Green synthesis of *Tribulus terrestris* titanium nanoparticles: Study of Biological Effects on Reproductive System in Wister Rats

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Abstract

Background: Green synthesis of nanoparticles is becoming one of the strong techniques, which may be suitable alternatives for chemical and physical method and had many uses in optical, electrical, and thermal properties and almost all types of industries from textiles to medicine. Biosynthesis of nanoparticles is a bottom up approach where the main reaction occurring is reduction/oxidation by plant.

Objective: This study was carried out to evaluate the histological and biological activities in Reproductive System of Titanium, *Tribulus terrestris* Titanium nanoparticles (*Tt*-Ti-NPs) and aqueous extract of *Tribulus terrestris* fruits(Dirassa) on Wistar rats.

Methods: Dirassa extract was used for the biosynthesis of TiNPs. TiNPs was prepared and characterized by use UV-Visible spectroscopy (UV), x-ray diffraction (XRD), Energy dispersive X-Ray Spectroscopy (EDS), scanning electron microscopy (SEM) to determine different parameters such as particle size, shape, crystallinity, fractal dimensions, pore size and surface area and then tested for their effect on Wister rats to estimate the effect of TiNPs on reproductive system. Thirty Wister rats was divided to five groups, group one as control, group two *T. terrestris* fruits extract Ti*Tt*NPs, group three 50mg/kg/day (low dose), group fourTi*Tt* NPs 300mg/kg/day (high dose) and the last group five TiNPs 300 mg/kg/day given to rats respectively oral for 30 days. Body weight was measured weekly. Sexual hormones (Testosterone, Estrogen Progesterone, LH, FSH and Prolactin), Histopathological studies of testes and ovaries were performed.

Results: Nanoparticles were characterized and spherical shaped and had the average size 6 nm. After 2 weeks there was significantly change in body weight, lowering weight in treated groups when compared with control animals ($p < 0.05$) and after 4 weeks the weight is increase.

In hormonal result for male show decrease in FSH, LH in all treated group and decrease in testosterone except in group 5. About female there was increase in progesterone in group treated with green Ti*Tt*NPs and decrease in LH and FSH. Marked reduction of reproductive hormones was observed in both male and female rats when given Titanium nanoparticles. Degeneration of the seminiferous epithelium as well as reduction of produced sperms in males, Lack of normal antral follicles, enlarge cystic follicles and presence of atretic follicles in females. Vaculation and fibrosis in some cells in rats.

Key words: Green synthesis, Titanium, Nanoparticles, Dirassa, *Tribulus terrestris*, Histopathology

Introduction

Human dreams and imagination often make to new science and technology. Nanotechnology, a 21st-century was born out of such dreams; the prefix ‘nano’ is referred to a Greek prefix meaning ‘dwarf’ or something very small and depicts one thousand millionth of a meter (10−9m).

Green synthesis of nanoparticles is becoming one of the strong techniques, which may be suitable alternatives for chemical and physical method and had many uses in optical, electrical, and thermal properties and almost all types of industries from textiles to medicine. Biosynthesis of nanoparticles is a bottom up approach where the main reaction occurring is reduction/oxidation by plant.

Due to many applications of Titanium nanoparticles TiNPs like chemical process, antimicrobials agent, and

precursor chemical agent for biomaterial synthesis are thought-about for several completely different analysis fields. Looking on the scale, morphology, and distribution, the Ti nanoparticles exhibit new or improved properties. It has been proven that TiNPs can be toxic to human and animal. Oral administration of this nanoparticle to the rats increases inflammation and disrupts the function of the liver, kidney and reproductive system (Jia *et al*., 2017) and also increases plasma glucose level (Hu *et al* .,2018). Previous studies also have indicated that TiNPs administration leads to aggregation in the vital organs such as, liver, brain, lung, spleen, and kidney (Gui *et al*., 2011).

Long term TiNPs administration (90 days) to mice can be accumulated within ovarian cells, resulting in ovarian dysfunction, mating and pregnancy rate reduction, ovarian inflammation and follicular atresia and It also has been shown that TiNPs was transferred to the brain of mice which can affect hormonal release from pituitary gland that leads to imbalance of sex hormone (Zhao *et al* 2013). Nowadays, many studies have shown that this nanoparticle is able to decrease sperm counts and motility and increases the number of abnormal sperms in epididymis (Khorsandi *et al*., 2017). Other studies indicated that TiNPs inhibits follicular growth and oocyte maturation of rat (Juan H *et al*., 2009), and also induces genotoxicity in Chinese hamster ovary cells in vitro (Di Virgilio *et al*., 2010).

Hong and colleague demonstrated that oral administration of 100 mg/kg TiNPs to pregnant mice can cross the blood fetal barrier and placental barrier and suppresses embryonic development and also induces fetal skeletal malformation (Hong F *et al*., 2017). A considerable body of evidences in laboratory animal models have shown that TiNPs administration can cause toxicity in the male reproductive system and induced impairment in testicular morphology and function.

In this study, we used a simple, effective, low cost and environmentally safe method to synthesis of TiNPs from Titanium tetra chloride ($Ticl_4$) solution using aqueous extract fruits of *Tribulus terrestris*. The characterization of the nanoparticles was carried out using different techniques including UV, XRD, EDS and SEM; moreover, the effect of the nanoparticles in reproductive system was investigated.

Materials and methods

Preparation of fruits extract:

The plant of *Tribulus terrestris* was collected from its natural habitat in National Botany Garden (Khartoum, Sudan). The plant's fruits was washed, dried and extracted with sterile distilled water. The extract was prepared by taking 20 g of the plant part material with 100 mL of deionized water, the mixture was boiling for 60 min in80c^{o}. The mixture was cooled and this extract is filtered by whatman no. (1) Filter paper this filtrate was used as the extract for the preparation of titanium nanoparticles (TiNPs).

Biosynthesis of Titanium nanoparticles:

Eighty ml of *Tribulus terrestris* fruits extract was added to 80 ml of 0.5 M aqueous $TiCL_4$ solution, with stirring magnetically at room temperature. The mixture of titanium tetra chloride and leaf extract at the end time was light yellow, after 1 hour the color was changed to brown.

The mixture was subjected to shaking on the stirrer for 4 hrs. At the end of reaction time, the mixture color was creamy. In this process nanoparticles were formed, after those add ammonia drop to drop to achieve pH of solution became 7. The nanoparticles were dried at 100°Cfor overnight and calcined at 450°Cfor 3 hours.

Characterization techniques of Titanium nanoparticles:

Characterization of nanoparticles is the most important factor to understand and control of nanoparticles synthesis and applications. The characterization is performed using a variety of different techniques such as scanning electron microscopy (SEM), X-ray diffraction (XRD), Energy dispersive X-Ray Spectroscopy (EDS) and UV−Visible spectroscopy. These techniques are proper to determine of different parameters such as particle size, shape, crystallinity,

fractal dimensions, pore size and surface area. Moreover, orientation, intercalation, and dispersion of nanoparticles could be determined by these techniques. For instance, the morphology and particle size could be detected by SEM.UV–Vis spectroscopy is used to confirm sample formation by showing the Plasmon resonance.

Experimental design

Thirty Rats was used for Ti NPs treatment. Group I was maintained as normal control. Group 2, *T. terrestris* aqueous fruits extract, Group 3 and Group 4animals were treated with 50 and300 mg/kg /day Ti*Tt* nanoparticles and group 5 Titanium nanoparticles 300 mg/kg/day given to rats respectively oral. Water and basal diet were added for all groups. All rats received their designated experimental oral doses for 4 weeks. Initial and final body weight and body weight gain for each group were recorded at the first day of experimental dosing every weeks until the end of experiment. Blood samples were collected at slaughter. At necropsy, all rats were examined to identify gross lesions and specimens of the testes and ovaries were fixed in 10% neutral buffered formalin and processed for histopathology.

Statistical analysis

The results were examined by independent Sample`s t-test using SPSS software (Statistical Package for the Social Sciences, version 20 , SPSS Inc, Chicago, Illinois, USA). All results were shown as means±standard deviation (SD) and a $p<0.05$ was determined as statistically significant(Snedecor, 1989).

Results

Characterization of Tio$_2$NPs

Nanoparticles were characterized and spherical shaped and had the average size 6nm.

UV-Vis Spectroscopy

UV-Vis min 1240 spectrometer was used to record the absorption spectrum of TiO_2 in range from 280nm to 350nm as shown in fig (1), the maximum value of absorption of pure $TiO_2$1.134 (a.u) wile for TiO_2 with plant equal 1.008 (a.u) at 300 nm for both samples corresponding to photon energy 4.133 eV, the absorption edge was also calculated using Urbach's equation at wavelength 324 nm with energy band gap 3.8 eV, it was observed that TiO_2 increase the absorption value of plant extraction.

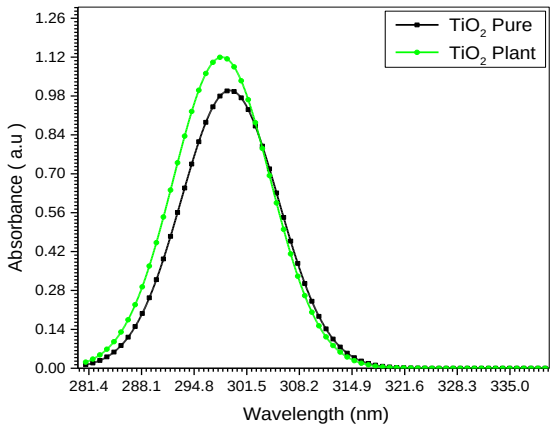

Fig (1): UV-Vis Spectroscopy of synthesized Titanium dioxide nanoparticles and Titanium *T. terrestris* nanoparticles

XRD Characterization

The XRD analysis was done to confirm the crystalline nature and particle size of the biologically synthesized TiO_2 NPS using *T. terrestris* fruit extract plant, (fig.2) represent the XRD pattern of TiO_2. The formation of titanium dioxide nanoparticles. The formation of TiO_2 was confirm with tetragonal body-centered structure with diffraction angels 25.27º, 37.96º 48.07º, 53.7º, 55 º, 62.7 º,75.3 º at planes (101, 004, 200, 105, 211, 204, 215) respectively, the particle size was calculated using scherrer's equation as follow

$$D = \frac{0.9\lambda}{\beta cos\theta}$$

Where D is particle size, λ is wavelength of X-ray (1.546 Å), β is full width at half maximum and θ is diffraction angle, the particle size of properad TiO_2 equal 6.6 nm and this size is suitable for bio applications.

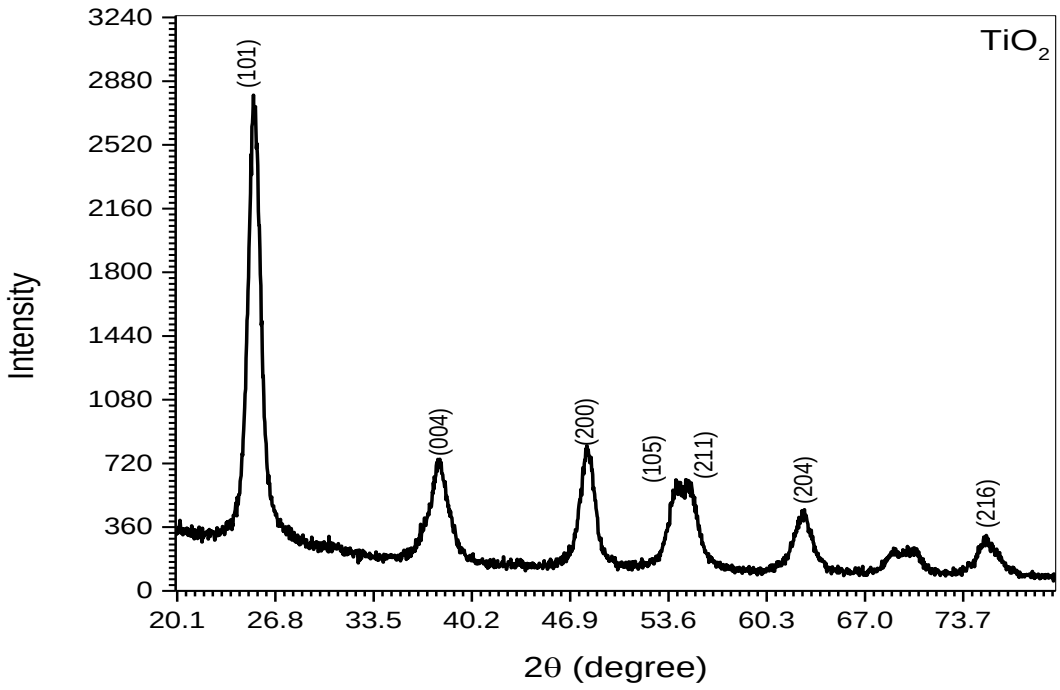

Fig (2): X-ray diffraction pattern of green synthesized anatase TiO_2 NPs.

The SEM and EDX Characterization

The SEM image of TiO_2 NPs was shown in (fig3). It clearly that the samples particles have ununiform shapes with unhomogenized surface, EDS result show that the sample consist of Titanium and oxygen.

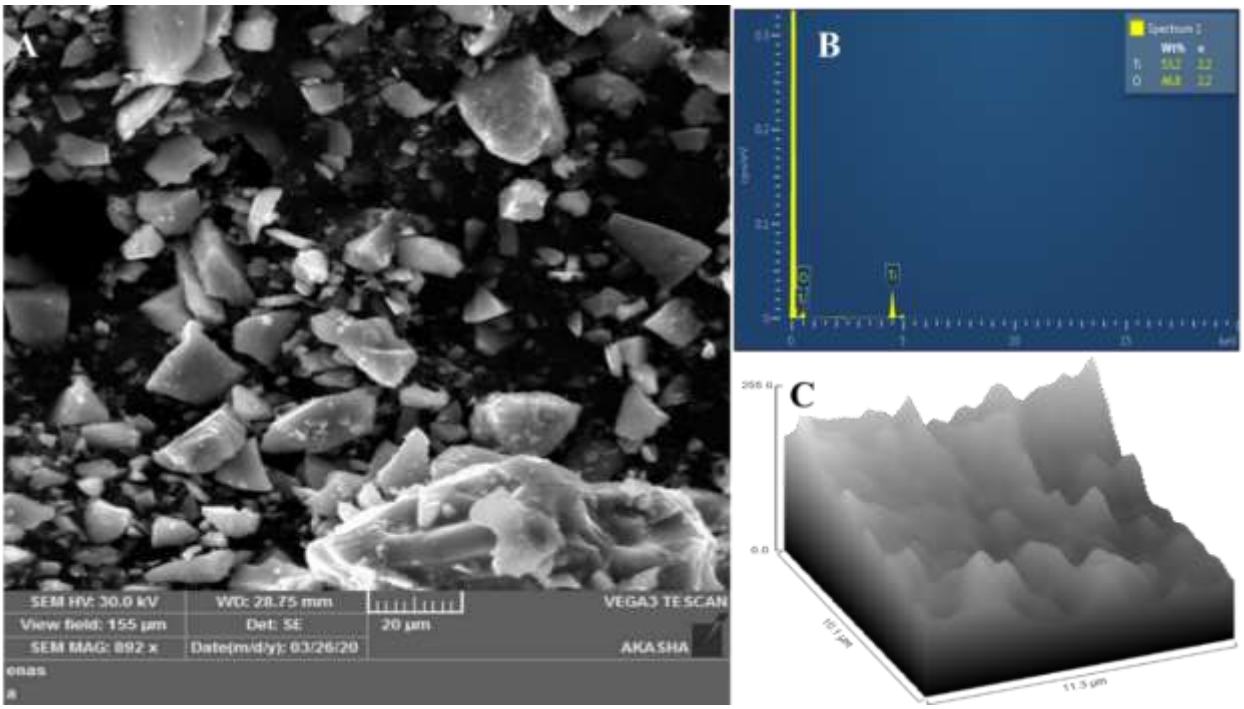

Fig (3): (a) SEM images of green synthesized Ti NPs. (b) EDX spectrum of green synthesized TiO_2NPs. (c) Non homogenous surface of TiO_2NP

Body weight change

After 2 weeks there was significantly change in body weight, lowering weight in treated groups when compared with control animals ($p < 0.05$) and after 4 weeks the weight is increase as showing in table (1).

Table.1. Changes in body weight constituents of rats given Titanium nanoparticles and aqueous extract of ***T. terrestris*** fruits orally for 4 weeks

Treatment groups	**Parameters**		
	Pretreatment Body weight (g) 0 week	**Body weight gain (g) After 2 weeks**	**Body weight gain (g) After 4 weeks**
1.Control (normal diet)	148.1±12.1	10.5±10.2	0.7±10.6
2. TiTt NPs(50 mg/kg/day)	152.5±2.6	10.3 ± 2.1^{NS}	$1.9\pm2.1^{*}$
3. TiTt NPs(300 mg/kg/day)	157.3±8.6	$0.5\pm8.1^{*}$	$10.0\pm8.1^{*}$
4.TiNPs (300mg/kg/day)	153.8±5.8	$4.8\pm5.9^{*}$	$2.5\pm3.5^{*}$
5.*T. terrestris* (300 mg/kg/day)	155.5±7.8	$2.5\pm6.8^{*}$	0.5 ± 9.1^{NS}

Values are expressed as means ± S.E. *(Standard Error).* (n = 6 rats in each group); NS = not significant;* Significant= (P<0.05) Ti*Tt* NPs = Titanium *Tribulus terrestris* nanoparticles; TiNPs = Titanium nanoparticles (Student's t test).

Serobiochemical change

In hormonal result for male show decrease in testosterone in all treated group except in group *Tt* aqueous fruits extract show in table (2). About female there was increase in Estrogen in group treated with green *Tt* aqueous fruits extract and decrease in LH and FSH presented in table (2). Marked reduction of reproductive hormones was observed in both male and female rats when given Titanium nanoparticles.

Table.2 .Effects of Titanium and *T. terrestris* fruits Titanium nanoparticles and aqueous extract of *T. terrestris* fruit on male and female reproductive hormones orally for 4 weeks.

Parameters	***Groups***				
	1.Control (normal diet)	**2.Ti*Tt*NPs (50mg/kg/day)**	**3.Ti*Tt*NPs (300mg/kg/day)**	**4. *T. terrestris* (300mg/kg/day)**	**5.TiNPs (300mg/kg/day)**
Male					
FSH	0.10±0.01	$0.04\pm0.02^{*}$	$0.04\pm0.02^{*}$	0.09 ± 0.01^{NS}	0.95 ± 0.01^{NS}
LH	0.90±0.01	$0.06\pm0.04^{*}$	$0.20\pm0.10^{*}$	$0.09\pm0.01^{*}$	$0.11\pm0.01^{*}$
PRL	1.01±0.10	$0.09\pm0.01^{*}$	0.70 ± 0.10^{NS}	0.80 ± 0.30^{NS}	$0.50\pm0.30^{*}$
Testosterone	7.60±0.05	$0.18\pm0.13^{*}$	$1.16\pm0.01^{*}$	$16.01\pm13.90^{*}$	$0.80\pm0.20^{*}$
Progesterone	0.54±0.46	$0.25\pm0.05^{*}$	0.48 ± 0.02^{NS}	$0.99\pm0.91^{*}$	$0.03\pm0.01^{*}$
Estrogen	43.10±37.0	$39.9\pm30.50^{*}$	$45.22\pm35^{*}$	$33.40\pm27.0^{*}$	$250\pm20.22^{*}$
Female					
FSH	0.95±0.85	0.60 ± 0.40^{NS}	$0.05\pm0.04^{*}$	$0.10\pm0.01^{*}$	$0.05\pm0.01^{*}$
LH	0.70±0.03	$0.07\pm0.03^{*}$	$0.10\pm0.90^{*}$	$0.08\pm0.02^{*}$	$0.10\pm0.09^{*}$
PRL	0.90±0.10	0.80 ± 0.40^{NS}	$0.50\pm0.40^{*}$	0.70 ± 0.10^{NS}	$0.50\pm0.40^{*}$
Testosterone	0.28±0.22	0.40 ± 0.20^{NS}	0.38 ± 0.32^{NS}	$0.09\pm0.01^{*}$	$0.80\pm0.30^{*}$
Progesterone	1.15±0.05	$3.90\pm3.30^{*}$	$6.50\pm3.70^{*}$	$0.03\pm0.01^{*}$	$0.03\pm0.01^{*}$
Estrogen	39.80±30.3	38.00 ± 32.20^{NS}	40.90 ± 39.50^{NS}	$160.70\pm39.50^{*}$	$235.00\pm5.10^{*}$

Values are expressed as means ± S.E. *(Standard Error).* (n = 6 rats in each group); NS = not significant;* Significant= (P<0.05) Ti*Tt*NPs = Titanium *Tribulus terrestris* nanoparticles; TiNPs = Titanium nanoparticles.

Histopathological effect of Titanium nanoparticles on ovarian and testes tissue

Degeneration of the seminiferous epithelium as well as reduction of produced sperms in males, Lack of normal antral follicles, enlarge cystic follicles and presence of atretic follicles in females. Vaculation and fibrosis in some cells in rats presented in figure (4,5 and 6).

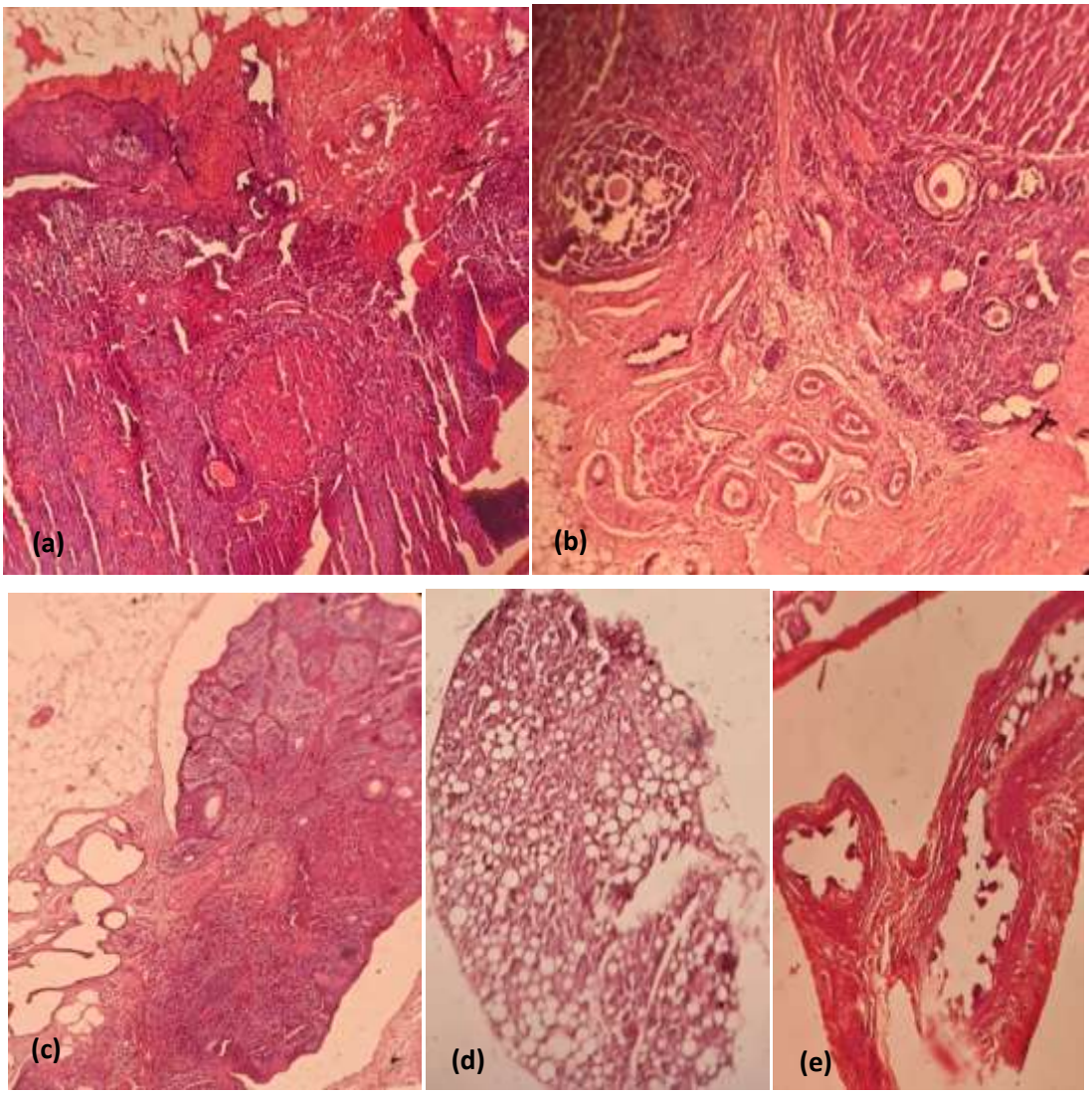

Fig.4 Ovary section of rat treated with *T. terrestris* fruit Titanium nanoparticles (Ti-NPs and aqueous extract of *T. terrestris* fruit at 50 and 300 mg/kg/day, show different size and different shape. H& E x100

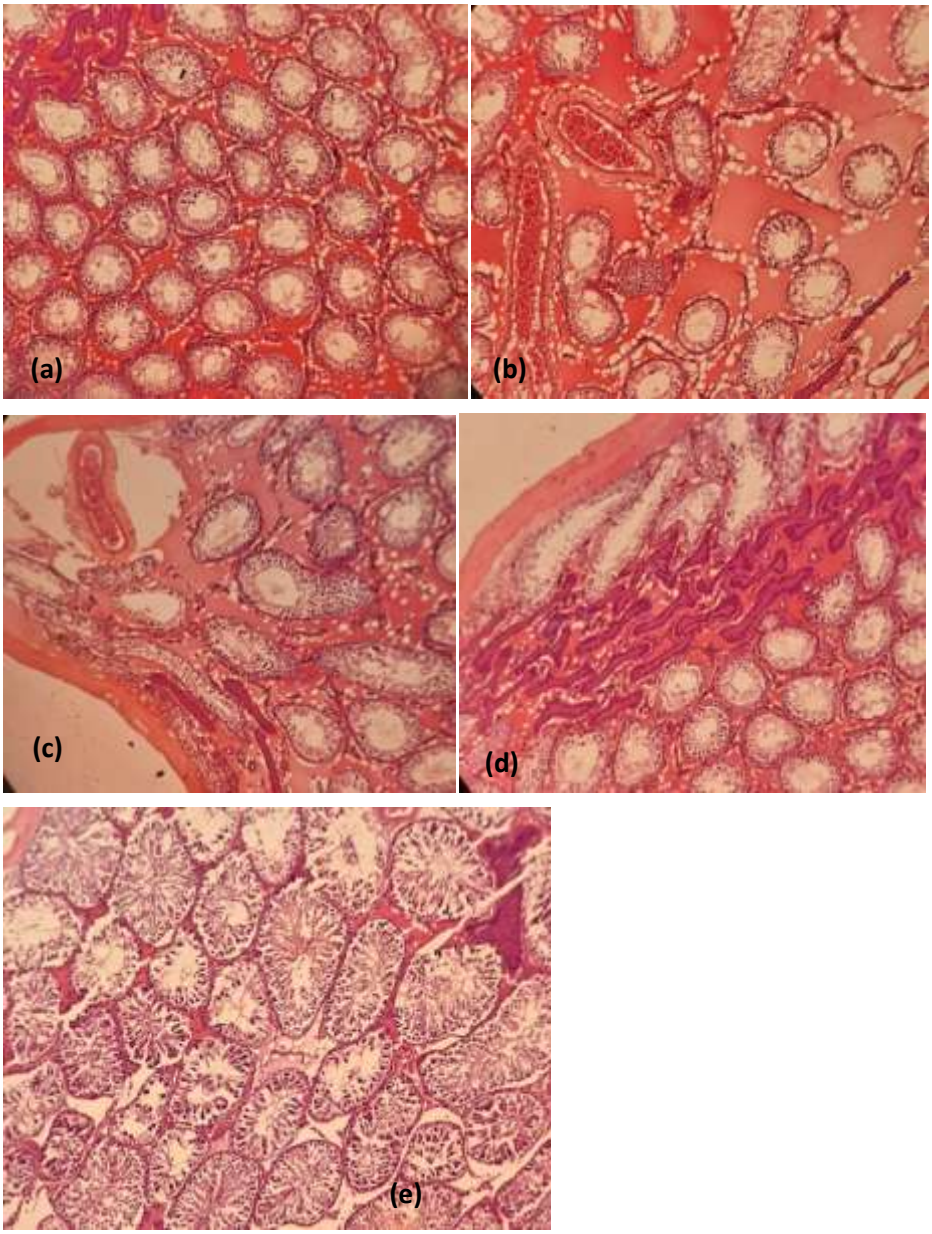

Fig 5. Shows a comparison of sperm of rats received daily oral doses of Ti NPs, for 4 weeks showing, (a) different size and different shape(b),sperm abnormality(c)reduction in sperm count compared to the control rats (d) and control (e) (H & E) ×100.

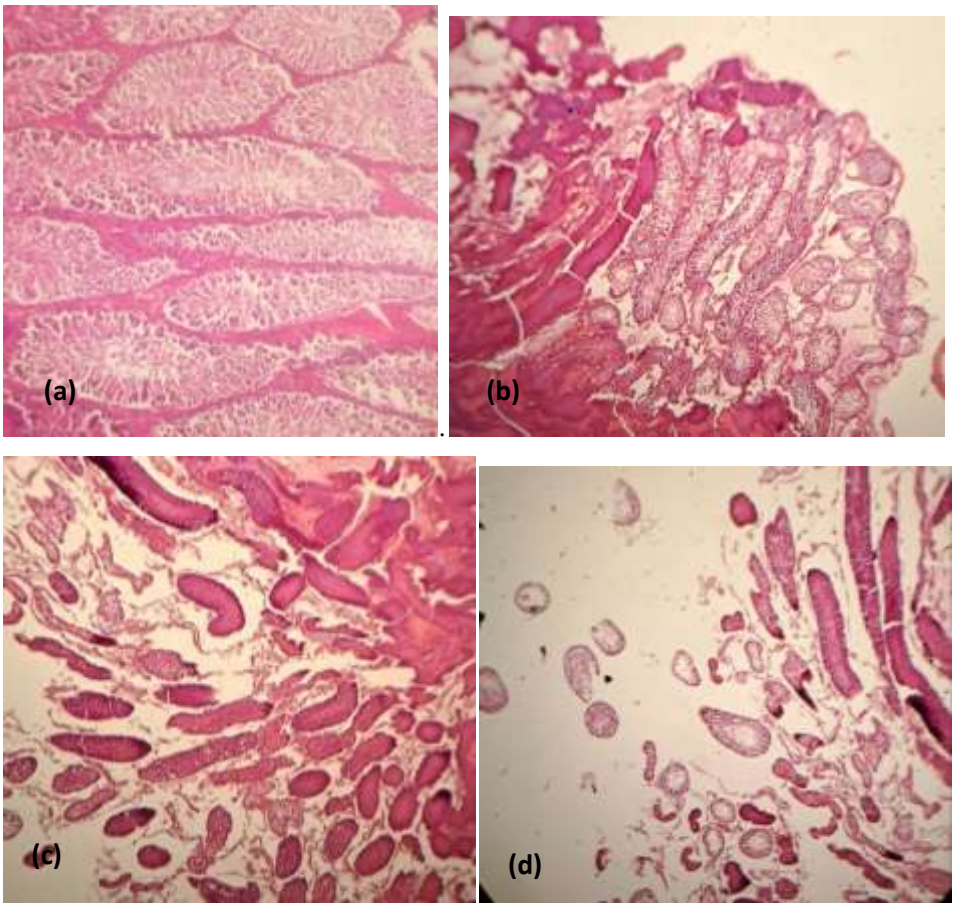

Fig 6. Shows a comparison of sperm of rats received daily oral doses of Ti NPs, for 4 weeks showing, (a) different size and different shape, (b) sperm abnormality, (c)reduction in sperm count compared to the control rats (d) (H & E) ×100.

Discussion

T. terrestris is known for its aphrodisiac properties and as a traditional medicine for treating male infertility (Gauthaman *et al*., 2002). A formulation of the saponins from *T. terrestris* was developed for veterinary application this formulation was effective in stimulating the sexual system (spermatogenesis, libido). Using *T. terrestris* extracts, an increase in sexual function in rats was demonstrated and attributed to an increase in testosterone, dihydrotestosterone, and dehydroepiandrosterone (Gauthaman and Ganesan, 2008) this agree with study in increasing testosterone in group that treated with *T. terrestris*. Different formulations containing *T. terrestris* extracts are marketed in USA and Europe as food supplements for stimulation, vigor (De Combarieu*et al*., 2003) and for other multiple ailments.

Green TiNPs characterization White colored titanium dioxide nanoparticles were prepared using the extract of *T. terrestris* and characterized using UV, XRD, SEM and EDS.

UV-vis spectral the presentstudy has described the synthesis of TiO_2 NPs by the *T. terrestris* furits extract mediated reduction of the aqueous titanium ions. Formation of TiO_2 NPs in aqueous solution was confirmed by using UV–vis spectral analysis. Results showed that the reduction of titanium ions and the generation of TiO_2 NPs were completed after overnight incubation at room temperature. The formation of creamy color indicated the reduction of titanium ions. The absorption spectra of the TiO_2 NPs formed in the solution had absorbance peaks at around (280-350) nm for the plant extract solution exposed to TiO_2 (Ambika and Sundrarajan, 2016) Fig (1).

The XRD analysis was done to confirm the crystalline nature and particle size of the biologically synthesized

Tio_2 NPS.XRD patterns of green synthesized TiO_2 nanoparticles were observed at 25.27°, 37.96°, 48.07°, 53.88°, 55°, 62.7°and 75.3° can be attributed to the 101, 004, 200, 105, 211, 204, and 215 crystalline Anatase structures of synthesized titanium dioxide nanoparticles (Fig. 2). These results were confirmed using Joint Committee on Power Diffraction Standards (JCPDS) No. 21 1272.

The calculated crystallite was found to be 6.6 nm for TiO_2 nanoparticles. The diffraction peak of the green synthesized TiO_2 nanoparticles is comparatively sharp (Varahalarao and Mohan, 2014). This phytochemical coating may enhance the stability and the dispersibility of the nanoparticles, which in turn may enhance their bioavailability, making them suitable for biological applications (Hariharan *et al*., 2017).

SEM and EDS the image was observed with in magnification of 20 μm. The TiO_2 nanoparticles were observed with irregular particle structure. The size was 6.6 nm nanoparticles. The nanoparticles were dispersed evenly on the surface with development of aggregate nanoparticles which revealed that powder particles are marginally agglomerated showing the view of spherical nanoparticles (Zahir *et al*., 2015).

The SEM image of Tio_2 NPs is shown in (Fig.3a). It clearly shows that the particles consist of agglomerated and nearly spherical in shape. The EDS spectrum image shows the elemental composition, which is present in the Tio_2 NPs and is showed in (fig 3b). It displays three strong peaks which are identified as titanium and oxide molecules (Dhaneswar*et al*., 2013).

The results of the present study showed that oral administration of TiNPs with 50 and 300 mg/kg for 4 weeks caused reproductive system toxicity. The findings indicated that TiNPs administration led to fibrosis and sperm abnormality. It has been proven that orally administration with Tio_2NPs accumulated in rats ovary lead to ovarian dysfunction such as atresia of primary and secondary follicles and also ovarian apoptosis induction (Gao *et al*., 2012). In this study, adult female and male was treated for 1 month. Our findings indicated that exposure to TiNP resulted in an increase in the serum Estrogen concentration that was agreed with previous studies which also showed significant reduction of levels of luteinizing hormone, testosterone, progesterone, and follicle-stimulating hormone (Zhao *et al*.,2013).

Decreased pregnancy rate in the present study may be related to imbalance of sex hormone concentrations. It is well known that there is a direct relationship between normal fertility and ovarian follicular development with the levels of sex hormones (Gao*et al*., 2012). In females, progesterone plays a key role in ovulation, implantation, and pregnancy (Graham and Clarke, 1997) and also has been proven to be important for ovulation through increasing of proteolytic enzymes production (wamasa *et al.*, 1992). Furthermore, LH and FSH are the most important hormones of hypothalamic-pituitary-gonadal axis, which regulates the production of gametes and fertility. Estrogen hormone also facilitates induction of receptor systems for FSH and LH in the granulosa cells (Lyon and Glenister, 1980). Thus, reduction of folliculogenesis and fertility by TiNPs administration may be due to impairments of sex hormones release such as Estrogen, FSH, LH, and progesterone (Gao *et al.*, 2012). Increased Estrogen levels in the current study and in previous studies may be associated with the activation of cytochrome p450 aromatase which is responsible for converting testosterone to Estrogen, but to prove this weneeds more investigation (Khorsandi *et al.*, 2017). Our findings also indicated that the FSH and LH with TiNPs were lower than those of the control rats and also the progesterone was significantly higher after TiNPs and lower in TiNPs with *T.t* administration. The previous studies indicated that oral exposure of TiNP for 30 consecutive days led to significant accumulation in the ovaries and testes also altered the ovarian causing Vaculation and fibrosis in some cells and sperm abnormality in male. Therefore, the reduction in rat's fertility potential following TiNPs administration may be due to disturbances in the system of the ovarian and testes cells and also hormonal imbalance. In the current study, we observed histological alterations in ovarian

and testes tissue caused by TiNPs administration including impairment in fibrosis and degenerating of follicles resulted in decrease of mature oocytes.

Conclusions

In this study, we have shown that Titanium nanoparticles can be synthesized via *Tribulus terrestris* fruits extract at an ambient temperature and characterized these nanomaterial's with several different techniques. This study concluded that Titanium nanoparticles pure is a toxic substance on Reproductive System, this effect was evident on the Sexual hormones and vital organs (ovaries and testes). The Titanium nanoparticles and Dirassa fruit aqueous extract reduced the loss of bodyweight in titanium rats. Ti nanoparticles can disrupt the system in male rats by decreasing intestosterone and decreasing in LH hormone concentration. This study provides experimental evidence that the orally administered Titanium nanoparticles Dirassa fruits extract on rats, improving thechange in histopathological structure in ovaries and testes. The aqueous extract of Dirassa fruitsexhibited significant activities in rats. This study supports the traditional use for Dirassa fruits andaqueous extract of *T. terrestris* (Dirassa) is good source to rising testosterone hormone.

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