated to these plants, which is found to have many pharmacological properties used to cure infertility.

The result of the fertility experiments showed that high concentration of 200ppm fluoride treated group animal pregnancy rate is decreased compared with control after 15d treatment of three medicinal plants extract pregnancy rates slightly increased in *S. acuta* plant groups compare with other *T. procumbens* and *T. indica* seed coat extract. We observed that the reproductive function of female mice exposed high concentration of fluoride was markedly damaged the ovary. The rate of pregnancy is decreased in fluoride-treated groups. This effect is one of the reasons for uterine and ovarian abnormalities of fluoride exposure effect (Zhou et al., 2013). Urine samples of fluoride exposure ($P < 0.05$) (Figs. 8, 9) and plant extracts treated groups the urea and creatinine levels are increasing and decreasing in mg/dl vary depending on fluoride contamination water consumption. The higher concentration of urea and creatinine affects kidney injuries that lead to kidney failure.

The ovary is main organs of reproduction, one of the main targets of toxicants affect this reproductive organ. Therefore we concentrate the reproductive organ of the ovary and the surface ovarian epithelial cell is important for infertility. In the present study, we found that both ovaries were analyzed fluoride exposure and after treatments of medicinal plants. We decided to study whether damaged reproductive function in female mice was caused by fluoride and we observed the ovarian secretion hormones of Estradiol, FSH and LH slightly decreased when inducing fluoride, $F^*(P < 0.05)$ after three medicinal plants *T. procumbens*, *S. acuta* root extracts and *T. indica* seed coat ethanolic extracts may reduce the fluoride toxicity and reverse effect of hormone level normal in *S. acuta* plant, and also beneficial effect significantly in *T. procumbens* and *T. indica* treatment ($P < 0.05$). The pituitary gland secretes FSH and LH hormones (Kumar et al., 1997). The main function of FSH is to stimulate the ovarian growth and follicle cell maturation. LH promotes the ovarian regulation, plays a role in follicular cell maturation, ovulation, and corpus lutetium development, and intervenes in the synthesis of steroid hormones (Campbell et al., 1995; Niswender et al., 2000; Rao, 2001; Hunder et al., 2004). Previously it has been reported that *T. prohumbens* and *T. indica* are used as bio-carbon for absorption of fluoride (Singanan, 2013). In this result showed that it takes just 3h to remove 98%

of fluoride with just 2g of the biocarbon filter (Mundada and Shivhare, 2010). Recently, researchers in India have developed a filter system based on a herbal plant (*S.acuta*) it easily removes fluoride from water (Trivedi and Vediya, 2013). In the light microscopic keen observation of the present study, the follicular cell development of different stages had inhibited in fluoride treated animal's ovary. The number of medium and large follicle decreased significantly in the groups exposed to 50 and 200ppm concentration of fluoride. Three medicinal plant extract eliminate the fluoride in the serum it belongs to increase the hormone level LH and FSH it stimulates follicle cell development going to be a normal ovulation at the end of the treatment.

Surface epithelial cells are covered by an ovary, which act as a transporter of material from the peritoneal cavity. It is a transporter. Surface ovarian epithelial cells are easily affected by toxicants 90% of epithelial ovarian cancer arises from this type of cell. Surface epithelial cells participate in follicle maturation and ovulation. At the time of ovulation surface epithelial cell layer cleaved and expel the oocyte after that regenerate cell and wound repair the layer. Which is affected by toxicants, it will not regulate the ovulation and follicle cell development. Fluorides are bind with G-protein receptor via apoptosis caused and directly stimulate the surface epithelial cell membrane (Agalakova and Gusev, 2012). Fluoride continuously injured the OSE cells from abnormal cell layer that is malignation after that arise cancer. Animal receiving plant extracts also revealed beneficial effect in the ovarian surface epithelial cell.

Conclusion

Fluoride highly contaminated ground water due to the drainage water exit from the various industries, Factories and Metamorphic Rocks is the main source. Fluoride damages the tissues of the various organs was clearly observed by Histopathology especially in the ovary. Among the three plants extract, the *S. acuta* root extract was found to be more effective than *T.indica* seed coat and *T. procumbens* root extract. This study concludes that the toxicity of fluoride affects the normal functioning of the ovary, fluoride groups' animal receiving plant extracts also revealed beneficial effect in the ovary, Follicle cell count, hormone levels, and ovarian epithelial cell damage in plant treated groups. Finally, the adverse effects on fertility are reversible when the extract treatment is observed in all three plants.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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