



Effect of aqueous extract of *Medicago sativa* and *Salvia officinalis* mixture on hormonal, ovarian and uterine parameters in mature female mice

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Abstract

This study was designed to investigate the effect of aqueous extract of mixture of *Medicago sativa* (MS) and *Salvia officinalis* (SO) on the reproductive system of mature female mice. Six groups of mature mice (12 each) received the aqueous extract of the plants mixture which was given orally with water supplement for two different periods (two and four weeks) and with two different doses (100 and 200 mg /kg/ day). Body and reproductive system weights were recorded and vaginal smears were taken. Only animals in estrus phase were anesthetized and blood samples were taken for hormonal analysis of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E₂). The ovaries and uteri were dissected out, weighed and studied histologically. A significant increase in body weight in all treated groups compared to the control groups and an increase in reproductive organs weight especially in the groups received higher doses was detected. Hormonal levels at the estrus phase revealed a significant increase in LH and E₂, while FSH was decreased in all treated groups. The histological preparation showed a remarkable increase in number of ovarian follicles and corpora lutea (CL). There was an increase in endometrial glands diameter especially in groups received the extract for long duration, while the uterine epithelial cells height increased significantly in all treated groups. It is concluded from the present results that the effects obtained may be attributed to the phytoestrogen constituents of both plants on female reproductive system.

Keywords: *Medicago sativa*, *Salvia officinalis*, reproductive system, female mice.

1- Introduction

Some evidence suggests that complementary and alternative medicine has found increased utilization among patients seeking infertility treatment. Infertile patients are vulnerable group that often seek a non- medical solution for their failure to conceive [1].

Medicago sativa (alfalfa) belongs to the family Fabaceae. Its leaves contain isoflavonoids which have shown to have phytoestrogenic properties; consequently alfalfa has been suggested to be useful in the management of menopause and menstrual discomfort [2], premenstrual tension and fibroid [3]. De Leo *et al.*, 1998[4] found that alfalfa is an effective agent in treatment of menopausal symptoms. Alfalfa can show both agonistic and antagonistic estrogenic effect [5]. It balances hormones and promotes pituitary function [6]. It may potentiate activity and increase risk of bleeding because of coumarin content in the plant [7]. Skidmore – Roth, 2004 [8] have cautioned that alfalfa may act as a uterine stimulant, and should not be used during pregnancy and lactation.

Salvia is a large polymorphous genus of the family Lamiaceae, comprising about 900 species [9]. A report from the United Kingdom proves that herbalist there employed *Salvia officinalis* (Sage) to treat symptoms of menopause such as hot flashes [10]. A combination of sage and alfalfa extract was found to be valuable as a therapy for postmenopausal women who suffer from estrogen deprivation such as dizziness, headache, hot flashes, insomnia, and palpitation [4]. Sage is reputed to be one of the richest sources of potent antioxidant [11]. The present study was designed to investigate the effect of aqueous extract of mixture of *Medicago sativa* (MS) and *Salvia officinalis* (SO) on the reproductive system of mature female mice.

2- Materials and Methods

2-1 Animals

Mature female Swiss Balb/ c Albino mice (n = 72), 10 weeks old and 17 – 20 gm body weight (BW), were obtained from the animal house of the High Institute for Infertility Diagnosis and ART, Al-Nahrain University. They were kept in an air – conditioned room (22 – 24 °C) with an automatically controlled photoperiod of 14 hr. light and 10 hr. darkness. Animals were housed in plastic cages measuring (28 x 15 x 14 cm), with a wire grid covers, four mice were kept in each cage containing bedding material of fine wood which was kept dry and changed every other day. Cages were washed regularly with hot water and disinfected with 70 % ethyl alcohol once a week. Mice were fed with the standard balanced pellet diet and given tap water *ad libitum*.

2-2 Plant materials

The dried plants powder (aerial parts) was obtained from Herbalist in Baghdad. The powder consists of a mixture of two plants, *Medicago sativa* and *Salvia officinalis* 1:1. Sample of the plants extract was authenticated by Herbal Medicine Department / Ministry of Health.

2-3 Preparation of the aqueous extract

Twenty five gm. of the dry powder were steeped in 250 ml of boiled water in a closed vessel for 20 min. The crude extracts were filtered by piece of gauze and the filtrates were added to the water supplement of the mice after calculating the daily water intake (10 ml/day/mouse).

2-4 Calculation of the dose

The dose used for human by (herbalist) was estimated to be about 100 - 200 mg/ kg/ day (1 to 2 tea spoonful three times daily). According to that, the same dose was calculated for mice.

2-5 Methods

Animals were divided randomly into six groups, 12 mice each and treated as follows:

G₁ & G₂ received 100 & 200 mg/kg/day respectively for two weeks and G₃ received tap water only (control).

G₄ & G₅ received 100 & 200 mg/kg/day respectively for four weeks and G₆ received tap water only (control).

By the end of the two periods, the following parameters were evaluated:

2-6 Body weight

For each mouse the BW was recorded at the beginning and end of the experimental work, using a mechanical balance (Tefesa, Germany).

2-7 Vaginal smear

At the end of treatment periods, phases of estrous cycle of mice were checked daily and recorded using vaginal smears. The materials for vaginal cytology were obtained by inserting a sterilized loop gently into the vagina and allow it to touch vaginal wall by rolling the loop smoothly. Smear then spread on a clean slide and fixed on a flame before staining with 1% aqueous methylene blue (Merk, Germany) for 3 – 5 min., stained smears were then washed with tap water, air dried and examined under a light microscope (Olympus, Japan) to determine estrous cycle phases [12].

2-8 Collection of blood sample

Only mice in the estrous phase were anaesthetized by means of intra-peritoneal injection of pentobarbital sodium (Nembutal[®], Sanofi, France) at a dose of 60 mg/kg BW. Blood was collected through cardiac puncture using a 22 gauge needle, left for 30 minutes, then centrifuged (Griffin Laboratory Centrifuge, Britain) at 3000 rpm for 10 minutes for preparation of sera which were stored at (- 20°C) until use for hormonal determination.

2-9 Histological preparation

Incision in the abdominal wall of anesthetized 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 (VMF 4X, Japan) using fine surgical scissor and weighed (after drying from normal saline using filter paper) by using an electrical balance (Sartorius, Switzerland), both ovaries were dissected out. Then the uterus was dissected out slightly below the tubo-uterine junction from one end and above the cervix from other end and immediately fixed with ovaries in 10 % formalin

solution (BDH, Chem. Ltd, England) for 24 hr., before transferred to 70 % ethyl alcohol (Prolabo, CE) and kept their until use for preparations of histological section .

The fixed tissues were then processed for routine paraffin – wax embedding. This procedure includes: dehydration, clearing, infiltration and embedding as described by Junquera *et al.*, 2005b [13]. Serial 5 μ m cross sections were made through the ovaries and uterine horns using glass knives on a rotary microtome (LEICA-RM- 2145, Germany). Serial sections were stained with hematoxylin and eosin and examined microscopically. Various ovarian and uterine measurements were done using eye piece micrometer (WF 10X / 18, Japan). The following parameters were determined:

Ovaries:

1. Number of primordial and primary follicles,
2. Number of growing follicles,
3. Number of corpora lutea.

Uterus

1. Endometrial cells lining height.
2. Endometrial glands diameter.

2-10 Hormonal assay

The following hormones were assayed using prepared kits of Radio-immunoassay technique (RIA).

LH (mIU / ml). Using h LH [125 I] IRMA kit, Budapest.

FSH (mIU / ml). Using h FSH [125 I] IRMA kit, Budapest.

3.17- β estradiol (pg/ml). Using Immunotech /A Beckman Coulter Company (RIA), France.

- 1.
- 2.

2-11 Microscopic photography

By using a light microscope (Olympus, Japan), photographs of the histological sections were taken, using konca film, Yashika camera, Japan.

2-12 Statistical analysis

Data from treated and control groups were expressed as mean \pm standard error (M \pm S.E.), and analyzed using the students' t – test and ANOVA table. P value <0.05 was considered significant and less than 0.01 considered highly significant [14].

3- Results and Discussion

*3-1 Effect of daily administration of 100 & 200mg /kg/day of aqueous extract of mixture of *Medicago sativa* and *Salvia officinalis* to mature female mice:*

3-1-1 Weight changes (Table 1):

A. Body weight: The results showed that there was no significant change in body weight of mice treated with 100 mg/Kg/day of plants extract (G₁) after 2 weeks compared to control group (G₃) whereas, a significant (P<0.05) increase was found in those treated with 200 mg/kg/day of plants extract (G₂) compared to that of the control group.

After four weeks of treatment, a significant (P<0.05) increase in all treated animals (G₄ and G₅) was obtained in comparison with the control group G₆.

A significant (P<0.05) increase in BW of mice received 100 mg/ kg/day for four weeks (G₄) was found compared with mice received the same dose for two weeks (G₁). A highly significant (P<0.01) increase was observed in those treated with 200 mg/kg/day for four weeks (G₅) compared to mice received the same dose for two weeks (G₂).

B. Reproductive system weight: After two weeks, a highly significant (P<0.01) increase appeared in the weight of ovaries, oviducts and uteri in both G₁ and G₂ in comparison with the control group G₃, on the other hand, after the second duration of the experiment, the reproductive system weight revealed a significant (P<0.05) increase in G₄ and a highly significant (P<0.01) increase in G₅ in comparison with that of the control group G₆.

G₄ showed a significant (P<0.05) increase compared to G₁, whereas a highly significant (P<0.01) increase was obtained in G₅ in comparison with G₂.

Table 1: Body weight and reproductive system weight associated with daily administration of mixture of *Medicago sativa* and *Salvia officinalis* mixture extract to mature female mice.

Parameter	2 weeks treatment			4 weeks treatment		
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
B.W. (g)	21.58 ±0.51	22.51 ^a ±0.22	21.09 ±0.32	23.47 ^{a,c} ±0.47	25.51 ^{a,d} ±0.37	22.34 ±0.22
Rep. Sys. wt.(mg)	11.18 ^b ± 0.40	11.71 ^b ± 0.51	8.31 ± 0.34	15.1 ^{a,c} ± 0.91	17.61 ^{b,d} ±0.74	11.04 ± 0.42

Values are mean ± SE. n = 12

^aP<0.05 in comparison with the control group.

^bP<0.01 in comparison with the control group.

^cP <0.05 in comparison with G₁.

^dP<0.01 in comparison with G₂.

The increase in body and reproductive organs weight is due to the fact that both plants are known to be a good appetite stimulant [15]; promote weight gain and treat digestive disorders [9]. Both herbs have hypoglycemic activity [16], hypoglycemia is among stimulators of growth hormone (GH) secretion from the anterior pituitary gland [17]. The GH is necessary to stimulate skeletal and muscle growth, regulate lipolysis, and promote cellular uptake of amino acids. Alfalfa contains amino acids especially L- arginine [16], which is an essential amino acids and well known potent GH secretagogue in man.

The plants used in this study contain steroid and isoflavonoid "coumestrol, daidzein and genistein" [15] which are considered as phytoestrogens. The metabolites of phytoestrogens exert an estrogenic effect on central nervous system which induces estrus and stimulates cell division and growth of the genital tract of female animals [18]. This may explain the increase in body weight and reproductive organs weight, in addition to direct effect of E₂. The expression of IGF-I is up- regulated in the reproductive tissues of animals fed the diet with relatively high phytoestrogen content. The IGF- I is a critical regulator of uterine growth, and a locally produced IGF-I could mediate the effects of E₂ on growth and cellular proliferation [19].

3-1-2 Histological changes:

3-1-2-1 Ovaries (Plates 1, 2, 3):

Changes in ovarian follicles numbers (Table 2): After two weeks, the number of primordial and primary follicles in mice treated with 100 and 200 mg/kg/day (G₁ and G₂) was statistically not significant compared to control group G₃. A statistically significant (P<0.05) increase in the number of growing follicles and in number of CL in both G₁ and G₂ was observed in comparison with that of G₃. After four weeks, there is a statistically significant (P<0.05) increase in the number of primordial, primary, growing follicles and CL in both G₄ and G₅ in comparison to control group G₆. The present study showed a statistically significant (P < 0.05) increase in number of primordial, primary and other growing follicles in both G₄ and G₅ compared with G₁ and G₂ respectively. There is a non-significant difference in the number of CL between all treated groups.

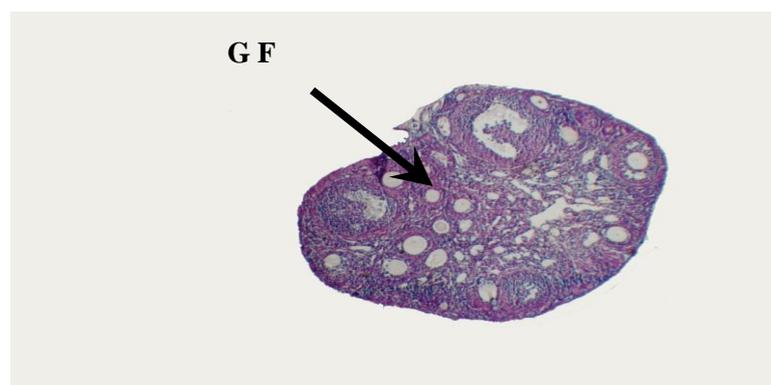


Plate 1: Ovarian section of a mature female mouse (control group), (H & E x 40)

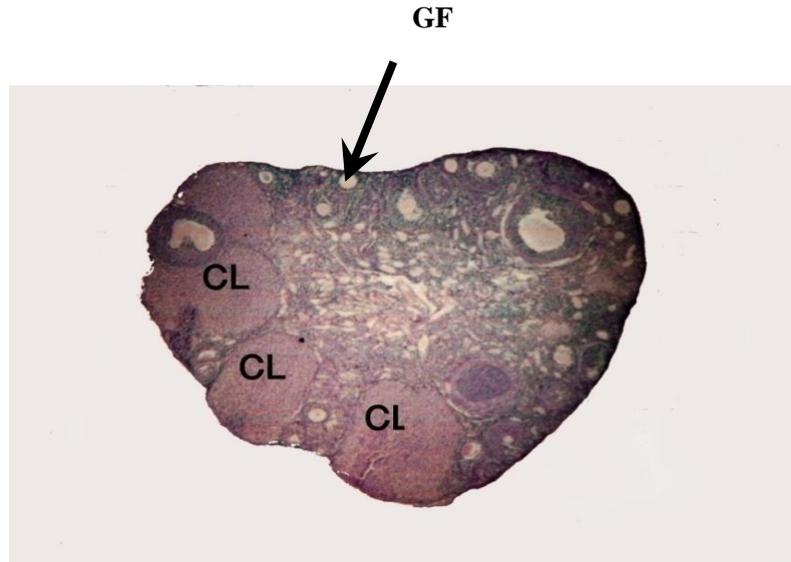


Plate 2: Ovarian section of a mature female mouse treated with 200 mg / kg / day for two weeks (H & E, x 40). Note the increase in number of follicles and corpora lutea.

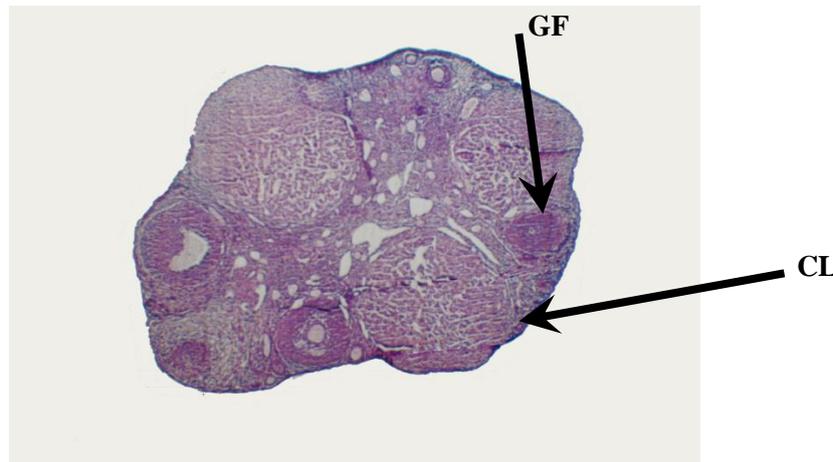


Plate 3: Ovarian section of a mature female mouse treated with 100 mg/ kg /day for four weeks (H &E, x 40). Note the increase in number of follicles and corpora lutea.

Table 2: Changes in the numbers of ovarian follicles associated with daily administration of *Medicago sativa* and *Salvia officinalis* mixture extract to mature female mice.

Follicle number	2 weeks treatment			4 weeks treatment		
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
Primordial & Primary Follicles.	13.28 ±0.61	13.425 ±0.99	13.32 ±0.68	14.28 ^{a,b} ±0.81	14.57 ^{a,c} ±0.57	13.29 ±0.47
Graf. Follicles.	21.58 ^a ±1.13	22.75 ^a ±0.91	19.71 ±0.42	23.27 ^{a,b} ±0.83	23.81 ^{a,c} ±0.92	20.14 ±0.51
Corp. Lut.	2.13 ^a ±0.22	2.38 ^a ±0.43	1.71 ±0.28	2.25 ^a ±0.40	2.38 ^a ±0.36	1.86 ±0.26

Values are mean ± SE. n = 12

^aP < 0.05 in comparison with the control groups.

^bP < 0.05 in comparison with G₁.

^cP < 0.05 in comparison with G₂.

The structural changes in the ovaries, including the increase in number of follicles and increase in number of CL can be attributed to the effects of estrogen like substances present in both plants, in addition to their effects in increasing the level of E₂ and LH.

The constituents of the two plants extract may promote the secretion of FSH at the follicular phase and the LH at preovulatory phase. The FSH is the fundamental component that controls ovarian follicle growth and maturation of early development stages. Furthermore, FSH stimulates the granulosa cell (GC) aromatase system that catalyzes the conversion of androgens into estrogens [20]. The LH receptors are expressed on GC membranes of the preovulatory follicle, and the survival of the latter in the late follicular phase despite declining FSH levels is sustained by increasing levels of LH [21]. Most of FSH actions on GCs, including the stimulation of the aromatase system, can be exerted by LH once its receptors are expressed [22]. The LH is also critically involved in the physiologic events that lead to the development of a competent preovulatory dominant follicle [23]. The expression of LH receptors in mature GCs is a fundamental feature of LH physiology as this is the likely mechanism that allows dominant follicle selection in the mid and late follicular phase of the normal spontaneous MC, at a time when serum FSH concentrations decline and LH concentrations increase [24]. Consequently, the elevation of E₂ and LH in this study was accelerating the growth of the ovary and increases the number of ovarian follicles and CL, despite the decrease in FSH in estrous phase of the estrous cycle. The hormonal levels will be accelerated after two weeks of treatment and continue for the same level after four weeks of treatment.

The data of the present study showed that the number of CL increased following two and four weeks of treatment with plants extract. This finding can be explained by the increase in the production of FSH at the follicular phase leading to increase in the number of CL and in turn the E₂ production was elevated. Estrogen production by the CL is largely a function of the theca – lutein or the small cells, which also produce androgens [25]. On the other hand the plants may trigger the effect of GH on E₂ production. The GH stimulates the CL to increase progesterone production. These effects can be mediated by IGF-I Rebar *et al* [26], which appears to play an important role in stimulating follicular growth and maturation as well as in augmenting steroid production [27].

3-1-2-2 Uteri (Table 3, Plates: 4, 5, 6):

During the first period of the experiment, the epithelial cells lining height and endometrial glands diameters elevated significantly (P<0.05) in G₁ and G₂ compared to that of control group G₃. The second period of the experiment revealed a significant (P<0.05) increase in uterine epithelial cells lining height and a highly significant (P< 0.01) increase in both G₄ and G₅ compared to G₆. A highly significant (P<0.01) increase in epithelial cells lining height was found in G₄ and G₅ compared to G₁ and G₂, whereas G₄ and G₅ showed a significant (P<0.05) increase in endometrial glands diameters compared to G₁ and G₂.

Table 3: Structural changes in the uteri associated with daily administration of *Medicago sativa* and *Salvia officinalis* mixture extract to mature female mice.

Parameter	2 weeks treatment			4 weeks treatment		
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
Epi. Cells Lin. Height(µm)	10.5 ^a ±0.31	10.4 ^a ±0.40	6.4 ±0.34	12.64 ^{a,c} ±0.30	12.45 ^{a,d} ±0.31	8.9 ±0.60
Endom. Gland Diam. (µm)	375.7 ^a ±28.22	366.2 ^a ±12.60	293.11 ±15.40	412.2 ^{b,e} ±15.10	493.73 ^{b,f} ±24.60	319.44 ±8.72

Values are mean ± SE n = 12

^aP<0.05 in comparison with the control groups.

^bP<0.01 in comparison with the control group.

^cP<0.01 in comparison with G₁.,

^dP<0.01 in comparison with G₂.,

^eP<0.05 in comparison with G₁.,

^fP<0.05 in comparison with G₂.,

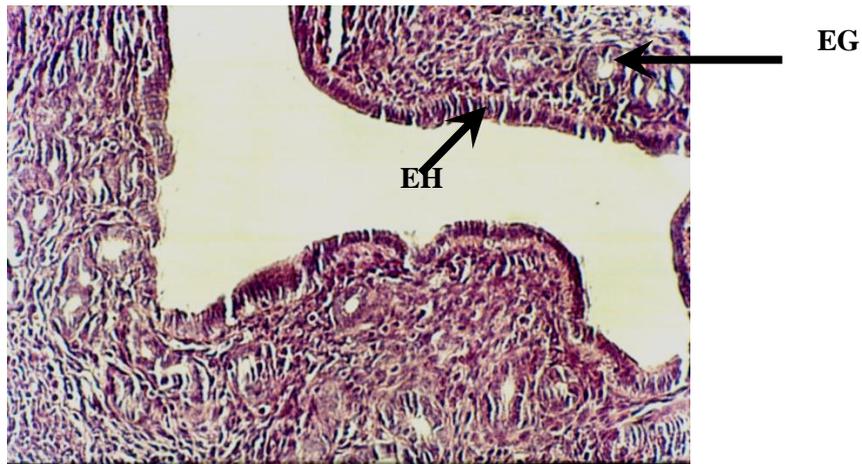
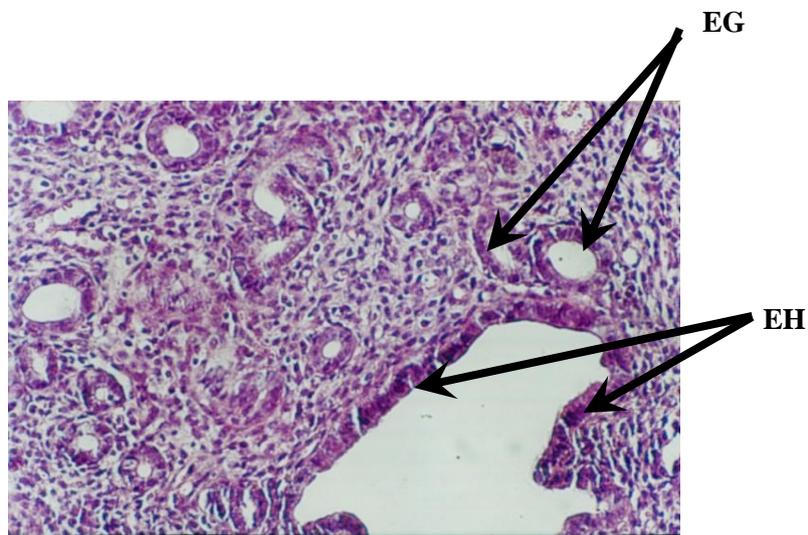
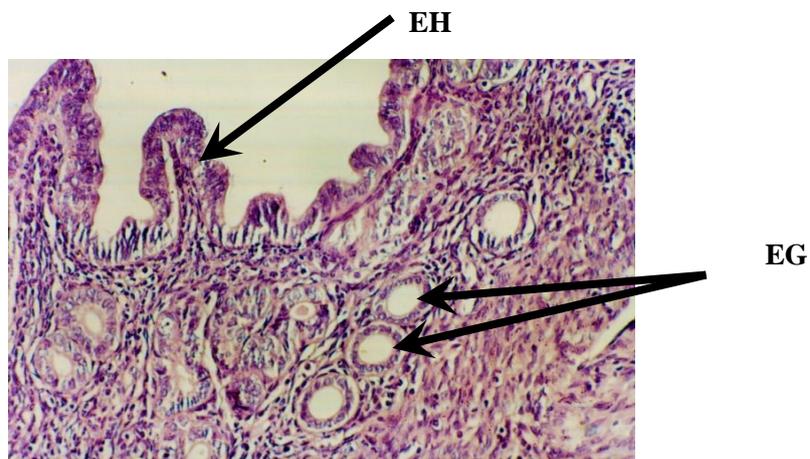


Plate 4: Longitudinal section in uterine horn of mature female mouse (control group), showing the EH height and EG diameter (H & E, 200 X).



Picture 5: Longitudinal section in uterine horn of mature female mouse treated with 200 mg / Kg /day for two weeks, showing increase in EH height and EG diameter (H & E, X 200).



Picture 6: Longitudinal section in uterine horn of mature female mouse treated with 100 mg/ Kg /day for four weeks, showing increase in EH height and EG diameter (H & E, X 200).

The increase in height of uterine epithelial cells lining and diameter of endometrial glands seems to be due to increase in E₂ production. Proliferation and differentiation of the endometrium are stimulated by estrogen secreted by the developing follicles [25]. During estrus, increased estrogenic secretion stimulates the endometrial cells to increase in height and show intense mitotic activity and the glandular elements of the endometrium secrete fluid mucus which flushes the tract [28]. Part of the effect of estrogens is to induce the synthesis of growth factors such as IGFs also called somatomedins, TGFs, epidermal growth factor (EGF). These autocrine and paracrine mediators are necessary for maturation and growth of the endometrium [25]. Isoflavones increase the endometrial cell proliferation and have estrogenic potency *in vitro* [29]. Isoflavones alone behave as weak estrogenic factors by increasing proliferation and alkaline phosphatase activity in endometrial and glandular cells [30]. Thus the result of plants mixture extract in uteri of the treated animals may be correlated with increase in the E₂ because the uteri are extremely sensitive to exogenous estrogen.

3-2 Hormonal changes (Table 4)

After two weeks, Serum level of FSH showed a significant (P<0.05) decrease in both G₁ and G₂ compared to that of G₃, while serum level of LH and E₂ showed a significant (P<0.05) increase in G₁ and G₂ in comparison to control group G₃. After four weeks, Serum level of FSH showed a significant (P<0.05) decrease in both G₄ and G₅ compared to G₆. Serum levels of LH showed non significant difference in G₄ and a significant (P<0.05) increase in G₅ in comparison to G₆. The serum level of E₂ showed a significant (P<0.05) increase in both G₄ and G₅ compared to G₆. Serum levels of FSH and LH showed non significant difference, while serum level of E₂ showed a significant (P<0.05) increase in both G₄ and G₅ compared to G₁ and G₂

Table 4: Hormonal changes associated with daily administration of *Medicago sativa* and *Salvia officinalis* mixture extract to mature female mice.

hormones	2 weeks treatment			4 weeks treatment		
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
FSH mIu/ml	3.24 ^a ±0.07	3.12 ^a ±0.12	3.61 ±0.14	3.27 ^a ±0.14	3.18 ^a ±0.23	3.69 ±0.18
LH mIu/ml	2.624 ^a ±0.24	2.75 ^a ±0.97	2.4 ±0.26	2.67 ±0.16	2.8 ^a ±0.67	2.535 ±0.19
E₂ pg/ml	9.07 ^a ±1.30	11.223 ^a ±0.83	7.585 ± 0.26	10.604 ^{a,b} ±1.92	13.187 ^{a,c} ±1.96	8.81 ±0.33

Values are mean ± SE. n = 7

^aP<0.05 in comparison with the control group.

^bP<0.05 in comparison with G₁.

^cP<0.05 in comparison with G₂.

The results of hormone profile in this study showed a statistically significant increase in serum E₂ level following two weeks of treatment and did not change through the other period. This observation might be related to increase in estrogens synthesis due to effects of plants extract on increasing the number of growing follicles and CL. Also the results showed an increase in the level of LH which may be resulted from increased E₂ synthesis. Estrogen is produced predominantly in the ovarian follicle by the GCs aromatization of thecal androgens [31]. The LH stimulates the biosynthesis of androgens from cholesterol by the theca cells; the androgens then diffuse across the basement membrane of the follicular wall and portion of these androgens reach the antrum.

The increase in E₂ may be attributed to the fact that MS and SO contain steroid, isoflavones and isoflavonoids [15]. This phytoestrogen which can be active at low or high concentration affects different target tissues and acts as agonist or antagonist to E₂. The phytoestrogens can influence basic cell biology to their effect on cellular enzymes including cytochrome P – 450 enzymes, as well as their ability to influence growth factors such as cytokines and to regulate genes and exert antioxidant actions even at low level [32]. The cytochrome P- 450 enzyme known as the " side – chain – cleavage " enzyme catalyzes the conversion of cholesterol to pregnenolone. This reaction is the rate – limiting step in estrogen production. Ovarian cells then convert pregnenolone to progestins and estrogens [25].

The increase in serum LH level after oral administration of the plants extract can be attributed to high plasma concentrations of estrogen for 1 to 2 days, as occur during the estrogen peak of the late follicular phase, which acts upon the pituitary to enhance the sensitivity of LH releasing mechanism to GnRH. The high estrogen may also stimulate further increase in the secretion of GnRH by the hypothalamus, although this remains controversial [33]. Moreover preovulatory secretion of progesterone, although limited, may exert a positive feedback on the estrogen – primed pituitary to augment LH release [33].

Although the study showed a decrease in serum level of FSH in estrous phase of the estrous cycle, this level did not affect ovulatory process. This can be explained as in the late follicular phase, FSH level decrease in relation to the rise in E₂ levels which mean that there is no need to increase FSH level in the late follicular or ovulatory phase which in turn did not affect the number of CL. At the same time, it has been noticed that the dominant follicle survival ($\geq 10\text{mm}$) is mainly LH – dependent [34]. The negative feedback of E₂ is predominantly exerted on FSH secretion at the pituitary level [35]. Just before ovulation, after the GCs acquire LH receptors, LH also stimulate the production of inhibin by these cells. The inhibins inhibit FSH production by the gonadotrophs [25]. After ovulation, the CL secretes E₂, progesterone and inhibin A under the control of LH [36]. The estrogens exert negative feedback at both low and high concentrations, whereas progestins are effective only at high concentration [25]. These dramatic events lead to decrease in FSH level.

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