

## Purification of some active ingredients of alcoholic extract of anise seed (*Pimpinella anisum*) and assessment their estrogenic effect on some physiological traits of immature female mice

تنقية بعض المواد الفعالة للمستخلص الكحولي لبذور الينسون ودراسة تأثيرها الأستروجيني على بعض الصفات الفسلجية في اناث الفئران غير البالغة

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### Abstract

This experiment was conducted in order to purify some active ingredients of 70% alcoholic extract of aniseed by adsorption chromatography on silica gel and gel filtration on sepharose CL-6B column, using phosphate buffer (0.1M, pH 7.0) as eluent, as well as gel filtration on sephadex LH-20 where chloroform : methanol (3:1) was used as an eluent. Thin layer chromatography on silica gel was used as a criterion for the identification of purified fractions. Ultra violet transilluminator as well as vanillin-H<sub>2</sub>SO<sub>4</sub> reagent were used to identify and develop of separated fractions, respectively. However, the fractions which purified on sephadex LH-20, showed an R<sub>f</sub> value (0.71) which corresponded to the R<sub>f</sub> values of anethole. To assess the estrogenic activity of purified fractions a dose of 150 mg/kg of body weight of collected fractions were given to immature female mice at three weeks age and administered orally for four weeks period. Twenty immature female mice were divided randomly into five groups. First group received crude extract of aniseed, while the second, third, and fourth groups received fractions purified on silica gel, sepharose CL-6B and sephadex LH-20 columns, respectively. Group five served as control, such treatments were continued until maturity, and then the animals were sacrificed for studying weight changes in ovaries and uterus. The results of this study revealed that there is a significant increment (P<0.01) in the weights of ovaries in the treated groups as compared to the control, while fractions obtained from sepharose CL-6B had no significant effect (P>0.01). Also there is a significant increment (P<0.01) in the weights of uterus in the treated group with respect to

## fractions obtained from sephadex LH- 20 as compared to other treated groups and control.

### المستخلص

استهدفت هذه التجربة تنقية بعض المواد الفعالة الموجودة في المستخلص الكحولي لبذور الينسون باستخدام كروماتوغرافيا الادمصاص على عمود من هلام السليكا silica gel والترشيح الهلامي على عمود من sepharose CL-6B باستخدام دارئ الفوسفات بتركيز 0.1 مولاري وبأس هيدروجيني 7.0 لإسترداد النماذج ، كما تم استخدام الترشيح الهلامي أيضا على عمود من sephadex LH-20 واسترد النموذج باستخدام مزيج من الكلوروفورم والميثانول بنسبة 1:3 بعدها تم تحديد نوع المركبات الموجودة في الأجزاء المفصولة باستخدام كروماتوغرافيا ألطبقه ألرقيقه وعد البنزين كطور متحرك . تم الكشف عن البقع الموجودة في الصفيحة باستخدام الأشعة فوق البنفسجية والكاشف (vanillin-H<sub>2</sub> SO<sub>4</sub>) وحددت أبقعه في تراكيز الأجزاء المفصولة من عمود sephadex LH-20 . وكانت قيمة R<sub>f</sub> 0.71 والذي يقارب R<sub>f</sub> مركب الأنيثول anethole وهذه تمثل نسبة 80-90% من مجموعة الزيوت الطيارة والتي تعد من أهم المواد الفعالة الموجودة في بذور الينسون والتي لها تأثير استروجيني فعال . لقياس الفعالية الحيوية للأجزاء المفصولة تم تجريع 20 فأرة غير بالغه بعمر ثلاثة أسابيع وبجرعة (150ملغم/كغم من وزن الجسم ) ، قسمت عشوانيا إلى خمسة مجاميع .المجموعة الأولى جرعت بالمستخلص الكحولي لبذور الينسون وجرعت المجموعة الثانية بالأجزاء المفصولة من عمود هلام السليكا أما الثالثة فقد جرعت بالأجزاء المفصولة من عمود هلام sepharose CL-6B وجرعت المجموعة الرابعة بالأجزاء المفصولة من عمود sephadex LH-20 وعدت المجموعه الخامسة كمجموعة سيطرة واستمرت المعاملة لحين البلوغ الجنسي بعمر سبعة أسابيع عند ذلك تم التضحية بها لدراسة التغيرات ألوزنيه في كل من المبايض والأرحام . أظهرت النتائج زيادة معنوية عالية (P<0.01) في معدلات أوزان المبايض في جميع المجاميع المعامله باستثناء المجموعة المعاملة بالأجزاء المفصولة من عمود sepharose CL-6B , كما أظهرت النتائج زيادة معنوية عالية (P<0.01) في معدلات أوزان الأرحام في المجموعة المعاملة بالأجزاء المفصولة من عمود هلام sephadex LH-20 والتي سجلت أعلى ارتفاعا مقارنة مع المجاميع المعاملة بالأجزاء المفصولة لهلام السليكا silica gel . و sepharose CL-6B ومجموعة السيطرة .

## Introduction

Herbs have been used for nutritive and medicinal purposes for centuries. The World Health Organization estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components [1]. Anise (*Pimpinella anisum*) is an annual herb indigenous to Iran, India, Turkey and many other warm regions in the world. Chemical analysis of anise oil showed the presence of the following active ingredients: anethole which represented (85%) of the essential oil as well as eugenole methylchavicol, anisaldehyde and estragole. Moreover, anise was employed in folk medicine in many parts of the world for stimulating digestion and give antiparasitic [2], antibacterial [3, 4] antifungal [5] and antipyretic effects [6]. Additionally, the plant and especially its fruit has been used to cure a variety of diseases including seizures and epilepsy [7, 8]. Aniseed (*Pimpinella anisum*) is a plant, which has been used as estrogenic agents for millennia, specifically to increase milk secretion regulate menstruation, and facilitate birth [9]. The galactogoual role of crude extract of aniseed was proved by [10] when the crude alcoholic extract was administrated during the last trimester of pregnancy; on the other hand, [11] found that aniseed extract stimulates mammary gland growth when given at prepubertal time. Aniseed oil possess an estrogen and progesterone like action on the rat's mammary gland [12]. [13] concluded that the administration doses (43 mg, 86mg /kg B.W.) of anise oil to male mice resulted in amarked improvement in reproductive capacity, which

explained by the effect of treatment on spermatogenesis, probably by hormonal regulation across hypothalamic-pituitary-testicular axis. However this experiment was aimed and designed to evaluate the estrogenic performance of the active ingredients of aniseed in albino immature female mice, by using chromatographic purification of bioactive components of alcoholic extract of aniseed on silica gel, sepharose CL-6B and sephadexLH-20, and identification of purified fractions.

### Materials and methods

The experiment was take place at Institute of genetic engineering and biotechnology for post graduate of Baghdad University in January-August/ 2007, where seventy percent of alcoholic extract was prepared according to the procedure of [14]. Purification of 70% alcoholic extract of anis seed was carried out using column chromatography technique: -

1. Column chromatography on silica gel, a glass column of (20x2.2 cm I.d.) was prepared according to the [15]. Silica gel (270-300 mesh) 0.1M phosphate buffer pH, 7.0 was used as eluent .
2. Column chromatography on sepharose CL-6B gel, glass column of (56x1.5 cm I.d.) was prepared according to the directions of the supplied company (Pharmacia-Sweden) phosphate buffer 0.1M, pH 7.0 as eluent.
3. Column chromatography on sephadex LH- 20, glass column of (38x 1.6 cm I.d.) was prepared according to the directions of Pharmacia-Sweden Company. Amixture of chloroform: methanol at ratio of 3:1 was used as eluent.
4. Thin layer chromatography technique, silica gel type G in the form of thin layer on aluminum plate supplied from Fluka Company benzene used as a mobile phase [14,16]. Five groups of a weanling female mice at 3 weeks age including four mice/group were divided randomly into:-

**Group one:** Animals in this group received orally 150 mg/kg B.W. of alcoholic extract of aniseed suspended in 50  $\mu$ L 0.01 M pH 7.0 phosphate buffer [10].

**Group two:** received orally 150 mg/kg B.W. fractions of 70 % alcoholic extract purified on silica gel column.

**Group three:** received 150 mg/kg B.W. fractions of 70 % alcoholic extract purified on sepharose CL-6B column.

**Group Four:** received 150 mg/kg B.W. fractions of 70 % alcoholic extract purified on sephadex LH-20 column.

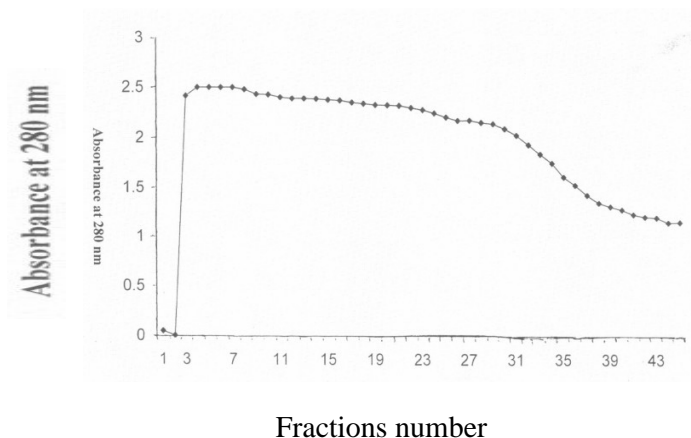
**Group five:** Animals in this group served as control under similar conditions and administered orally 50  $\mu$ L of 0.01 M, pH 7.0 phosphate buffer / animal daily. Parameters studied involved relative weights of ovary and uterus.

Statistical analysis: Completely randomized design (CRD) program [17] was used to test the effect of the treatments on traits involved in this study. The least significant difference (LSD) test was also used to compare significance between the means [18].

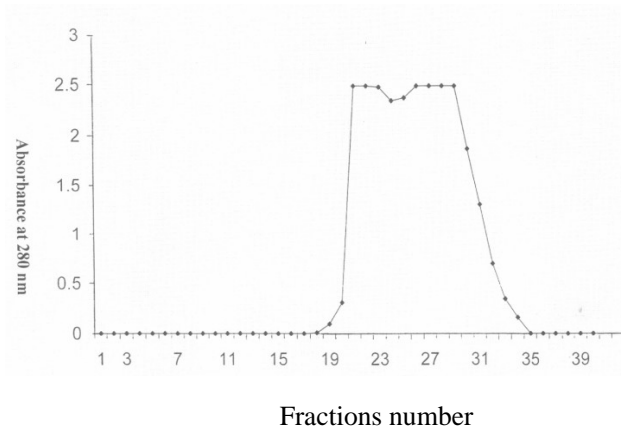
### Results

The result of this experiment showed that chromatography of 70% alcoholic extract of anis seed on sephadex LH-20 column yield to some extent a homogenous symmetrical peak Figure (3). On the other hand neither silica gel nor sepharose CL-6B column yields

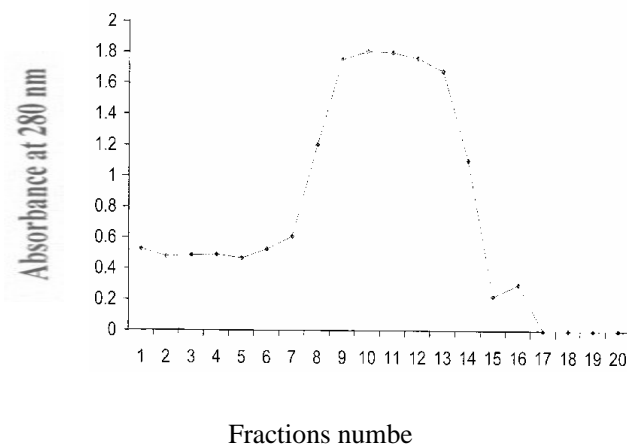
pure fractions Figures (1,2). Thin layer chromatography technique revealed that the bioactive fractions obtained from sephadex LH-20 column appears as rose color spot seen by using UV transilluminator and vanillin-H<sub>2</sub>SO<sub>4</sub> reagent, which has R<sub>f</sub> value equal to 0.71 .This R<sub>f</sub> value identical to R<sub>f</sub> value of anethole reported by [14]. Furthermore, fractions yield from silica gel and sepharose CL-6B column had not given any spots on the TLC plate Figure (4). The results clearly showed Figures (1, 2, 3 ) that sephadex LH-20 was a superior matrix for the fractionation of 70% alcoholic aniseed extract, and the symmetrical single peak was identified on TLC plates.



**Fig (1): purification of 70 % alcoholic extract of aniseed on silica gel column (20x2.2), phosphate buffer 0.1 M at pH 7.0 was used as an eluent at flow rate of 60 ml/hr, fraction size 5 ml.**



**Fig (2) : purification of 70% alcoholic extract aniseed of on sepharose CL-6B column (56x1.5 cm) phosphate buffer 0.1 M at pH 7.0 was used as an eluent, at flow rate of 60 ml/hr, fraction size 5 ml.**



**Fig (3): purification of alcoholic extract of aniseed on sephadex LH-20 column (38x1.6 cm) chloroform:methanole (3:1) was used as an eluent at flow rate of 30 ml/hr, fraction size 5 ml .**

**Fig (4): chromatogram TLC analysis using benzene as mobile phase and vanillin-  $H_2SO_4$  for the development of the spots.**

**Number 1 and 2 represent the fractions obtained from silica gel column.**

**Number 3 and 4 represent the fractions obtained from silica gel column and decolorized by charcoal.**

**Number 5 and 6 represent the fractions obtained from sephadex LH-20 column and decolorized by charcoal.**

**Number 7 represent the fractions obtained from sepharose CL-6B column and decolorized by charcoal.**



The results exhibited in table (1) revealed that there were no significant differences, ( $P > 0.01$ ) in ovaries weight (gm/100 gm B.W.) of immature female mice treated for 4 weeks with 150 mg/kg B.W. in fractions purified on sepharose CL-6B ( $0.024 \pm 0.001$ ) as compared to the control ( $0.028 \pm 0.005$ ). On the other hand, there was a significant increment ( $P < 0.01$ ) in ovaries weight in groups of mature female mice treated with 70% alcoholic crude extract ( $0.051 \pm 0.005$ ). Fractions eluted by silica gel ( $0.037 \pm 0.001$ ) and sephadex LH-20 ( $0.051 \pm 0.011$ ) as compared to the control, respectively. The relative weight of uterus in immature female mice after 4 weeks of oral administration of 70% alcoholic crude extract of aniseed and fractions purified on adsorbent or gel filtration chromatography columns were showed in table (1). Fractions of 70% alcoholic aniseed crude extract purified on sephadex LH-20, chromatography column caused significant increment ( $p < 0.01$ ) in the uterus weight of immature female mice ( $0.617 \pm 0.135$ ) as compared to the control group ( $0.153 \pm 0.012$ ). Furthermore, other groups of animals treated with crude extract and fractions eluted from silica gel and sepharose CL-6B columns recorded no significant increment ( $P > 0.01$ ) in the weight of the uterus which being ( $0.287 \pm 0.097$ ), ( $0.192 \pm 0.030$ ) and ( $0.225 \pm 0.029$ ), respectively as compared to the control ( $0.153 \pm 0.012$ ).

**Table (1): Effect of purified fractions of 70% alcoholic crude aniseed extract on ovaries and uterine weight (gm/100gm B.W.) of immature female mice at 3 weeks aging and treated for 4 weeks.**

Treatments	Mean $\pm$ SE	
	Mean% of ovary weight	Mean% of uterus weight
Control	$0.028 \pm 0.005^b$	$0.153 \pm 0.012^b$
Crude Extract	$0.051 \pm 0.005^a$	$0.287 \pm 0.097^b$
Silica gel	$0.037 \pm 0.001^{ab}$	$0.192 \pm 0.030^b$
Sepharose CL-6B	$0.024 \pm 0.001^b$	$0.225 \pm 0.029^b$
Sephadex LH-20	$0.051 \pm 0.011^a$	$0.617 \pm 0.135^a$
LSD	0.0188 **	0.2326 **

\*\*  $p < 0.01$

Values are expressed as the Mean  $\pm$  SE, n=5/group

Mean in the same column with no common superscript differ significantly ( $p < 0.01$ ).

## Discussion

Sephadex LH -20 is lipophilic cross-linked dextran derivative, used for the fractionation of organic- soluble natural products such as steroid, fatty acids, and porphyrin and carotenoid pigments. [19,20]. Anethole was eluted as a symmetrical peak on sephadex LH-20 confirming the suitability of this matrix for the superior separation rather than silica gel and sepharose CL-6B.

TLC was used in this work to detect anethole extracted from aniseed. The  $R_f$  value was approximately the same as recorded by [14] which was 0.71 using benzene as mobile phase, while  $R_f$  recorded by [16] was 0.69 using the anethole same mobile phase, on other hand [21] reported that the  $R_f$  value of anethole was 0.74 using chloroform /ethyl-acetate /

formic acid (85:15:1) as mobile phase. It is important to mention that the normal range for  $R_f$  value of anethole is between 0.69-0.74.

Anethole is the most active ingredients of aniseed and represented 85% of essential oil, and the estrogenic potency was attributed to the polymer forms of anethole (dianethole, photoanethole), this estrogenic activity was discussed on the bases of the structural similarity of anethole and dopamine. Dopamine acts to inhibit the secretion of prolactin, but that effect could be repressed by successful competition of anethole at the appropriate receptor sites [9].

The results of this experiment Table (1) indicated that fractions of 70 % alcoholic purified aniseed extract on sephadex LH- 20 column which contains mainly anethole exert an estrogenic effect in immature female mice reproductive system through some remarkable observations such as weight increment of the ovaries and uterus. Growth and maturation of ovaries under the control of gonadotropic hormones (FSH and LH) secreted from pituitary gland during sexual puberty. FSH stimulates cells of ovarian follicles to synthesize estrogen, and stimulates the development and growth of the primary follicles to secondary follicles and then to graafian follicles [22]. LH is responsible for induction of ovulation and formation of corpus luteum which stimulated to secrete progesterone. [23, 22].

The ovarian hormones play an important role in uterine weight increment through estrogen hormone activity which stimulates protein synthesis in the myometrium and increases divisions of endometrial cells. Progesterone increase growth and divisions of active uterine glands, and increase blood supply to the endometrium, all these changes will lead to increase of uterine weight. [24, 25]. Ovarian and uterine weight increment in prepubertal animals considered as one of the criteria that can be used in the bioassay of these hormones [26, 27]. However, the ovarian weight increment in this study may be attributed to the stimulatory effects of the anethole on granulosa cells of the ovarian follicles inducing their numbers and growth development, as well as accelerating maturation processes at different follicular stages, in addition to increasing blood supply and metabolic rates[22].

However, the significant increment in the weight of the uterus may be attributed to the direct effect of anethole on cell division and proliferation induction of the uterine cells. On the other hand, the crude extract of aniseed showed a significant increment in the weight of ovaries of immature female mice. Similar results concerning to the effect of 70% alcoholic aniseed crude extract on the weight of ovaries and uterus of immature female rats was reported by [11]. Moreover, the estrogenic potency of anethole recorded in this experiment was confirmed histologically through an increment of endometrium layer thickness of uterus. [2].

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