



## The Effectiveness of Super Ovulation and Multiple Pregnancies in Sprague Dawley Rat using *Morus alba* Linn. Fruit

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### ABSTRACT

The white mulberry, *Morus alba* L. has been used by several Asian societies for the treatment of infertility. However, there is no evidence that products of the plant can influence ovarian health. Thus, the aim of this study was to determine the effect of the *Morus alba* fruit extract on the ovarian function of nonpregnant and pregnant rats. The study showed that in rats *Morus alba* fruit extract stimulates follicular stimulating (FSH) and luteinizing hormones (LH), estrogen, and progesterone productions that peaked at 8 days of treatment. The effect was dose-dependent with hormone production increasing with increase in dose of the extract. The Graafian follicles were fully matured, and the number increased with increase in dose of the extract. The uterus of pregnant rats contained several embryos that gave birth to the full-term offspring without abortion or embryonic abnormalities. In conclusion, the *Morus alba* fruit extract can be used to induce superovulation to cause multiple pregnancies. Thus, the *Morus alba* fruit extract has potential to be developed into a compound for the treatment of female infertility.

**Keywords:** Ovulation induction, Multiple pregnancies, Hormone levels, Natural products, Rat model

### INTRODUCTION

The Chinese has a long history of traditional use mulberry as herb medicine [1]. They have used the fruit for treating many ailments from premature greying of hair to food poisoning and hypertension [2,3]. The *Morus alba*, also known as white mulberry (Figure 1), is a fast-growing, small to medium-sized flowering tree belonging to the family Moraceae. The tree is widely distributed and cultivated in most Eastern Asian countries [4] and certain temperate regions of the world, including Kurdistan region-Northern Iraq. The tree is a good source of food for humans and animals and the leaves are used to feed silkworms [1]. The mulberry juice or mulberry pomace is used as a diet supplement by virtue of its isocaloric and isonitrogenous properties similar to corn grain and cottonseed meals [5]. Although clinical trials on the use of the *Morus alba* contract has been conducted, the full potential of the tree as a source of therapeutic compounds has yet to be fully realized [6].



Figure 1(a) *Morus alba* fruit on the tree



Figure 1(b) *Morus alba* fruit in dried form

The various biological and pharmacological activities attributed to the mulberry fruit, leaves and roots include antioxidation [7-10], macrophage activation [11], antimicrobial infection [12-14], antiviral infection [15], antiparasitic [16], anti-inflammation [17-19], cytotoxicity and anticancer [17-22], anti-diabetes [23-25], anti-ulcer [26], anti-hyperlipidemia [27], anti-atherosclerosis [28-31], anti-obesity [32,33], anti-aging [34], anti-hypertension [35], cardioprotection [36], cognitive enhancement, neuroprotection [37], and hepatoprotection [38,39]. Other pharmacological properties of *Morus alba* include anti-platelet [40], anxiolytic, anti-asthmatic [41], antidepressant, and immunomodulatory activities [6].

Mulberry leaves have flavonoids as major constituents with polyphenols in lesser amounts [42]. The root bark of the *Morus alba* contains flavonoids, alkaloids, and stilbenoids [43,44] and the mulberry fruit is rich in linoleic, palmitic, oleic, and stearic acids [45].

The treatment of choice for anovulation in infertile women has been clomiphene citrate (CC), which is a relatively safe and effective oral drug. However, this drug is known to have side effects such as psychological disorder [46], hot flashes and ovarian cysts [47], and is implicated a cause of visual disturbance [48].

In Iran, *Morus alba* is listed as one of the plants in Iranian traditional medicine used in the treatment of infertility in men [49]. On the contrary, in India the juice of the *Morus alba* leaves is taken orally as a contraceptive. In the rat diabetic model, it was shown that oral treatment with cooked pulverized mulberry leaves increased the number of germinal, Leydig, and Sertoli cells, diameter of seminiferous tubules, testosterone level, and testis weight [50]. Currently, there is no reported evidence on the effect of products of the mulberry plant on the female reproductive system. Thus, our aim is to determine the effect of the *Morus alba* fruit extract on the reproductive activities of female rats.

## MATERIALS AND METHODS

### Fruit Extract Preparation

The fruit of *Morus alba* L. (Moraceae) collected from uncultivated fields in the vicinity of Sulaimani city, Kurdistan region, Northern Iraq during fruiting season of May to June 2016. The harvested fruit authenticated in the Department of Field Crop, College of Agricultural Sciences, University of Sulaimani with voucher number UNIVSUL/AGRI/

FC/0011. The fruit was sun-dried to brown in color and grounded into coarse powder. Exactly 250 g of *Morus alba* fruit powder was subjected to the maceration extraction method [51]) using 500 mL ethanol. The ethanol filtrate was evaporated for 48 hours using the Buchi vacuum rotary evaporator (Sigma-Aldrich) to obtain a semi-solid mass, which was kept in an air-tight glass container and stored at 4°C.

### Experimental Design

Thirty-two 7 to 8 weeks old virgin female Sprague-Dawley rats weighing between 150 g and 200 g and were obtained from the Animal House of Department of Clinic and Internal Medicine, College of Veterinary Medicine, University of Sulaimani, Iraq. The rats were kept in polystyrene cage and acclimatized for 5 days under room temperature of  $25 \pm 1^\circ\text{C}$  with a 10 to 14-hour light-dark cycle and normal rat diet and water were provided ad libitum. Rats were grouped into four (A to D, n=8 each). Group A served as control negative and received normal rat diet and water, whereas groups B, C, and D rats received 300 mg/kg, 600 mg/kg, and 900 mg/kg of freshly prepared semisolid *Morus alba* fruit extract in 1 mL of distilled water daily. The extract was administered orally using force feeding needle once daily for 14 days.

On day 7 of treatment, 2 fertile male rats were introduced to each cage for 24 hours to allow mating. Two rats from each group were euthanized on day 7 to obtain the ovaries and another 2 on day 15 to obtain the developing embryos. The embryos were macroscopically examined. The rest of the pregnant rats were allowed to carry to term to deliver their pups. Euthanasia was conducted humanely using chloroform inhalation anesthetic in a tightly closed glass chamber and the ovaries and embryos collected. The study was approved by the Animal Care and Use Committee, College of Veterinary Medicine, University of Sulaimani (UNIVSUL/ACUC/2014-0099).

### Histopathological Examination of the Ovaries

The Ovaries were fixed in 10% of formalin for at least 48 hours before placing in plastic cassettes and dehydrated using an automated tissue processor (Leica, ASP300, Germany). The tissues were embedded in paraffin wax, and the blocks sectioned using a semi-automated microtome (Leica, RM2155, Germany). Subsequently, the tissue sections mounted on the glass slides were deparaffinized and rehydrated. The sections were stained with Harris's hematoxylin and eosin (H&E) [52]. Finally, tissue sections mounted with cover slips using Distrene-Plasticizer Xylene (DPX) were examined using a light microscope image analyzer (Olympus BX51TF Japan).

### Hormonal Measurement

Approximately 3 mL of blood was collected from each rat via cardiac puncture, while under Di-ethyl ether anesthesia, on days 0, 8 and 15 of the experiment in EDTA. Plasma was obtained for FSH, LH, estrogen and progesterone measurement using the ELISA method [53].

### Statistical Analysis

The results of this experiment expressed as mean  $\pm$  SD and analyzed statistically using SPSS version 20.0 (SPSS Inc., Chicago, USA). Significance level was determined at  $\alpha=0.05$ .

## RESULTS

The effect of treatment with *Morus alba* fruit extract on the rat plasma LH, FSH, and estrogen was peak and most significant ( $p<0.05$ ) after 8 days (Figures 4-7). The level of these hormones decreased again after 15 days. The effect of the *Morus alba* fruit extract was dose-dependent with the concentration of hormones positively correlated with concentration of extract used in the treatment. The plasma LH and FSH peaked at  $1.56 \pm 0.01$ ,  $0.53 \pm 0.08$ , mIU/mL, respectively while estrogen at  $101.03 \pm 0.6$  pg/mL at Day 8 after treatment with 900 mg/kg of extract. The effect of treatment with *Morus alba* fruit extract on plasma progesterone increased significantly ( $p<0.05$ ) both with treatment concentration and period of treatment. The highest plasma progesterone was  $99.67 \pm 0.25$  pg/mL after treatment with 900 mg/kg extract at Day 15. The extract appears to have a delayed effect on the plasma progesterone (Table 1).

**Table 1(a) Mean values of FSH, LH, estrogen and progesterone levels following administration of *Morus alba* extract on Day 0**

Group	FSH (mIU/ml)	LH (mIU/ml)	Estrogen (pg/ml)	Progesterone (pg/ml)
A (Control Negative)	0.18 ± 0.44	0.88 ± 0.11	56.07 ± 0.3	18.20 ± 0.10
B (Low Dose)	0.15 ± 0.31	0.75 ± 0.55	52.31 ± 0.1	19.32 ± 0.73
C (Mid Dose)	0.16 ± 0.17	0.82 ± 0.15	49.03 ± 0.3	17.16 ± 0.51
D (High Dose)	0.19 ± 0.30	0.79 ± 0.01	54.39 ± 0.9	20.31 ± 0.90

Values are given as mean ± SEM for each group; There is no significant difference ( $p < 0.05$ ) in all treatment groups compared to Group A; Statistical level of significance was determined by one-way ANOVA

**Table 1(b) Mean values of FSH, LH, estrogen and progesterone levels following administration of *Morus alba* extract on Day 8**

Group	FSH (mIU/ml)	LH (mIU/ml)	Estrogen (mIU/ml)	Progesterone (mIU/ml)
A (Control Negative)	0.19 ± 0.09	0.86 ± 0.07	54.06 ± 0.35	19.20 ± 1.2
B (Low Dose)	0.28 ± 0.05*	1.10 ± 0.15*	77.36 ± 1.2*	22.4 ± 0.8
C (Mid Dose)	0.41 ± 0.12*	1.34 ± 0.11*	89.07 ± 0.3*	25.52 ± 0.5
D (High Dose)	0.53 ± 0.20*	1.56 ± 0.01*	101.03 ± 0.6*	29.71 ± 0.07*

Values are given as mean ± SEM for each group; \* indicates a significant difference ( $p < 0.05$ ) in treated groups compared to group A (control group); Statistical level of significance was determined by one-way ANOVA

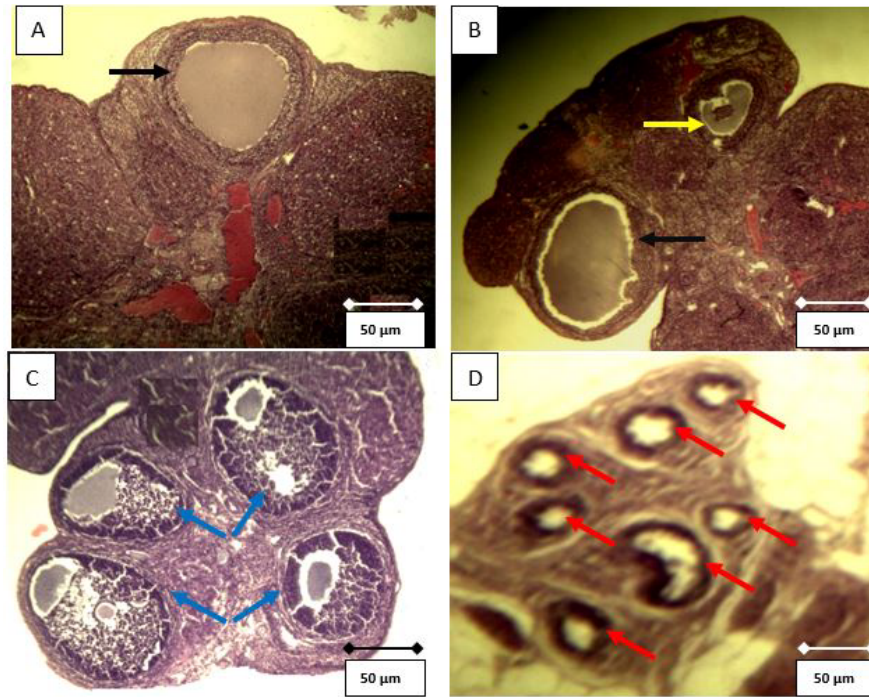
**Table 1(c) Mean values of FSH, LH, estrogen and progesterone levels following administration of *Morus alba* extract on Day 15**

Group	FSH (mIU/ml)	LH (mIU/ml)	Estrogen (mIU/ml)	Progesterone (mIU/ml)
A (Control Negative)	0.17 ± 0.01	0.85 ± 0.91	56.07 ± 0.9	18.21 ± 1.07
B (Low Dose)	0.20 ± 0.05	0.90 ± 0.15	60.36 ± 1.2	38.21 ± 0.01*
C (Mid Dose)	0.24 ± 0.12	0.94 ± 0.11	64.07 ± 0.13	63.44 ± 0.08*
D (High Dose)	0.29 ± 0.30*	0.99 ± 0.01*	70.03 ± 0.04*	99.67 ± 0.25*

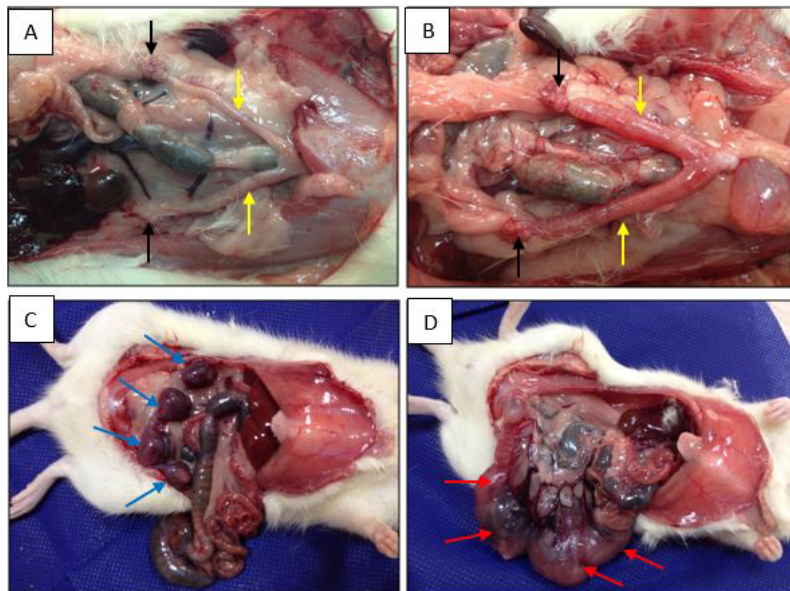
Values are given as mean ± SEM for each group; \* indicates a significant difference ( $p < 0.05$ ) in treated groups compared to Group A; Statistical level of significance was determined by one-way ANOVA

The histopathological examination showed that non-treated immature virgin rats had follicles at intermediate stage of development (Figure 2A). However, the ovaries of rats treated with 300 mg/kg *Morus alba* fruit extract had both fully matured and secondary Graafian follicles. The matured follicles contained fluid filled cavities (Figure 2B). The Graafian follicles was highest in number in rats treated with 900 mg/kg extract suggesting the effect directly dose-dependent (Figures 2C and 2D).

The ovaries and uterine horns of non-treated virgin rats were normal in size and gross morphology (Figure 3A). Pre-mated rats sacrificed on day 8 of treatment showed enlarged ovaries with clear, brilliant, good blood supply, healthy uterus and uterine horns (Figure 3B). After mating on Day 15 of treatment, pregnant rats showed multiple embryos (Figure 3C). By Day 21 of treatment with *Morus alba* fruit extract the embryos were still healthy and well-nourished (Figure 3D).



**Figure 2** The effect of treatment with *Morus alba* on oocyte maturation and superovulation in Sprague-Dawley Rats. A) Control negative group without treatment. Black arrow: follicle at intermediate stage of development. B) 300 mg/kg *Morus alba* treatment group. Black arrow: Mature follicle. Yellow arrow: follicle at intermediate stage of development. C) 600 mg/kg *Morus alba* treatment group. Blue arrow: Multiple Graafian follicles. D) 900 mg/kg *Morus alba* treatment group. Red arrows: Multiple follicles at various stages of development



**Figure 3** Viscera of Sprague-Dawley rats treated with 900 mg/kg *Morus alba*. A) Normal control negative group without treatment showing normal-sized ovaries and oviducts. B) Virgin rat treated for 1 week showing enlarged ovaries and good blood supply in both ovaries. Black arrows: Ovaries. Yellow arrows: Oviducts. C) Pregnant rats treated for 2 weeks. Blue arrows: multiple embryos. D) Pregnant rats treated for 3 weeks. Red arrows: multiple developed embryos

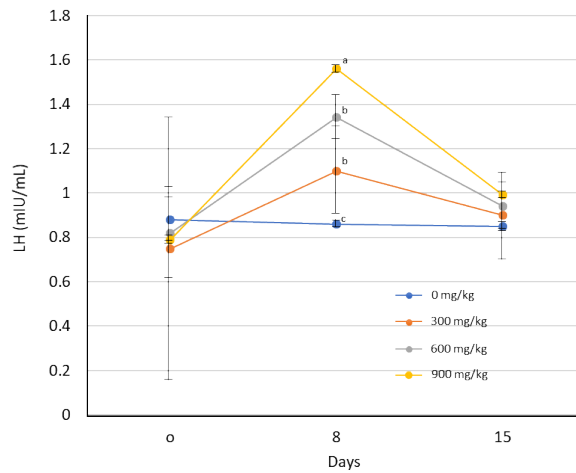


Figure 4 Plasma luteinizing hormone level in rats treated with *Morus alba* extract. Each point represents mean with standard deviations. For each time period, means with different letters are significantly different at  $p < 0.05$

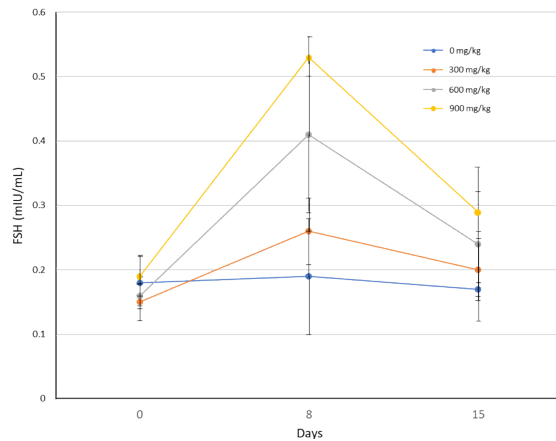


Figure 5 Plasma follicle-stimulating hormone level in rats treated with *Morus alba* extract. Each point represents mean with standard deviations. For each time period, means with different letters are significantly different at  $p < 0.05$

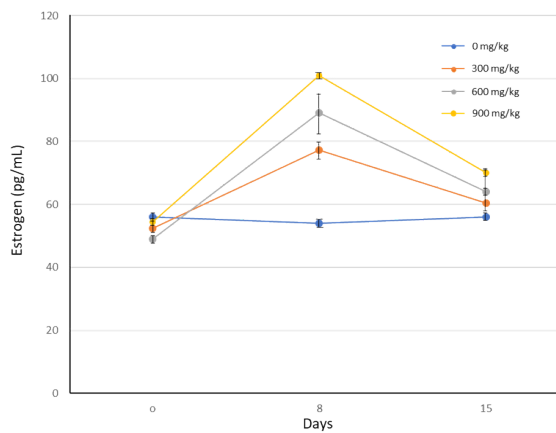
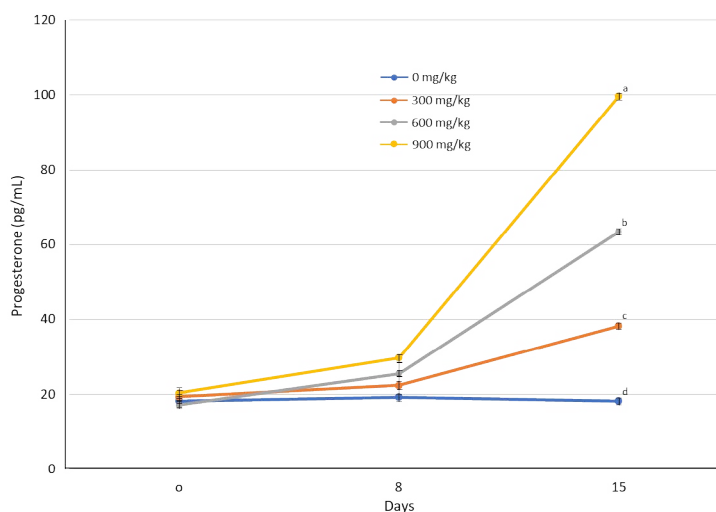


Figure 6 Plasma estrogen level in rats treated with *Morus alba* extract. Each point represents mean with standard deviations. For each time period, means with different letters are significantly different at  $p < 0.05$



**Figure 7 Plasma progesterone level in rats treated with *Morus alba* extract. Each point represents mean with standard deviations. For each time period, means with different letters are significantly different at  $p < 0.05$**

## DISCUSSION

The first choice of treatment in the management of infertility in normally estrogenized, an ovulatory woman is clomiphene citrate (CC). The drug improves reproductive hormonal milieu by competing with estrogen at the hypothalamic level. Clomiphene citrate causes an increase in gonadotropin-releasing hormone (GnRH) from the hypothalamus resulting in increased secretion of FSH and LH from the anterior pituitary gland [54].

Among the Kurdish, fresh and dried mulberry fruit are used as a means to balance sex hormones, treat infertility, and as support for embryo health. Although the fruit is purported to be of health benefit, its effectiveness has not been verified through controlled investigations. Currently, it is not known as to the effect of the plant extract on the female reproductive hormones. In this study, the rat was used as a model to determine the effectiveness of the *M. alba* fruit extract in the maintenance of pregnancy. Our study showed that consumption of the mulberry fruit extract did not cause any ill-effects on pregnant rats.

The pregnant rat is an excellent model for the investigation on the female reproductive cycle by virtue that the rat has a short estrous cycle lasting approximately four days. The proestrus and estrus phases of the estrous cycle are characterized by increases in plasma FSH and LH levels that are associated with folliculogenesis and ovulation. The FSH and LH stimulate theca cells during folliculogenesis to stimulate production and release of estradiol [55]. Steroidogenesis in preovulatory follicles occurs via LH receptors on the theca cells and FSH receptors, possibly also involving LH receptors, on the granulosa cells. This shows that FSH and LH play important roles in follicle development. In the present study, treatment with *Morus alba* fruit extract had increased production of FSH and LH after 8 days. The level of these hormones decreased again after 15 days of treatment.

FSH stimulates production of estrogens by the granulosa cells of the ovarian follicles and corpus luteum. The increase in production of ovarian FSH in rats as the result of treatment with *Morus alba* fruit extract had enhanced the secretion of estrogen hormone from the granulosa cells. However, the effect of FSH on significant progesterone production as the result of *Morus alba* fruit extract treatment occurs later than the effect of estrogen production; most probably approximately one week later. It has also been suggested that increased FSH production stimulates follicular growth, and this can occur without the contribution of LH [56].

Histopathologically, *Morus alba* fruit extract treatment did not affect the size or morphology of Graafian follicles. However, the *Morus alba* fruit extract had increased the number of fully matured follicles and this effect is dose-dependent, that is, a greater number of follicles with treatment with higher doses of extract. This phenomenon is expected to result in superovulation and multiple pregnancies. This result indicates that both increasing levels of FSH and LH hormones played a critical role in both superovulation and multiple pregnancies. The increase in follicle number as the result of treatment with extract was accompanied by enlargement of the ovary, increase in blood supply, activation and

engorged uterus, which are conditions conducive for multiple pregnancy. The maintenance of the superovulated ovary is supported by increase in estrogen and progesterone production.

### CONCLUSION

This study shows that *Morus alba* fruit extract can potentially be used to stimulate ovulation that will facilitate multiple pregnancies while contributing to the maintenance of embryo health. In conclusion, *Morus alba* fruit extract may be used to increase sex hormone production in females, improve ovulation that could lead to superovulation and multiple pregnancies. The extract has potential to be developed as a safe alternative source in the treatment of infertility in females.

### DECLARATIONS

#### Conflict of Interest

Authors declared that there is no conflict of interest. The authors alone are responsible for the content of this manuscript.

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