THE MAMMOGENIC AND LACTOGENIC EFFECTS OF ANSISE OIL IN RATS

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ABSTRACT

This study include two experiments: the first experiment was designed to determine the effects of aniseed oil administration during the last trimester of gestation on mammary gland development and milk synthesis during lactation period. Twenty pregnant rats, at the 15th day of pregnancy, were randomly divided into two equal groups as: control group (10 animals) received tap water orally and treated (10 animals) group received aniseed oil orally (0.043 gm kg^{-1} B.w) during the last week of gestation, at parturition, five animals from each group were scarified and their mammary gland weight (MGwW%) was recorded. The remaining animals (5 animals group^{-1}) were allowed to suckle their litters for the first eleventh days of lactation period. At eleventh day of lactation the litters weight gains (LWGs%) and litters stomach weight (LSwW%) was calculated. The result revealed significantly increased of the three parameters (MGwW%; LWGs%; LSwW%) in treated group as compared to control. The second experiment was designed to investigate the mechanism of action of aniseed oil. Fifteen female rats, at five weeks of age, were randomly divided into three equal groups, treated for two weeks as follow: group I ovariectomized (OA) rats received aniseed oil (0.043 gm kg^{-1} B.w.); group II intact (IA) rats received aniseed oil (0.043 gm kg^{-1} B.w.); group III sham operated (S) control rats received tap water. Four parameters; mammary gland weight%, uterine weight%, follicular stimulating hormone and lutenizing hormone (MGwW%, UwW%, FSH and LH levels) were employed. This investigation showed that MGwW% and UwW% were highest in IA, while the OA rats showed the highest levels of FSH and LH. In conclusion, aniseed oil it induces mammogenesis, lactogenesis and galactopoieses, most probably by its indirect action on the mammary glands.

Key wards: mammary glands, ovaries, uterus, gonadotropin hormones.

INTRODUCTION

A galactagogue is an agent that promotes secretion and flow of milk (Mahmood *et al.*, 2012). In several parts of the world, particularly in the developing countries with a heritage of folklore, herbal medicine has been practiced by the practitioners of traditional medicine, for enhancing the milk secretion in lactating mothers (Zapantis *et al.*, 2012).

Many plants and herbs are known to be galactagogues, among these, is the plant aniseed, which has been employed as a folk remedy to increase milk production since ancient centuries (Wang *et al.*, 2014). The main constituent of essential oils of anise is anethole, which makes up to 80-90% of anise volatile (Hamed and Abdel Gawad, 1990 ; Jankovsky *et al.*, 1993). Structurally, it is simillar to catecholamines and possesses similar activities, such as bronchodilation and weight loss (Vasudevan *et al.*, 2000 ; Ostad *et al.*, 2001). Anethole has been considered to be the active estrogenic agent and has two isomers, transisomer which is the active form, while the toxic form is cis isomer (Claudia, 1993 ; Sema *et al*., 1995). Anise has been used for benefit of milk production and fat content, promote menstruation, facilitate birth, alleviate the symptoms of male climacteric (Schulz and Hansel, 1996). Researchers suggest that the actual pharmacologically active agents are polymers of anethole, such as dianethole and photoanethole, which may influence secretion of prolactin, anise seeds are often part of galactophoric and chalagogic preparation (Wang *et al.*, 2014). The galactogogual role of crude extract of anise seeds was proved by Al-Saadi (1997). Who concluded that aniseed extract administration (0.152 gm kg^{-1} B.wt during the last trimester of gestation in rats) has a positive effect on rat's mammary gland development and performance. On the other hand, Al-Jubori (1999) found that anise extract stimulates mammary gland growth when given at prepuberal time. This effect was extended both at postpubertal time and during lactation. However, the effects of anise oil on mammogenesis and mechanism of action remain in few unproved speculations. Therefore, this effort was made to throw light on the effects of anise oil on:

1. Some physiological parameters including; a. MGwW% at first day of lactation. b. LWGs% during the first eleven days of lactation. c. LSwW% at eleventh day of lactation.

2. Mechanism of action: This study also highlighted some aspects on pathways of the effects of aniseed oil under presence and absence of ovaries. The following parameters were employed:

1. MGwW % and UwW %. 2. The plasma concentrations of Gonadotropins FSH and LH.

MATERIALS AND METHODS

Animals' colony: A total number of 70 Norway albino mature female rats and 14 male were used in this investigation. They were fed ordinary pellet diet. The animals were kept at a temperature between 23-28 °C. The animals were housed as one male for each five females in wire meshed stainless steel cages (56, 40 and 17) for mating, after that the pregnant animals kept in cages (33, 15 and 13) individually until parturition and others allowed to suckle their litters for the first eleventh days of lactation, at the Iraqi Center for Cancer and Medical Genetic Researches. The light and dark cycle was (12:12hr). Animal had free access to food and water. Care was taken to avoid unnecessary stress as noise and cage crowding.

Preparation of aniseed oil: The seeds of anise were purchased from the local market and authorized in the "Iraqi National Herbarium". The seeds of herb were cleaned and ground in a grinder and pressed by mechanical hydraulic press (H. Fisher and Co. norf, Germany) without heating under pressure of 400 Bar. The yield was 80 ml of aniseed oil from 5 kg of aniseeds.

Experiment one: The role of aniseed oil on mammary glands performance.

Twenty, Norway albino female rats, at last trimester of gestation, were used in this experiment their average weight ranged between 220-270 gm. The aniseed oil was given orogastrically to the experimental rats and in a dose of (0.043 gm kg^{-1} B.w) daily for one week (last week of gestation), at parturition five animals were killed to obtain mammary glands samples while the remain five animals were allowed to suckle their litters till eleventh day of lactation, the litters weight recorded at first and eleventh day of lactation then killed to obtain their stomach (milk) weight; control rats received the same volume of tap water as a placebo, under similar condition.

1. **Mammary gland weight (MGwW%) ratio:** At day of parturition, five animals were randomly isolated from each group, and their B.wt and MGw were recorded. MGw were normalized per 100g B.wt as follows:

$$(\text{MGwW\%}) = \frac{\text{MGw}}{\text{B.wt}} \times 100$$

2. **Litter weight gains (LWGs%) ratio:** The remaining animals, which were allowed to suckle their litters for the first eleven days of lactation period. During this period, daily LWGs% were calculated as follows:

$$\text{LWGs\%} = \frac{\text{Final L B.wt- Initial L B.wt}}{\text{Initial L B.wt}} \times 100$$

3. **Litter's stomach content weight (LSwW%) ratio:** The amount of milk obtained (stomach contents) used as lactation performance (Weber, 1998). At tenth day of lactation, each litter was isolated from its mother for overnight, at the end of isolation, mother and young were reunited and the litter permitted to nurse for one hour. After the nursing period, the litter was weighted, then sacrificed by ether, and stomach contents weighted. The weight of milk (stomach) obtained was then expressed in percentage as follows:

$$\text{LSwW\%} = \frac{\text{LSW}}{\text{LB.wt}} \times 100$$

Experiment two: Effect of aniseed oil on mammary gland and uterine development in intact and ovariectomized rats

This study was designed to localize the site of action of aniseed oil, either directly on mammary gland or indirectly through pituitary gland, under presence and absence of ovaries. Fifteen rats were divided equally into three groups:

1. Group I ovariectomized (OA): Rats of this group were orally administrated aniseed oil (0.043 gm kg^{-1}) daily for two weeks.
2. Group II intact (IA): Rats of this group were orally administrated aniseed oil (0.043 gm kg^{-1} B.wt) daily for two weeks.
3. Group III sham operated: (S) served as control under similar condition, and received tap water.

Ovariectomy: A median abdominal incision was performed for each deeply ether anaesthetized rat at five weeks of age. Following both cornu of the uterus, the ovaries could be identified at the tip of each. Both ovaries were excised and then the abdomen was sutured. Animals were kept in a clean cage with no medication; i.e. ovareictomized rats received no prophylactic antibiotics orally. Aniseed oil

treatment began at six weeks of age, which extend to eight weeks age; and at the end of treatment. The blood and tissue samples were collected.

Blood sampling: After deep anesthesia by diethyl ether (BDH Chemicals Ltd, England), blood samples (4 ml) were obtained via cardiac puncture from each anaesthetized rat (control and experimental) using disposable syringes washed with heparin (Leo pharmaceutica products, Denmark). Samples were centrifuged (Gallenkanp, England) at 3000 rpm for 15 minutes, and then plasma samples were stored in deep freeze till used for gonadotropins hormonal assay FSH and LH.

Tissues sampling: After longitudinal abdominal opening of animals, the mammary tissue was then carefully dissected from the overlying skin. From each rat, four pieces of mammary glands (right and left thoracic, right and left inguinal) were excised and weighted, also the uterus was isolated and weighted. All tissue samplings were carried out between 9.00 to 12.00 AM. Four parameters (MGwW%, UwW% and the concentration of Gonadotrpins FSH and LH) were employed in this investigation to assess the response to aniseed oil treatment.

1. $\frac{\text{MGw}}{\text{B.wt}} \times 100 = \text{MGwW\%}$
2. $\frac{\text{Uw}}{\text{B.wt}} \times 100 = \text{UwW\%}$
3. Gonadotropins (FSH and LH) hormones assay RIA.

Statistical analysis: Statistical analysis of data was performed on the basis of T-test and one way analysis of variance (ANOVA), depending on the experimental design. Specific group differences were determined using least significant difference (LSD) test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Experiment one: The role of aniseed oil on mammary gland performance

Mammary glands weight%: the results in table 1 explain that aniseed oil treatment lead to a significant increase ($p<0.05$) in mammary glands weight% (4.78±0.16) at parturition as compared to control (3.7±0.14). This may be attributed largely to up-regulation activity of receptors for estrogen and/or progesterone hormones which they are responsible for alveolar and tubule of mammary glands development (Ganong, 1995). Yet, it has been mentioned that crude anise seeds extract increase DNA & RNA concentration in the mammary gland (Al-Saadi,

1997). An increased DNA & RNA concentrations indirectly reflects enhancement in the process of protein synthesis to match the increased demand of milk and secretion in the lactating mothers (Patel *et al.*, 2016).

Litters weight gain% ratio: values of litters weight gain of control group and aniseed oil treated, are illustrated in table 1. Litters weight gain % increased significantly ($p<0.05$) at eleventh day of lactation in treated group (55±1.99) as compared to control (35 ±2.01). The effect of aniseed oil 0.043 gm Kg^{-1} B.wt lead to litter weight gain of treated rats (Table 1). The increased MGwW% means increasing the mass of mammary tissues (DADF, 2015), and as a results increase the site of milk synthesis (mammary cells) and extraction (from blood stream) of main milk constituents such as proteins, lipids, carbohydrates, minerals and water (Tsuda and Sekine, 2000 ; Ollivier, 2002). Thus, the LWGs% had come indirectly from elevation of milk quantity and/or quality, which lead to increase growth rate of newborns (Ismail, 2016).

Litters stomach weight% ratio: the data pertaining to litters stomach weight of control and aniseed oil group are depicted in table 1. Litters stomach weight showed a significant increase ($p<0.05$) at eleventh day of lactation in treated group (2.6±0.42) as compared to control (1.7±0.22). Aniseed oil caused a significant increase in LSwW% at the eleventh day of lactation in treated rats as compared to control rats (Table 1). The weight of stomach content (which reflects the milk obtained from nursing mothers) is used as an index of lactation performance (Galbat *et al.*, 2014). After isolation and reuniting the mother and young, during this interval any milk present in stomachs of young was digested and the mammary glands of the mother rat became turgid with milk. Therefore, the excessive developments of mammary glands lead to increase area where milk synthesis and secretion take place.

Table 1. Effect of daily oral dose of aniseed oil (0.043 gm kg^{-1} B.wt) during the last week of gestation on mammary glands weight at parturition, litter weight gain and litters stomach weight at eleventh day of lactation in rats

Parameters / Groups	Mammary glands weight % at parturition	Litters weight gain% at eleventh day of lactation	Litters stomach weight% at eleventh day of lactation
Control	3.7±0.14 B	35 ±2.01 B	1.7±0.22 B
Treated	4.78±0.16 A	55±1.99 A	2.6±0.42 A

Values represent mean ± SE. (n=5 mother $group^{-1}$, 7 pups $mother^{-1}$) Values are expressed as mean ± SE. n=5/group. Capital letters denote between group differences, $p<0.05$ vs. control.

Experiment two: Effect of aniseed oil on mammary gland and uterine development in ovariectomized and intact rats

Mammary gland weight (MGwW%): The effect of (0.043 gm kg^{-1} B.w) aniseed oil administration to OA and IA female rats, for two weeks on MGwW% are shown in table 2. In particular, there was a marked increase ($p<0.05$) in MGwW% in IA group as compared to OA and control S. The mean values of MGwW% were 1.082±0.036, 1.348±0.156 and 1.22±0.058 in OA, IA and S groups, respectively. In Europe, the sexual maturity of rats, is achieved at 6.5-7.5 weeks (45-52 days) and female rats have their first estrus cycle at 6-7 weeks i.e. 42-49 days (Komarek, 2000). However, in Iraq sexual maturity of rats occurs one week earlier i.e. 5.5-6.5 weeks (Sakran *et al*., 2000).

Therefore, the age of rats used in this experiment was chosen to be close to puberty. Furthermore, their mammary glands and uteri were not previously sensitized by sex hormones. Ovariectomized rats were chosen to study some aspects of mode of action of aniseed oil. Ovariectomy was performed to remove ovarian influence i.e. getting rid of the main site of sex hormone synthesis and secretion (Ganong, 1995 ; Guyton, 1996). The mammary gland is one of the few organs in mammals that complete their morphological development postnatally (Ball, 1998). The weight of organs basically depends upon the organic and bio-constituents in addition to fluid, besides the numbers of cell which it's contains. Al-Saadi (1997) found that crude seed extract of Pimpinella anisum caused increase in DNA and RNA of the rat's mammary gland. Therefore, the elevation of mammary gland weight in this study may attributed to the elevation of cell number and its contents, because the elevation of DNA concentration mean increase in the number of cell in tissue (DADF, 2015). Since, the DNA is limited and constant per cell, therefore any increase in its concentration in mammary gland refers to the increase in its cell numbers. In addition the elevation of DNA concentration means increase in cellular division which led to increase in the number of cell and as a result increase in mammary weight.

Uterine weight (UwW%): Table 2 also demonstrates UwW% of OA and IA aniseed oil (0.043 gm kg^{-1} B.w) treated rats. There was a significant increase ($p<0.05$) in UwW% of IA 0.313±0.053 as compared to OA 0.185±0.041 and S 0.201±0.024 groups. Table 2 demonstrate that UwW% of IA group is higher than those of OA and S rats. The ovarian hormones estrogen and progesterone play a critical role in increase uterine weight through increase protein synthesis and

mitotic division of myometrium, while the progesterone in addition to the increase growth and activity of uterine gland it increase the blood supply of endometrium, all these changes lead to increase uterine weight (Alsherwany, 2015). Therefore, the elevated uteri weights and mammary gland weights in intact and control can attributed to that aniseed oil might contain substances affecting directly on the ovaries and activate them to secret steroid, or indirectly through the Gonadotropins and GnRH.

Table 2. Effect of daily oral dose of aniseed oil (0.043 gm kg^{-1} B.wt) administration for two weeks on mammary gland and uterine weight in ovariectomized and intact female rats

Groups / Parameters	OA	IA	S	LSD values
Mammary gland weight%	B 1.082 ± 0.036	A 1.348 ± 0.156	B 1.122 ± 0.058	0.201
Uterine weight%	B 0.185 ± 0.041	A 0.313 ±0.053	B 0.201 ±0.024	0.101

Values are expressed as mean ± SE. n=5/group. Capital letters denote between group differences, $p<0.05$ vs. control. OA= Ovariectomized+Aniseed oil. IA= Intact+Aniseed oil. S=Sham oparated (control).

Levels of Gonadotropins: Follicular stimulating hormone (FSH): The effect of aniseed oil administration (0.043 gm kg^{-1} B.w) for two weeks on FSH concentration (mU ml^{-1}) in OA, IA and control S animals are shown in table 3. The concentration of FSH was 14.70±0.826, 11.60±0.631 and 8.76±0.865 for OA, IA and control S, respectively. It was significantly ($p<0.05$) higher in the OA.

Lutenizing hormone (LH): Similarly, the levels of LH were higher 0.406±0.119 in OA rats as compared to those of IA 0.110±0.021 and S 0.082±0.010 (Table 3). OA rats revealed higher concentrations of FSH and LH than other groups (Table 3). The growth and maturation of gonads (ovaries) is under the direct effect of (FSH and LH) from pituitary. The first one stimulates granular cells of ovarian follicles to synthesis estrogen, besides the growth and maturation of primary follicles (Ganong, 1995). While the latter aids in final maturation and secretion of estrogen in addition to ovulation and corpus lutetium formation and progesterone secretion (Guyton, 1996). The estrogen and progesterone hormones increase uterine weight by increase protein synthesis and mitotic division of endometrium. The OA rats showed a slightly reduced MGwW% and UwW% and higher levels of FSH and LH than in IA and control S rats. This finding confirms the suggestion that in the absence of

ovaries, aniseed oil is unable to activate mammary glands and uteri. This also indicates that estrogenic activity of aniseed oil is not similar to that of endogenously produced estrogens. It also indicates that aniseed oil does not stimulate mammary gland and uterine growth directly. Furthermore, the ovariectomy lead to eliminate negative feedback inhibition of estrogen on the pituitary and/ or hypothalamic level, and as a result, the FSH and LH were elevated in OA. However, LH and FSH also increase in IA compared to S. This finding suggests that anise, somehow, enhances the release of Gonadotropins or GnRH. The higher concentration of FSH and LH in OA, therefore probably had come by two causes: the first is the removal of negative feedback inhibition of estrogen and progesterone on pituitary level, the second cause could be the postulated effect of aniseed oil on pituitary gland directly to increase the level of Gonadotropins secretion. Hence, these findings suggest a central mechanism of action of aniseed oil, through the hypothalmohypophyseal axis. However, it is beyond the scope of this experiment to elucidate the mode of action of aniseed oil. Further research is suggested to underline these mechanism.

Table 3. Effect of daily oral dose of aniseed oil (0.043 gm kg^{-1} B.wt) administration for two weeks on plasma Gonadotropins FSH and LH in ovariectomized and intact female rats

Groups / Hormones	OA	IA	S	LSD values
FSH (mU ml^{-1})	A 14.70 ±0.826	B 11.60 ±0.631	B 8.76 ±0.865	2.9
LH (mU ml^{-1})	A 0.406 ±0.119	B 0.116 ±0.021	B 0.082 ±0.010	0.211

Values are expressed as mean ± SE. n=5/group. Capital letters denote between group differences, $p<0.05$ vs. control. OA= Ovariectomized+Aniseed oil. IA= Intact+Aniseed oil. S=Sham oparated (control).

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تأثير زيت بذور نبات الينسون في نمو الغدد اللبنية وتكوين الحليب في الجرذان

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المستخلص

تضمنت الدراسة تجربتين الاولى: بيان تأثير زيت بذور نبات الينسون خلال الاسبوع الثالث (الاخير) من الحمل في نمو وتطور الغدد اللبنية وتكوين الحليب خلال فترة الرضاعة. استخدمت 20 انثى حامل في اليوم الخامس عشر من الحمل وقسمت عشوائيا الى مجموعتين متساويتين: مجموعة سيطرة (10 امهات) جرعت بالماء الاعتيادي خلال الاسبوع الثالث من الحمل، ومجموعة معاملة (10 امهات) جرعت بزيت بذور نبات الينسون بجرعة (0.043) خلال الاسبوع الاخير من الحمل. عند الولادة تم عزل خمسة امهات من كل مجموعة وتم التضحية بها لاستخراج اوزان غددها اللبنية نسبة الى اوزان اجسامها، اما الخمسة امهات المتبقية من كل مجموعة السيطرة والمعاملة سمح لها بإرضاع مواليدها الى اليوم الحادي عشر من ارضاعة عنده تم تسجيل اوزان المواليد واوزان معدتها نسبة الى وزن الجسم. اظهرت النتائج ان هناك زيادة معنوية في

اوزان الغدد اللبنية في يوم الولادة وزيادة اوزان المواليد واوزان معدتها في اليوم الحادي عشر من الرضاعة مقارنة مع مجموعة السيطرة. اما التجربة الثانية فقد استهدفت دراسة آلية عمل زيت بذور نبات الينسون بوجود وغياب تأثير المبايض، وتتلخص هذه التجربة اختبار فيما لو كان عمل الينسون مباشرة على الغدد اللبنية أم على محور النخامية- تحت المهاد. استخدمت في هذه التجربة 15 أنثى في عمر خمسة أسابيع قسمت عشوائياً إلى ثلاثة مجاميع متساوية وعوملت لمدة أسبوعين على النحو التالي: المجموعة الأولى: اجري لها عملية إزالة المبايض وعولجت بالزيت بجرعة غم كغم$^{-1}$ من وزن الجسم). المجموعة الثانية: سليمة وتناولت الزيت بنفس الجرعة والمجموعة الثالثة: اجري لها عملية فتح وخياطة البطن فقط وتناولت ماء الشرب الاعتيادي واعتبرت كمجموعة سيطرة. تم استخدام أربعة مؤشرات هي وزن الغدد اللبنية ووزن الرحم، قياس مستوى الهرمون المحفز للجريب والهرمون اللوتيني لتحقيق هدف هذه التجربة. أظهرت نتائج هذه التجربة إن الحيوانات السليمة والتي تناولت الزيت شهدت أعلى وزن للغدد اللبنية والرحم في حين بينت الحيوانات مزالة المبايض أعلى تركيز لقياس الهرمونين. إن عدم تحفيز الغدد اللبنية والرحم بعد العلاج بالزيت في الحيوانات مزالة المبايض مع ارتفاع تركيز الهرمونين يشير إلى فعل الزيت الغير المباشر على الغدد اللبنية والرحم وإنما يعمل على محور تحت المهاد.

الكلمات المفتاحية: الغدد الثديية، المبيض، الرحم، هرمونات الغدد التناسلية.