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Evaluation of the effect of aqueous extract of *Tribulus terrestris* on some reproductive parameters in female mice

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Abstract
The aqueous extract of *Tribulus terrestris* was evaluated for its activity on the reproductive system of mature albino female mice. Two experiments involved maternal treatment with the extract (orally) for two and four weeks with two dose levels (100 and 200 mg/kg/day) at each period were conducted. Different ovarian and uterine parameters were studied in addition to determination of follicle stimulating hormone (FSH), luteinizing hormone(LH) and estradiol hormone levels during the estrus phase. The two weeks period experiment revealed no significant differences in maternal body weight or reproductive organs weight. A significant increase in the number of growing follicles, diameter of mature follicles, endometrial lining cells height and endometrial glands diameter was obtained in both dose levels. The four weeks period experiment showed a remarkable increase in body weight and reproductive organs weight in both dose levels. No significant differences on the levels of ovarian parameters, whereas, a significant increase was obtained in the endometrial lining cells height on both dose levels and a significant increase in endometrial glands diameter in the highest dose level.
As concerning the levels of reproductive hormones, although the differences were not significant, but an obvious increase was obtained in FSH and LH and a decrease in estradiol was detected in both dose levels. In conclusions, although there was some fluctuation, a dose dependent activity was obtained with the two weeks period seems to be more effective on both ovarian and uterine parameters, whereas the four weeks period was more effective on uterine parameters.

Keywords: *Tribulus terrestris*, reproduction, female mice.

1- Introduction
Since the past decades, some plants have been playing important role in disease curing along with artificial medications commonly called medicinal plants. Iraqi public folks dealt with use of herbs in health and disease control, and Iraq possesses great potentiality of developing drug industry as some local plants which have wide application in medicine are growing in abundance in the wild state such as *TT*, which has been investigated for desirable benefits [1].
Many researchers focused on the benefit of *TT* and its effects on female reproductive organs [2]. *Tribulus terrestris* extract contains many compounds such as alkaloids, flavonoids oil, saponins, resins and nitrates [3, 4]. *Tribulus* was without significant side effects if used at the safe range "250-750 mg/day" [5].
The biological properties of *Tribulus* extracts include diuretic properties, increased release of nitric oxide from endothelium and nerve endings; it relaxes smooth muscles and increases angiotensin converting enzyme (ACE) inhibition [6]. It also has antioxidant properties [4, 7]. *Tribulus terrestris* possesses antihypertensive activity [8, 9].
Therefore, the aim of this study is to evaluate the effect of crude aqueous extract of *TT* on reproductive parameters in mature female mice as an animal model.

2- Materials and Methods

2.1 Animals

Sixty mature female Swiss white mice with an average age of 7-8 weeks and 16-19 gm body weight were obtained from the animal house of the Institute of Embryo Researches and Infertility Treatment, Al-Nahrain University. They were kept in an air-conditioned room (22-24°c) with a controlled photo period of 14 hours light and 10 hours darkness. The animals were housed in opaque plastic cages measuring (28×15×14) cm, five mice were kept in each cage containing a wooden shave. Tap water and diet (commercial food) were freely available for the animals *ad libitum*. The mice were kept at least one week for adaptation. During this period abnormal and sick mice were excluded from the experiment. The animal cages were cleaned and sterilized with 70% ethanol once a week regularly.

2.2 Plant materials

Tribulus terrestris was provided by the Herbal Medicine Department/ Ministry of Health. The fruits of the plant was collected and authenticated from Mosul-Iraq by the Department of Herbal Medicine / Ministry of Health. The classification of the plant was also certified by the Iraqi State Board for Seed Testing and Certification.

2.3 Preparation of the aqueous extract

Twenty- five grams of the dried plant powder were suspended in 200ml of cold distilled water in a closed vessel, shaking with magnetic stirrer for 24 hrs, centrifuged at 3000 rpm for 15 minutes and the supernatant was filtered using Whatman No.1 filter paper and the filtrate was then evaporated nearly to dryness (gummy residue). The yield was found to be 10%. The gummy residue was dissolved in appropriate volume of distilled water and stored in a labeled sterile screw capped bottle at (-20°C) until use [1].

2.4 Calculation of the dose

The dose level used for human by herbalist was estimated to be about 150mg/kg B.W. /day. According to that, the dose was approximately calculated for mice [10].

2.5 Determination of oestrous phase

By the end of treatment in both periods, phases of oestrous cycle were determined through vaginal smear technique [11]. Animals at the oestrous phase were anaesthetized for blood collection and dissection of reproductive organs.

2.6 Collection of blood sample

During the oestrous phase animals were anaesthetized by means of intraperitoneal injection of pentobarbitol sodium at a dose level of 40-50mg/kg B.W [12]. Blood was collected through cardiac puncture using a 23 gauge needle, left for 30 minutes, and then centrifuged at 3000 rpm for 10 minutes for preparation of sera which were kept at- 20°c until use for hormonal determination.

2.7 Histological preparation

Incision in the abdominal wall of anaesthetized mice was done, whole reproductive system was removed after sacrificed and immersed in a falcon petri-dish with few drops of warm normal saline to be cleared from surrounding adipose tissue under dissecting microscope using fine surgical scissor and weighed (after drying from normal saline using filter paper) by using a sensitive balance, both ovaries and uterus were dissected out slightly below the tubo-uterine junction from one end and above the cervix from other. The numbers of corpora lutea were calculated under the dissecting microscope. One side of ovary and uterus immediately fixed in 10% formalin solution for 24hr., before transferring to 70% ethyl alcohol and kept until use for histological preparation [13]. The other side of ovary and uterus fixed in Bouin's fluid for 18hr., before transferring to 50% ethyl alcohol for 2hr., then transferred to 70% ethyl alcohol and kept their until use for preparations of histological section. The different fixatives were used to choose the best one for histological sections.

The fixed tissues were then processed for routine dehydration, clearing and infiltration with paraffin- wax embedding ([11]. Serial 5µm cross sections were made through the ovaries and uterine horns using a rotary microtome [14]. The sections were stained with hematoxylin and eosin and examined microscopically [11].

Various ovarian and uterine measurements were done using eye piece micrometer [11]. The following parameters were determined:

Ovaries

- Number of growing follicles.
- Diameter of mature (graafian) follicles.

Uterus

- Endometrial lining cells height.
- Endometrial glands diameter.
-

2.8 Hormonal assay

Hormones namely: FSH, LH and 17 β-estradiol (E_2) were assayed by the end of the 2^{nd} & 4^{th} weeks of the experiment using prepared kits of radio-immuno assay technique (RIA). RIAs obey the principles of competitive protein binding assay and it relies on the availability of hormones of sufficiently high purity for labeling with radioisotopes [15].

2.9 Microscopic photography

Using a light microscope, photographs of the histological sections were taken, by means of a digital camera.

2.10 Statistical analysis

Data from treated and control groups were expressed as mean ± standard error (M ± S.E), and analyzed using the analysis of variance(ANOVA) with least significant test. P value < 0.05 was considered significant and P value <0.01 considered highly significant [16].

3-Results

3.1 Effect of oral treatment of aqueous extract of TT for two weeks on body weight, reproductive organs weight and number of corpora lutea

The results are shown in Table 1. No significant differences in body weight and reproductive organs weight were detected between the experimental groups G_1 and G_2 and control group G_3. At the same time, no significant differences in the number of corpora lutea were found in G_1 and G_2 in comparison with the control group G_3.

3.2 Effect of oral treatment of aqueous extract of TT for four weeks on body weight, reproductive organs weight and number of corpora lutea

After four weeks of treatment with aqueous extract of *TT,* no significant differences in body weight and reproductive organs weight between the experimental group G_4 and control group G_6 as shown in table -2. A significant ($P< 0.05$) increase in body weight was found in G_5 compared to the control group G_6.A significant ($P<0.05$) increase in reproductive organs weight was found in experimental group G_4 compared to control group G_6, a highly significant ($P< 0.01$) increase was found in experimental group G_5 compared to control group G_6 and a significant ($P< 0.05$) increase in G_5 in comparison with G_4.On the other hand, no significant differences in the number of corpora lutea were found in G_4 and G_5 in comparison with the control group G_6.

3.3 Effect of oral treatment of aqueous extract of TT for two weeks on number and measurements of some ovarian and uterine parameters

The results are shown in Table 3. No significant differences in number of growing follicles was found in experimental group G_1 compared to control group G_3, a highly significant ($P< 0.01$) improvement was exhibited in G_2 in comparison with the control group G_3 Plates (1,2) and a significant ($P< 0.05$) increase was found in G_2 compared to G_1.

There is a significant ($P< 0.05$) increase in diameter of mature follicles in G_1 and G_2 compared to G_3, and a significant ($P< 0.05$) increase in G_2 compared to G_1, Plates (3, 4).

As concerning the endometrial lining cells height, a highly significant ($P< 0.01$) increase was noticed in experimental groups G_1 and G_2 in comparison with control group G_3. At the same time, a highly significant ($P< 0.01$) increase was detected in G_2 compared to G_1.

On the other hand, G_1 and G_2 showed a highly significant ($P< 0.01$) increase in endometrial glands diameter compared to the control group G_3, Plates (5,6) and a highly significant ($P< 0.01$) increase was found in G_2 compared to G_1.

3.4 Effect of oral treatment of aqueous extract of TT for four weeks on number and measurements of some ovarian and uterine parameters

As shown in Table 4, No significant difference in number of growing follicles and diameter of mature follicle in experimental groups G_4 and G_5 in comparison with the control group G_6.

The endometrial lining cells height showed a highly significant ($P< 0.01$) increase in G_4 and G_5 in comparison with control group G_6, Plates (7,8), and a highly significant ($P< 0.01$) increase in G_5 was found in comparison with G4.

The endometrial glands diameter showed no significant difference in G_4 and G_5 compared to control group G_6, but a significant ($P< 0.05$) increase in G_5 was detected in comparison with G_4.

3.5 Effect of oral treatment of aqueous extract of TT for two and four weeks on some reproductive hormones

In Table 5, although statistically non-significant, but a remarkable increase was found in FSH and LH levels in the two experimental groups during the two periods of the experiment compared to the control groups. At the same time, a remarkable decrease in E_2 hormone was exhibited in the two experimental groups during the two periods of the experiment in comparison with the control groups.

Table 1: Effect of oral treatment of aqueous extract of *TT* for two weeks on body weight, reproductive organs weight and number of corpora lutea.

Parameters / Groups	Number of dams	Weight of animals (gm)		Reproductive organs weight (mg/100gm.B.W)	Number of corpora lutea
		Before treatment	After treatment		
G1 100 mg/kg/day	10	17.23 ±0.41	19.21 ±0.70	273.50 ±48.77	13.30 ±0.37
G2 200 mg/kg/day	10	15.87 ±1.10	18.18 ±1.13	246.17 ±75.47	17.20 ±1.29
G3 Control	10	16.63 ±1.11	19.58 ±1.35	274.49 ±48.63	15.20 ±1.38

Values are mean± SE.

Table 2: Effect of oral treatment of aqueous extract of TT for four weeks on body weight, reproductive organs weight and number of corpora lutea.

Parameters / Group	Number of dams	Weight of animals (gm)		Reproductive organs weight (mg/100gm.BW)	Number of corpora lutea
		Before treatment	After treatment		
G4 100 mg/kg/day	10	16.22 ±0.45	19.53 ±1.13	237.35 [a] ±60.18	15.70 ±1.42
G5 200 mg/kg/day	10	15.88 ±1.10	20.40 [a] ±0.78	269.92 [b,c] ±45.40	19.40 ±3.76
G6 Control	10	16.66 ±1.11	17.48 ±1.00	109.79 ±26.90	17.40 ±1.79

Values are mean± SE.

[a] *$P< 0.05$ in comparison with control.*

[b] *$P<0.01$ in comparison with control.*

[c] *$p<0.05$ in comparison with G4*

Table 3: Effect of oral treatment of aqueous extract of *TT* for two weeks on number and measurements of some ovarian and uterine parameters.

Parameters / Group	Number of dams	Number of growing follicles	Diameter of mature follicles (µm)	Endometrial lining cells height (µm)	Endometrial glands diameter (µm)
G1 100 mg/kg/day	10	12.00 ±1.53	286.00 [a] ±6.0	27.50 [b] ±1.96	52.91 [b] ±2.15
G2 200 mg/kg/day	10	15.50 [b,c] ±1.19	241.74 [a,c] ±8.72	22.00 [b,d] ±2.96	51.41 [b,d] ±2.88
G3 Control	10	10.25 ±0.85	195.647 ±8.65	13.78 ±0.67	38.85 ±1.27

Values are mean± SE.
[a] *P< 0.05 in comparison with control.*
[b] *P<0.01 in comparison with control.*
[c] *p<0.05 in comparison with G1*
[d] *P< 0.01 in comparison with G1*

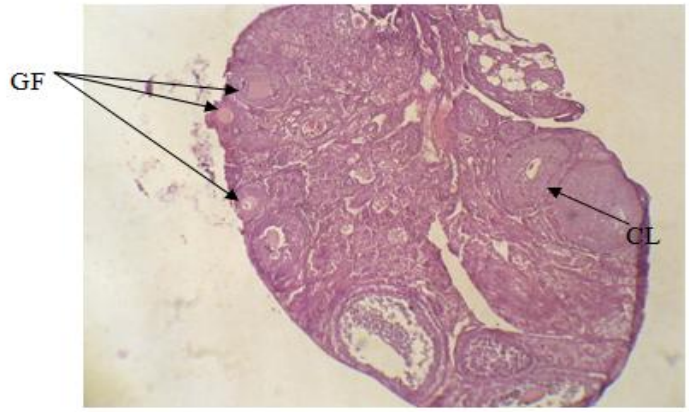

Plate 1: Cross section in the ovary of a mature female mouse (control group), (H & E, x100), showing the Corpus luteum (CL), Growing follicle (GF).

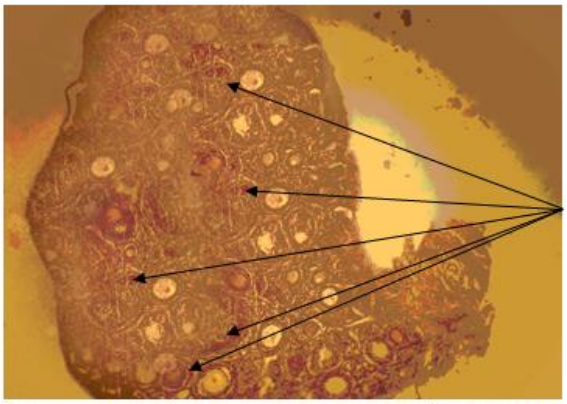

Plate 2: Cross section in the ovary of a mature female mouse treated with 200 mg/kg/day of *TT* for two weeks (H & E, x100), showing the increase in the No. of GF.

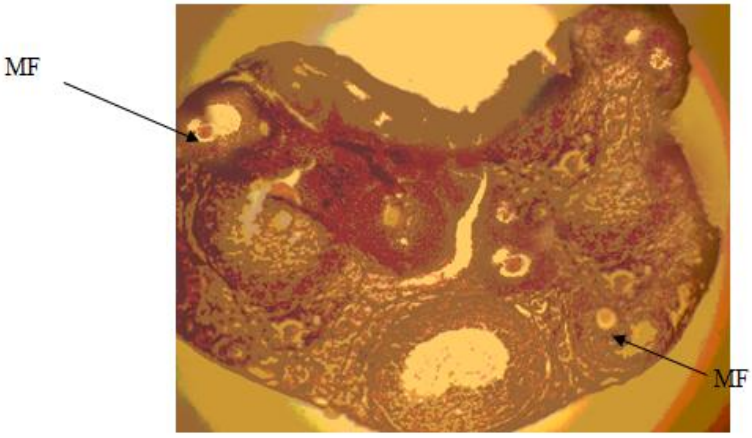

Plate 3: Cross section in the ovary of a mature female mouse (control group), (H & E, x100), showing the mature (graafian) follicle MF.

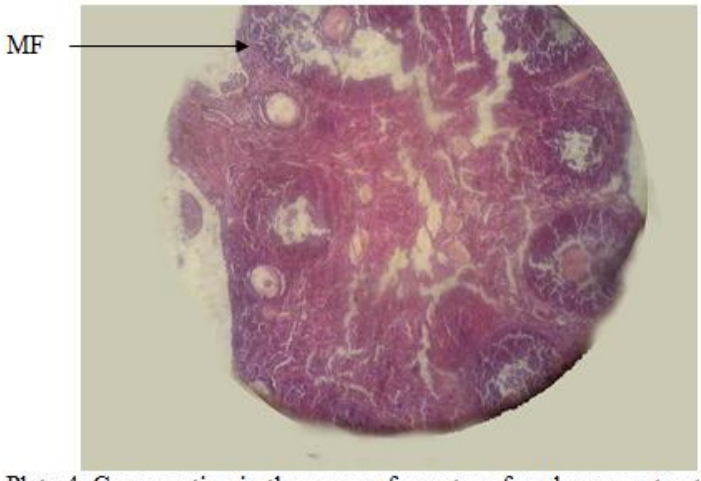

Plate 4: Cross section in the ovary of a mature female mouse treated with 200 mg/kg/day of *TT* for two weeks, (H & E, x100), showing the increase in diameter of MF.

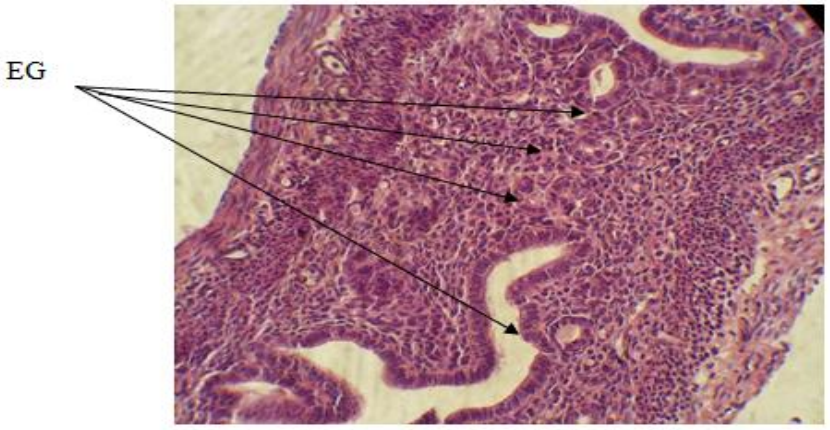

Plate 5: Cross section in the uterine horn of a mature female mouse (control group), (H & E, x400), showing the endometrial glands (EG) diameter.

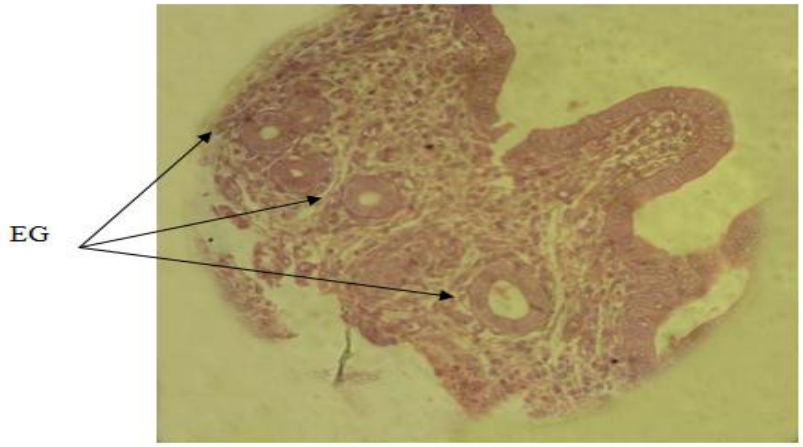

Plate 6: Cross section in the uterine horn of a mature female mouse treated with 200 mg/kg/day of *TT* for two weeks, (H & E, x400), showing the increase in EG diameter.

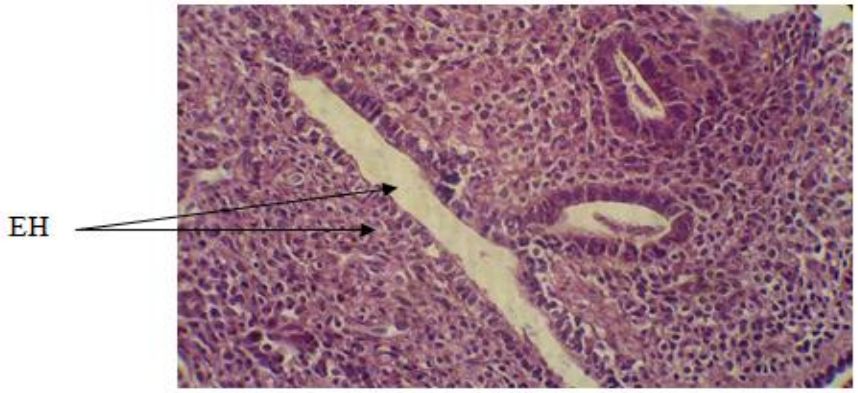

Plate 7: Cross section in the uterine horn of a mature female mouse (control group), (H & E, x400), showing the endometrial lining cells height (EH).

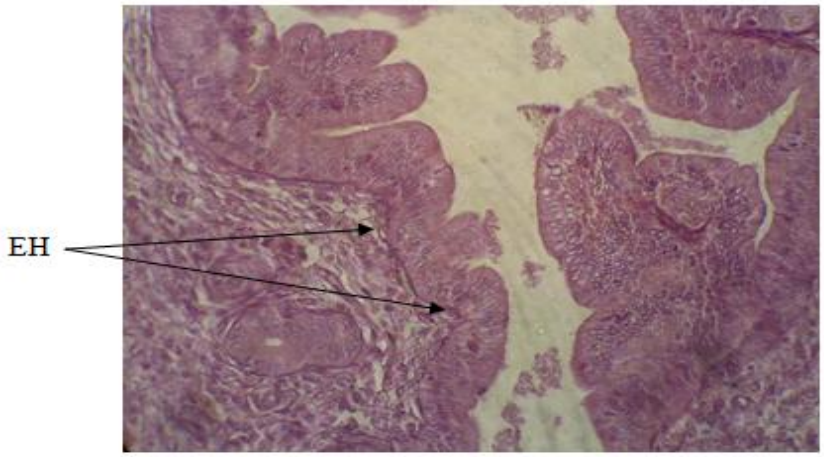

Plate 8: Cross section in the uterine horn of a mature female mouse treated with 200 mg/kg/day of *TT* for four weeks, (H & E, x200), showing the increase in EH.

Table 4: Effect of oral treatment of aqueous extract of *TT* for four weeks on number and measurements of some ovarian and uterine parameters.

Parameters / Group	Number of dams	Number of growing follicles	Diameter of mature follicles (µm)	Endometrial lining cells height (µm)	Endometrial glands diameter (µm)
G4 100 mg/kg/day	10	17.00± 1.39	323.29± 38.13	17.55 ± 1.19 [b]	36.73± 1.48
G5 200 mg/kg/day	10	16.43± 1.95	300.50± 38.79	31.14 ± 1.84 [b,d]	51.17 ± 2.70 [c]
G6 Control	10	16.00± 0.58	289.76± 23.32	12.00± 1.03	45.07± 4.00

Values are mean± SE.
[b] *P<0.01 in comparison with control.*
[c] *p<0.05 in comparison with G4*
[d] *P< 0.01 in comparison with G4*

Table 5: Effect of oral treatment of aqueous extract of *TT* for two and four weeks on some reproductive hormones

After two weeks				After four weeks			
Parameters / Group	**FSH (mIU/ml)**	**LH (mIU/ml)**	**E2 (P. mol/L)**	**Parameters / Group**	**FSH (mIU/ml)**	**LH (mIU/ml)**	**E2 (P. mol/L)**
G1 100 mg/kg/day	0.88± 0.24	0.70± 0.31	4094.0± 617.88	**G4 100 mg/kg/day**	1.30± 0.28	1.24± 0.18	3824.60± 923.85
G2 200 mg/kg/day	0.74± 0.12	1.00± 0.20	3708.2± 707.89	**G5 200 mg/kg/day**	1.03± 0.17	0.87± 0.11	6086.00± 735.15
G3 Control	0.42± 0.10	0.52± 0.15	5270.0± 619.93	**G6 Control**	1.08± 0.20	0.90± 0.13	6330.40± 849.16

Values are mean± SE.

4- Discussion

The results obtained from the present study revealed a remarkable increase in body weight and reproductive organs weight which were significantly higher than the controls after four weeks of treatment. This increase was possibly due to that treatment which affected the metabolic pathway towards hypoglycemic condition resulted in stimulation of growth hormone (GH) secretion from anterior pituitary gland that encourages secretion of insulin like growth factors "IGF-I and II" [17]. This hormone is necessary to stimulate skeletal muscle growth, regulate lipolysis and promote cellular uptake of amino acids [18]. This result is in agreement with other researchers who reported a significant increase in body weight of female mice [19], and male mice [20] treated with *TT* extract. Insulin like growth factors I and II play important role in increasing protein synthesis and decreasing protein catabolism [21].

The other possibility is that this increment may be due to the presence of some substance (s) in the extract that stimulates the regulation of fats and carbohydrates metabolism [22]. It has been reported that *TT* may be a good appetizer and digestion promoter [23].

On the other hand, the significant increase in reproductive organs weight collectively (ovaries, oviducts and uterus) is in agreement with Abid, 2010 [19]. This increment may be caused by antioxidant activity of the extract [24] or by some *TT* aqueous extract contents such as saponins (disgenin) and sterol (β-siosterol, stigma

sterol) which contain phytoestrogen [25, 26, 27]. The metabolites of phytoestrogen exert an estrogenic effect on central nervous system which induces estrus and stimulates cell division and growth of genital tract of female animals [28]. This may clarify the increase in body weight and reproductive organs weight, as well.
Furthermore the study revealed an evidence of remarkable increase in number of growing follicles which initiate the increase in reproductive organs weight [29]. The result of such increase is in agreement with Esfandiari *et al.*, 2011[30] who tested a purified extract of *TT* on rat. Though the present results revealed no statistically significant differences in female reproductive sex hormones, there was an obvious increase in FSH and LH and a decrease in estradiol, a result which is in agreement with Milanov *et al.*, 1985 [31] with respect to FSH and LH but not with estradiol. Rising of FSH and LH leads to increasing diameter of mature follicles [32], an effect which may also explain the increase in ovarian weight.
Sato *et al.*, 2002 [29] reported that systemic and local IGF-I play a major role in estrogen effect on growth and epithelial proliferation of mouse uterus, an effect which is reflected in the current study by the highly significant differences in endometrial lining cells height and endometrial glands diameter. On the other hand, this finding disagree with Martino- Andrade *et al.*, 2010 [33] who reported that low dose levels of *TT* purified extract given to castrated female rats for 28 days was unable to stimulate endocrine sensitive tissues such as uterus and vagina.
The elevation of FSH and LH level may explain the significant increase in the number of growing follicles mentioned before, a result which is resembling that obtained by Zarkova, 1983 [34] after treating human females with *TT* extract for the same period and getting the same results at day 13 onto 14 of menstrual cycle.
Steroidal saponins present in *TT* extract lead to a direct increase in LH [35], this hormone has many receptors in theca interna cells of ovarian follicles [18] and act via cAMP to increase conversion of cholesterol to androstendione, some of androstendione is converted to estrogen which enters the circulation. The relationship between dose and duration of treatment may play a role in the decrease of estrogen level in comparison with controls. The declining of E_2 was nearly at ovulation stage [36].
This effect was expressed in the present results by the evidence of a significant increase in endometrial lining cells height and that the growing follicles may reach maturity, all these events may lead to accelerate hormonal action especially FSH and LH [37].
In conclusion, treatment of female mice with the crude aqueous extract of *TT* showed obvious effects on the reproductive parameters studied. The two weeks period of treatment seems to be more effective on both ovarian and uterine parameters, whereas the four weeks period was more effective on the uterine parameters.

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