Effects of Herbs – Containing Phytoestrogens on Rat Testis: A Histological, Histochemical and Biochemical Study

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ABSTRACT:

BACKGROUND:

Estrogens has traditionally been known as the female hormone, but this idea has been challenged in early 1990's and an essential physiological role for estrogen in male fertility was identified. Phytoestrogens are naturally occurring non-steroidal plant chemicals that can act like the female hormone estrogen. The herbs (anise alfalfa and vervain) chosen in this study contain phytoestrogens.

OBJECTIVE:

Previous studies demonstrated controversy of the effects of phytoestrogens on the rat testes .Hence, the present investigation was undertaken to investigate the influence of typical dose of herbs containing phytoestrogen on the rat testis.

MATERIALS AND METHODS:

Twenty-four apparently normal mature male rats were divided into four groups of 6 animals each. The first "control" group received only 4ml of distilled water as a placebo. The second group received 40mg/kg of anise seed; the third group received 53.3 mg/kg of dried herb of vervain and the fourth group received 400 mg /kg of alfalfa seeds. All experimental groups received the doses through oro-gastric tube daily for fourteen days. Testicular histology was evaluated by light and enzyme histochemistry. Plasma FSH and testosterone concentrations were taken to support our results.

RESULTS:

Histological examination of anise, alfalfa and vervain – treated groups showed an increase in the height of germinal epithelia. There was marked lipoprotein lipase activity in the whole of the interstitial tissue which is more in amount in experimental groups than that in control group. Acid phosphatase granules were infiltered the seminiferous epithelia mildly in control group , moderately in anise and vervain groups and markedly in alfalfa group. The number of interstitial cells showing marked acid phosphatase activity was higher in all experimental groups than that in control group. Alkaline phosphatase exhibited intense activity in the boundary tissue of the seminiferous tubules in testes of control and experimental groups but it appeared thicker in the latter.

CONCLUSION:

The low dose and short duration of treatment used in our study made these phytoestrogen – containing herbs to have a stimulatory effect on leydig cell steroidogenesis. This study also demonstrated that aniseed being the most potent of the three herbs followed by alfalfa in stimulating testosterone synthesis. This is possibly attributed to the coumarin constituent of aniseed and alfalfa.

KEY WORDS: phytoestrogen-containing herbs, testis, acid phosphatase, alkaline phosphatase, lipoprotein lipase

INTRODUCTION:

Estrogens have traditionally been known as the female hormone, but this idea has been challenged in early 1990's and an essential physiological role for estrogen in male fertility was identified [(1)]. The demonstration that male fertility is impaired in mice lacking estrogen receptor-alpha (ER-α) along with the discovery of a second estrogen receptor-beta (ER-β),which is widely expressed in the male reproductive tract, has clearly showed the role of estrogens in male [(1,2)]. The importance of estrogen in the adult testis was also highlighted by phenotype of aromatase knockout (ArKO) mouse, where the inhibition of estrogen biosynthesis resulted in spermatogenetic abnormalities (3-5). Because the estrogen receptors are expressed in the developing reproductive tract from fetal life through adulthood and estrogen receptor-β is predominant in the seminiferous epithelium, estrogen may act directly on the seminiferous tubules to mediate spermatogenesis [(5)].

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Phytoestrogens are naturally occurring non-steroidal plant chemicals that can act like the female hormone estrogen.These compounds are able to bind to both estrogen receptors, particularly the estrogen receptor-β isoform, in agonistic fashion with high affinity and are thought to exert their estrogenic effects through mechanism similar to estradiol [6,7].Because the relative potency of phytoestrogens is significantly lower than that of steroidal estrogens, exposure to them has been regarded as no harmful, even beneficial.

The consumption of diets with high levels of soy has been related to multiple beneficial effects including chemopreventive activities against various hormone-dependent cancers including breast and prostate and alleviation of some of the adverse consequences of menopause [8]. They are also known to be protective in the prevention of cardiovascular disease and osteoporosis. However, they can cause impaired reproductive function in some animal species (9-11) and in the absence of endogenous phytoestrogens, they can act as partial estrogen agonists [5].

The herbs chosen for this study , contain phytoestrogens as shown from their chemical constituents. Anise or Aniseed, less commonly anis (pimpinella anisum) is a flowering plant in the family Apiaceae. It is used extensively as a spice and is listed by the Council of Europe as a natural source of food flavoring. It is stated to possess expectorant, antispasmodic, carminative, and parasiticide properties [12,13]. Anise has been reputed to increase milk secretion, promote menstruation, facilitate birth, alleviate symptoms of the male climacteric and increase libido [14]. The main constituents of aniseed are coumarins, flavonoid (flavonol and flavone, glycosides), volatile oils(trans-anethole,estrgole,anise ketone), carbohydrate and lipids (saturated and unsaturated) [12,13].

Vervain or verbena, pigeon's grass, herb of grace (Verbena officinalis) is a slender perennial plant in the family Verbenaceae. It is listed by the Council of Europe as a natural source of food flavoring. It is stated to possess sedative, antispasmodic, mild diaphoretic and reputedly galactogogue properties .The most characteristic chemical constituents of vervain are glycosides (iridoid glycosides, verbenaline ,hasatoside, verbenin, phenyl propanoid glycosides), volatile oils (monotrepene components) and other constituents like adenosibe, alkaloid, buitters, carbohydrates, β – carotene invertin, saponin and lannic acid [12,13].

Alfalfa is a genus of perennial flowering plant, Medicago, most commonly referring to M. sativa L. also called luceme. It is widely cultivated and is increasing in popularity for human consumption due to its promotion as a dietary supplement. It is stated to be a source of vitamins A, C, E and K and of minerals, calcium, potassium, and phosphorous. A steroidal saponin fraction is believed to play a role in the hypocholesterolemic and hemolytic activity of the leaves and sprouts . The alkaloids found in the seed possess emmenagogue and lactogenic activity. Alfalfa leaves also contain flavones, isoflavones, sterols and coumarin derivatives [12,13].

The present investigation was undertaken to investigate the influence of typical dose of herbs containing phytoestrogen on the rat testis. Testicular histology was evaluated by light and enzyme histochemistry. Plasma FSH and testosterone concentrations were taken to support our results.

MATERIALS & METHODS:

Twenty-four apparently normal mature male rats (Rattus Norvegicus), average weight between 170-200 gm were used in this study . They were housed in the Animal Breeding Centre ,College of Medicine /University of Baghdad under normal diurnal lighting conditions, kept at constant temperature (about 25C)and given free access to tap water and food.

Rats were divided into four groups of 6 animals each. The first "control" group received only 4ml of distilled water as a placebo through oro-gastric tube daily for fourteen days. The second group received 40mg/kg of anise seed [13]; the third group received 53.3 mg/kg of dried herb of vervain [13] and the fourth group received 400 mg /kg of alfalfa seeds [13]. All experimental groups received the doses through oro-gastric tube daily for fourteen days. All animals were weighted just before the experiment and before their sacrifice.

From each deeply ether anesthetized animal, 1ml blood was aspirated via cardiac puncture for hormonal assay of testosterone and FSH using the ELFA Technique (Enzyme Linked Fluorescent Assay), then the assay was completed by VIDAS apparatus.

Specimens processed for paraffin –embedding were immediately fixed in 10% formalin for 24 h. The fixed tissues were then processed for routine paraffin-wax embedding and sectioned serially at 5 micrometers thickness using electric microtome. From each paraffin tissue block, ten sections were prepared for routine haematoxylin –eosin stain [14].

Specimens for enzyme study were fixed in formal calcium at 4C for 18h [14] . Frozen specimens were cut at 6 micrometer thick. From each frozen

specimen, 3 sets of sections were prepared (ten sections each). The 1st set was processed for the demonstration of alkaline phosphatase using calcium –cobalt method; the second set for acid phoshatase using metal precipitation metod; and the third set for lipoprotein lipase using tween method [15].

For hormonal study, data analysis is done using mean +/- standard deviation, ANOVA and correlation to assess the significance between the studied groups.

RESULTS:

All experimental rats maintained good general health and showed rather hyperactivity and better appetite. Additionally, experimental rats revealed a slight, however, non-significant rise in body weight when compared with their controls.

Histological examination of the testes of control rats (Fig.1A) showed seminiferous tubules separated by interstitial tissues .The seminiferous epithelium consists of spermatogenic cells and Sertoli cells. The interstitial tissue is a loose connective tissue containing leydig cells, fibroblasts, macrophages and other connective tissue cells.

The testes of anise (fig.1B) ,alfalfa (Fig.1C) and vervain (fig.1D)- treated rats showed an increase in the height of germinal epithelia when compared with their controls. The number of spermatogonia was incresased and the tubular lumen is almost filled with spermatozoa. The interstitial tissues were relatively more abundant when compared with their controls and contained an increased number of interstitial cells.

Sections of control testes showed prominent Lipoprotein lipase activity in the interstium. More diffuse and apparently weaker staining reaction was seen in the germinal epithelium. The lumen of seminiferous tubules showed moderate enzyme activity in the form of diffuse brownish reaction. The cells of interstitial tissue exhibited different affinity towards lipoprotein lipase enzyme ranged from diffuse to heavy granular reaction (Fig.2 A).

Sections of anise (Fig.2B) and alfalfa (Fig.2C) –treated groups showed marked Lipoprotein lipase activity in the whole of the interstitial tissue which is more in amount than that of control group. Lipoprotein lipase activity took the form of coarse granular reaction which obscured the architecture of cells in the interstium. Diffuse and weak staining reaction was seen in the germinal epithelia. The staining reaction of the lumen of the seminiferous tubules was more than that in control group.

The activity of lipoprotein lipase in vervain –treated group (Fig.2D) ranged from fine "black"-coarse "brown" granular reaction in the interstitial tissue. The architecture of cells was obscured in the interstium. Diffuse and weak enzyme activity was noticed in the germinal epithelia. The lumen of seminiferous tubules shows diffuse enzyme activity which was less than that revealed in anise and alfalfa groups.

The testes of control (Fig.3A), anise (Fig.3B), alfalfa (Fig.3C) and vervain (Fig.3D) –treated groups showed an intense " coarse granular" acid phoshatase activity at the base of the seminiferous epithelium where the Sertoli cells were located. The seminiferous epithelia is mildly infiltered with Fine granules in control testis, moderately infiltered with fine – pin point granules in anise treated testis, heavily infiltered with fine - pin point granules in alfalfa and vervain treated groups . The interstitial tissue exhibited fine-coarse granular reaction . The number of interstitial cells which showed marked enzyme activity (coarse granules that obscure the outline of cells) was higher in all experimental groups than that in control group.

The control testes (Fig.4 A) showed an intense alkaline phosphatase activity in the boundary tissue of the seminiferous tubules. The interstitial tissue showed diffuse and apparently weak enzyme reaction and contained discrete areas with intense enzyme activity. The germinal epithelium showed diffuse and weak enzyme activity. However, the basal region of this epithelium demonstrated slightly stronger enzyme activity than the remainder.

All experimental testes (Fig.4 B, C&D) revealed intense alkaline phosphatase activity in the boundary tissues of the seminiferous tubules which appeared to be thicker than those in control group. The seminal epithelium revealed diffuse enzyme activity apparently slightly stronger than that in the control group. The basal cells showed slightly stronger enzyme activity than the remainder. The interstitial tissue showed diffuse and apparently weak reaction apart from discrete areas which showed intense enzyme activity. These areas appeared larger than those in the control group.

Serum FSH and Testosterone were significantly increased in all experimental groups (Tables - 1 & 3). Serun FSH was higher in aniseed followed by vervain and alfalfa. Serum testosterone was higher in aniseed followed by alfalfa and vervain (Table-1 and Fig. 5). Table- 2 showed positive correlation between FSH and testosterone.

DISCUSSION:

Histological examination of anise, alfalfa and vervain - treated groups showed an increase in the

height of the germinal epithelia. This increment in the height of the seminiferous epithelia represents a substantial increase in the germinal tissue. It had been found by Nathaniel *etal*. [16] that the increase in the diameter of seminiferous tubules and their lumens considered as a maturation event representing an initial differentiation of spermatogonia and progressive maturation of the germinal epithelia.

It had been noticed by Shaughnessy *etal*.[17] that androgen treatment did not affect Sertoli cell numbers and that testosterone and dihydrotestosterone increase the numbers of spermatogonia and spermatocytes. Additionally, it had been found by Alch and Manna [18] that the antispermatogenic activity of Busulphan or myleran cause a decrease in the seminiferous tubular diameter but Sertoli cells morphology and number remained normal. Therefore, we suspect that these herbs(anise ,alfalfa and vervain) may have an androgen like action which result in the increment of the height of seminiferous epithelia that occurred secondary to the increase in the number of sperrmatogonia, primary spermatocytes and spermatids but not Sertoli cell number.

Androgens are known to stimulate spermatogenesis through androgen receptors on the Sertoli cells and peritubular myoid cells and it had been found by Shaughnessy *etal*.[17] that stimulation of spermatogenesis requires direct androgen action on the Sertoli cells. Therefore, we suspect that anise vervain and alfalfa, through androgen receptors on Sertoli cells, may stimulate spermatogenesis.

It is well known that mesenchymal leydig cell precursors and progenitor leydig cells proliferate in the prepubertal testis and differentiate into androgen producing cells at puberty. These cells do not originate from fetal leydig cells but are newly differentiated leydig cells that arise from pluripotent mesenchymal precursors[19]. As postnatal leydig cells differentiate, they transition through three discrete maturational stages termed progenitor leydig cells, immature leydig cells and adult leydig cells[20] . These progressively more differentiated stages are characterized by decreasing proliferative rate and increasing testosterone biosynthetic capacity [21]. Testicular macrophages play an integral role in the interstitial tissue of the rat testis and secrete a number of cytokines, some of which are mitogenic to leydig cells [22]. Therefore, we suspect that the increase in the interstitial tissue in the testes treated with anise, alfalfa and vervain is attributed to the increased number of interstitial cells especially leydig cells and these cells is attributed to continued differentiation from precursor cells because differentiated leydig cells do not divide . These herbs may stimulate macrophages to secrete cytokines which are mitogenic to leydig cells.

It is well known that expression of lipoprotein lipase m RNA differs according to the type of tissue. The highest expression was found in the testis which may confirm the role of lipoprotein lipase in the process of spermatogenesis[23] . Lipoprotein lipase was expressed in normal seminiferous tubules and in the interstitial cells and Sertoli cells. This enzyme is also expressed by macrophages and smooth muscle cells [24]. It is well known that lipoprotein lipase converts chylomicrones and very low density lipoproteins to denser lipoproteins such as chylomicrone remnant; intermediate density lipoprotein and low density lipoprotein .Leydig cells acquire cholesterol from high density lipoprotein, low density lipoproteins and/or denova biosynthesis[25]. Therefore, we suspect that the increased enzyme activity, in the interstitial tissue of rats treated with anise, alfalfa and to lesser degree vervain, was attributed to increased number of interstitial cells particularly macrophages which showed marked lipoprotein lipase activity.

Testes of control and experimental rats showed an intense acid phosphatase activity in the Sertoli cell region. This finding indicates a prominent / significant autophagic function of this cell type in this tissue during the reproductive period as it had been demonstrated that acid phoshatase activity is present in heterophagic lysosomes of Sertoli cells[26] . It is well known that acid phoshatase activity – present in spermatogonial cells, primary and secondary spermatocytes, spermatids and spermatozoon bundles- may readily provide phosphate to meet their high energy requirements[27] and that this enzyme is involved in the absorption of residual spermatid cytoplasm and as well as in the removal of spermatozoa remaining after the reproductive period [26]. Therefore, we suspect that the granular infiltration of seminal epithelia with acid phoshatase: mild infilteration of fine granules (control) ; moderate infiltration of fine- pinpoint granules (anise); heavy infilteration of pin-point granules in alfalfa & vervain indicate that vervain, alfalfa and to lesser extent anise may stimulate Sertoli cell lysosomes to digest more residual bodies and cytoplasmic remains in the seminiferous tubules and that more acrosome and axoneme formation were found in these experimental groups.

The interstitial tissue in all experimental groups exhibited more intense acid phoshatase reaction in

the form of fine- coarse granules. The cells which exhibit this granular reaction are likely to be leydig cells and/or macrophages since it had been found by Bozzola & Russell [28] that acid phoshatase activity was seen in the inner cisternae of Golgi apparatus within lipofuscin pigment granules and autophagic vacuoles.

Testes of control and experimental rats showed an intense alkaline phosphatase activity in the boundary tissue of the seminiferous tubules. It is well known that each seminiferous tubule is surrounded by a basement membrane. Outside the basement membrane, there is a collagenous layer containing fibroblasts and other spindle –shaped cells representing the contractile myoid cells [29]. According to the authors Chapin *etal.*[30] and Gunawardana[31] peritubular localization of alkaline phosphatase is similar in all mammals and that the cell processes of fibroblasts showed enzyme activity on the cell membranes and in pinocytotic vesicles. The more peripheral myoid cell processes also show reaction product in their membranes. It is well known that alkaline phosphatase activity has been associated with the formation of fibrous proteins such as collagen [32]. According to the authors Anthony and Skinner [33], peritubular cells provide a site of androgen action and that alkaline phosphatase histochemistry selectively detects desmin-containing contractile cells in tubular and peritubular cultures. Therefore, we can suppose that the activity of fibroblasts and peritubular myoid cells in all experimental testes may be increased since the boundary tissue of seminiferous tubules in these groups exhibited intense enzyme activity and appeared thicker than in the control testes.

The interstitial tissue showed diffuse and weak alkaline phosphatase activity with discrete areas showing intense enzyme activity. These discrete areas composed mainly of transitional cells since it had been shown by Gaunawardana [31] that the vacuoles in transitional cells were lined by reaction products of enzyme activity where as the vacuoles present in the leydig cells were free of enzyme activity. Alkaline phospharase is an enzyme whose activity is reported to be influenced by steroid hormones [34]. Spironolactone, on the other hand, had been found to decrease the activity of alkaline phosphatase due to inhibition of testicular steroidogenesis by destruction of cytochrome P-450 which is the result from decrease steroid hydroxylase and decline lysosomal enzyme alkaline phosphatase [35]. Therefore, we postulated that anise; alfalfa and vervain stimulate testis steroidogenesis by increasing the activity of testicular 17 - α hydroxylase and the content of microsomal cytochrome P-450 and this result in increasing the activity of alkaline phosphatase.

Alkaline phosphatase is said to be a histochemical marker for primordial germ cells of various species including rat [36]. It is known that alkaline phosophatase enzyme is required for the synthesis of glycogen which in turn apparently participates in the metabolic process of spermatogenesis [37]. Mann [38] reported that some phosphatase including alkaline phosphatase disappear in the course of spermatogenesis. Alkaline phosphatase activity is considered to be associated with the passage of metabolites across cell membranes as well as with changes in the blood-brain barrier and blood testis barrier [39]. Therefore, we suppose that anise, alfalfa and vervain increase the transfer of material between Sertoli cells and spermatogonia since these herbs induced stronger alkaline phosphatase activity in the basal part of the seminal epithelia.

The three herbs used in our study significantly increase the level of testosterone which may indicate that these herbs may have gonadotropin – like action which produces leydig cell hypertrophy and hyperplasia and increased steroidogenic capacity. It is well known that testosterone is primarily synthesized in leydig cells. The number of leydig cells inturn is regulated by LH and FSH.In addition, the amount of testosterone produced by existing leydig cells is under the control of LH which regulates the expression of 17-b hydroxysteroid dehydrogenase . FSH acts on intratubular cells. It induces the production of androgen – binding protein by means of which testosterone can pass the Sertoli – Sertoli junctional complexes as well as the production of activin and inhibin by Sertoli cells which both influences the hormone release in hypothalamus and pituitary[40].

This study shows that phytoestrogens can stimulate spermatogenesis in mature rats. Most previous studies have reported suppressive effects of phytoestrogens on testosterone production. Perinatal as well as pubertal exposure to isoflavones has been found to decrease plasma testosterone levels [41].Ibrahim [42] demonstrated that anise oil in a dose 1ml/kg for 30 days induced several histopathological changes (inhibition in Sertoli cell numbers, necrotic spermatocyte cells, etc) on mature rats .Assinder *et al.* [43] concluded that exposure of the adult male rats to high phytoestrogen diet disrupts spermatogenesis and increases germ cell apoptosis. It is possible that the discrepancy between our study and previous ones is due to the different doses used and duration of treatment. This result was in agreement with the

study reported by Almstrup and colleagues who demonstrated that most phytoestrogens are aromatase inhibitors at low concentrations but estrogenic at higher concentrations [44].

Hence, it is plausible that the low dose and short duration of treatment used in our study made these phytoestrogen – containing herbs to have a stimulatory effect on leydig cell steroidogenesis. This study also demonstrated that aniseed being the most potent of the three herbs followed by alfalfa in stimulating testosterone synthesis. This is possibly attributed to the coumarin constituent of aniseed and alfalfa. It is well known that coumarins have higher binding affinities to ER- β than the other phytoestrogen compounds [45] and this estrogen receptor is predominant in the seminiferous epithelium(5).

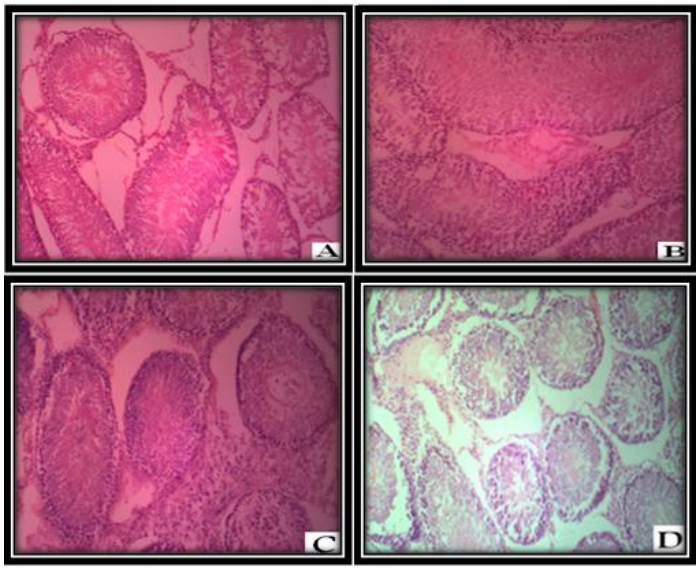

Fig. 1: Testis of control rat (A). Testes of anise (B),alfalfa (C) and vervain (D) –treated groups showed an increase in the height of germinal epithelia. Haematoxylin & eosin Stain. (A,B,C&D) X100

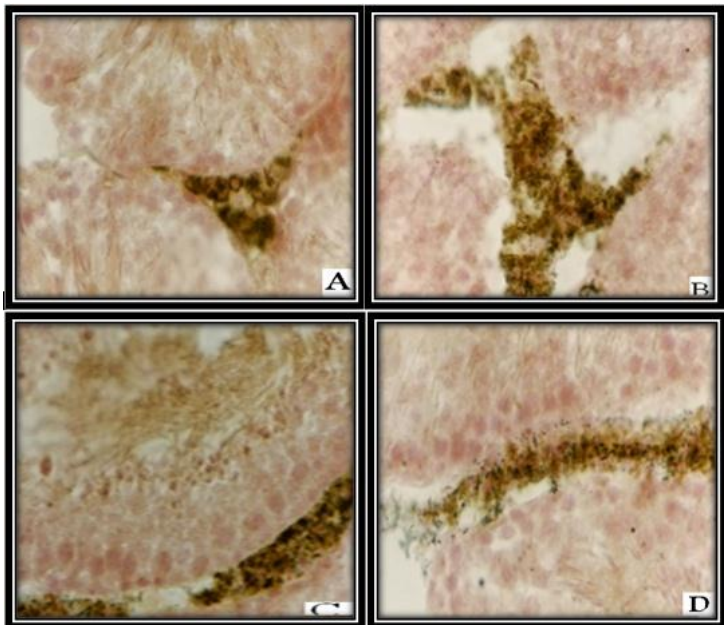

Fig.2: Testis of control rat exhibited (A) prominent enzyme activity in the interstitium. Testis of Anise (B) , alfalfa (C) and vervain (D) – treated groups showed marked enzyme activity in the whole of the interstitial tissue which is more in amount than that of control group . Lipoprotein lipase (A,B,C&D) x 400

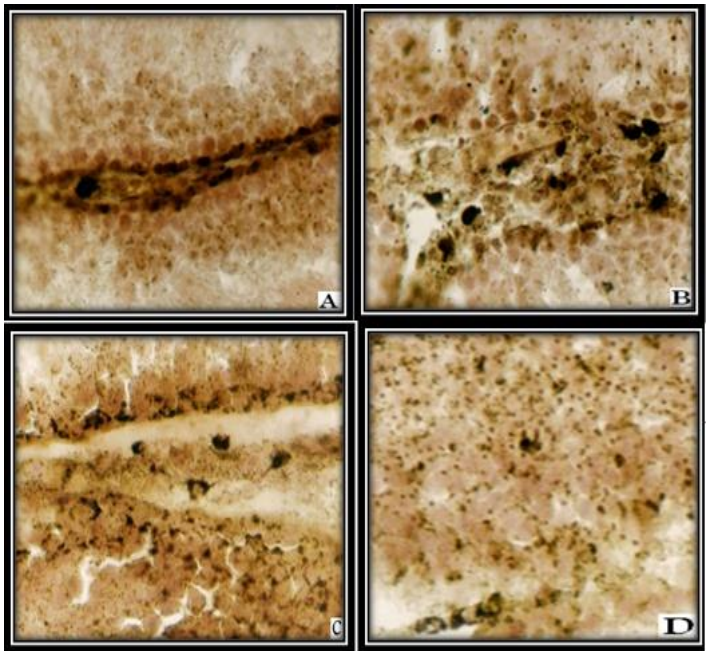

Fig. 3: The seminiferous epithelia were mildly infilterated with fine granules in control testis (A); moderately infiltered with fine – pin point granules in anise –treated testis (B); markedly infiltered with pin point granules in alfalfa-treated testis (C) and moderately infiltered with pin point granules in vervain –treated testis (D) . The number of interstitial cells showing marked enzyme activity was higher in all experimental groups than that in control group. Acid phosphatase (A, B , C, D) X 400.

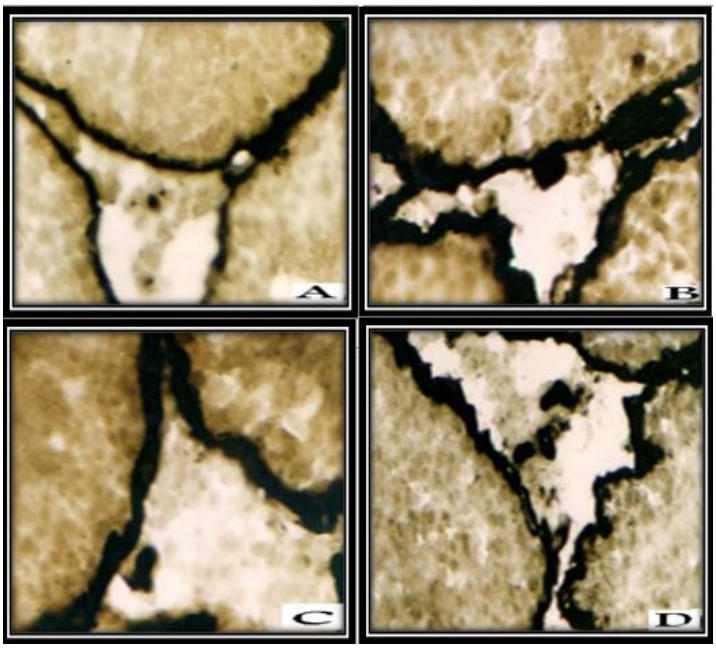

Fig. 4 : The boundary tissue of the seminiferous tubules showed intense enzyme activity in Testes of control (A) anise (B); alfalfa(C) and vervain (D) rats and it appeared thicker in all experimental groups . Alkaline phoswhatase (A, B, C, D) x 400

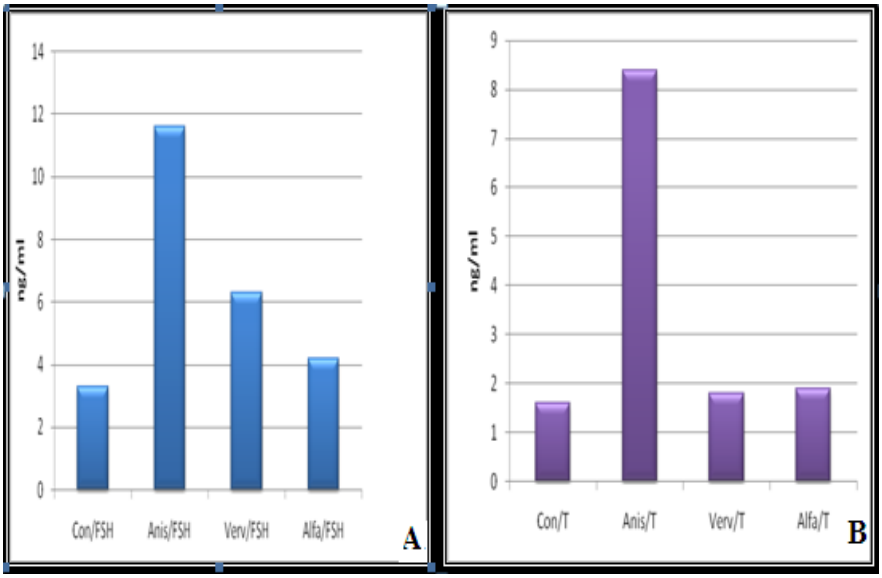

Fig.5 : The effect of aniseed, alfalfa and vervain on FSH (A) and on Testosterone (B)

Table 1: Serum FSH and Testosterone in control and ex[perimental groups. Data were expressed as mean +/- standard deviation(SD)

Groups	FSH ng/ml +/- SD		Testosterone ng/ml +/- SD	
Control	3.3833	.14720	1.5333	.08165
Aniseed	11.6000	.14142	8.4000	.14142
Vervain	6.3000	.18974	1.7833	.20412
Alfalfa	4.2000	.14142	1.9000	.14142

Table 2: Pearson Correlation showing positive correlation between FSH and Teestosterone

	FSH	Testosterone
FSH	1	949(**)
Testosterone	.949(**)	1

** Correlation is significant at the 0.01 level (2-tailed), N (24).

Table 3: Analysis of variance (ANOVA) of FSH and Testosterone among control and experimental groups.

dependent Variable	Group	Group	Mean difference	Sig.
FSH	1.00	2.00 3.00 4.00	-8.21667(*) -2.91667(*) -.81667(*)	.000 .000 .000
	2.00	1.00 3.00 4.00	8.21667(*) 5.30000(*) 7.40000(*)	.000 .000 .000
	3.00	1.00 2.00 4.00	2.91667(*) -5.30000(*) 2.10000(*	.000 .000 .000
	4.00	1.00 2.00 3.00	.81667(*) -7.40000(*) -2.10000(*)	.000 .000 .000
Testosterone	1.00	2.00 3.00 4.00	-6.86667(*) -.25000(*) -.36667(*)	000 .000 .000
	2.00	1.00 3.00 4.00	6.86667(*) 6.61667(*) 6.50000(*)	000 .000 .000
	3.00	1.00 2.00 4.00	.25000(*) -6.61667(*) -.11667	000 .000 .000
	4.00	1.00 2.00 3.00	.36667(*) 6.50000(*) 11667	000 .000 .000

* The mean difference is significant at the .05 level. 1.00 (control), 2.00(aniseed), 3.00 (vervain) and 4.00 (alfalfa).

CONCLUSION:

The low dose and short duration of treatment used in our study made these phytoestrogen – containing herbs to have a stimulatory effect on leydig cell steroidogenesis. This study also demonstrated that aniseed being the most potent of the three herbs followed by alfalfa in stimulating testosterone synthesis. This is possibly attributed to the coumarin constituent of aniseed and alfalfa

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