

## A STEREOLOGICAL STUDY: EFFECT OF POMEGRANATE HYDRO-ALCOHOLIC EXTRACT ON TESTIS OF RATS

Esrafil Masnouri<sup>1</sup>, Hassanali Abedi<sup>2\*</sup>, Mohammad Ali Izadi<sup>3</sup>, Mojtaba Fattahi Abdizadeh<sup>4</sup>

1. Department of Anatomical Sciences, Cellular and Molecular Research Center, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
2. Research Center for Noncommunicable diseases, Department of Physiology, Faculty of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran.
3. Department of Anatomical Sciences, Faculty of Medical Sciences, Isfahan University of Medicine, Isfahan, Iran.
4. Department of Microbiology, Faculty of Medicine, Sabzevar University of Medical Sciences, Sabzevar, Iran.

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### ABSTRACT

Pomegranate (*Punica granatum* L.) fruit is widely consumed as fresh fruit and juice. All parts of this plant were used to treat various ailments. The aim was a quantitative study on the rat testis after administration of pomegranate hydro-alcoholic extract. Eighteen adult male Wistar rats (180- 200 gr) were divided into three groups; each group containing six rats. One milliliter distilled water, 250 (mg/kg) and 500 (mg/kg) pomegranate hydro-alcoholic extract were given daily for eight weeks by gavage to rats in the first, second and third groups, respectively. After 8 weeks the rats were anesthetized and their testes removed and were studied using stereological methods. The results revealed that the testes weight, testis index and volume of testes were increased in the receiving groups extract as well as the volume of somniferous tubules and interstitial tissue in these groups in compared with the first group ( $p < 0.05$ ). However, no differences were observed between groups in length of the seminiferous tubules ( $p > 0.05$ ). The results revealed that pomegranate hydro-alcoholic extract not only had no adverse effects on the testes but also increased testicular weight, cell density of the tubules and interstitial tissue in tests of rats.

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### Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest fruits that is consumed in large quantities in the form of juice or fresh fruit. Muslims believe that the pomegranate is a fruit that has come from paradise [1]. The pomegranate tree widely cultivated in many countries such as Iran, India, Afghanistan, USA, China, Japan and Russia. Different parts of this plant have diverse therapeutic applications [2]. Also, it has been reported that extracts of different parts of this plant have variety biological activities such as antitumor, antibacterial, anti-diarrheal, antifungal, anti-ulcer and antifertility [3]. Different parts of pomegranate tree include; seeds, roots, bark, leaves, flowers, peel and juice. There is a notion that a combination of pomegranate seeds, juice, and peel could prevent abortion [4,5] reported *Punica granatum* L. peel can lead to healing of wound in vivo. Oil, juice and peel of this plant has weak estrogenic properties for the treatment of menopausal symptoms. Also, juice and peel of it has powerful antioxidant features [6]. Pomegranates contain arachidonic acid, a necessary polyunsaturated fatty acid very rare in plants, and protocatechuic acid; these compounds collaborate in the synthesis of prostaglandin, and consequently, play a definitive role in the reproductive system [2]. Its juice, peel and oil also have anticancer activities, such

**Corresponding Author:** Hassanali Abedi, Research Center for Noncommunicable diseases, Department of Physiology, Faculty of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran. E-mail: [hassanaliabedi@jums.ac.ir](mailto:hassanaliabedi@jums.ac.ir)

as intervention through invasion, cell cycle, proliferation of tumor cell and angiogenesis that likely accompanied with anti-inflammatory, pharmacological and phytochemical functions of all *Punica granatum* parts [6]. Flavonoids in the fruit juice have prevented activities on low density lipoprotein oxidation; therefore, this part of the plant has anti-arthrogenic effect [7]. *Punica granatum* flowers have used for the treatment of diabetes mellitus in Greek medicine. Hydro-alcoholic extract of pomegranate has a powerful antioxidant activity on some of tissues [8] and the antioxidant capacity of its juice and seeds is 2-3 times more of red wine and green tea [9]. Pomegranate extracts showed that have the property of scavenging of free radicals and also decrease macrophage oxidative stress and lipid peroxidation in animals [10], and moreover enhancement of plasma antioxidant content in old people [11]. Many studies have studied the effects of pomegranate on fertility in males and females. The pomegranate fruit is unavoidable connection with fertility, birth and undying life due to their many seeds. However, there is no evidence about the positive and/or negative effects of pomegranate and/or its extracts on male fertility [12]. Also, there is no evidence about quantitative studies of pomegranate effects on the testis. Therefore, aim of the present study was using stereology methods on testis after administration of pomegranate.

## Material and Method

Pomegranate fruit purchased from local market was dried and powdered before extraction. To obtain hydro-alcoholic extract of pomegranate, 2 liter of ethanol (70%) was added to the powder (500 gr) of the pomegranate. After 3 days, the solution was filtered by a funnel blocked with cotton wool. The filtrated solution was evaporated at room temperature. The obtained powder was further extracted with 600 ml of hot water then evaporated to dryness [8]. Finally, powder dissolved in distilled water before administration.

Eighteen male Wistar rats (180- 200 gr) were prepared from animal house center of the Ahvaz Jundishapur University of medical sciences. All animals were housed in cages with 12/12 h light/dark cycle at  $21 \pm 2$  °C and were carried out in accordance with Ahvaz Jundishapur University Ethical Committee (AJUEC). Rats were divided into three groups of 6 rats each. Just 1 ml distilled water was administered to rats in the first group, and they served as control (I). The second and third group received 250 and 500 mg/kg pomegranate fruit powder dissolved in 1 ml distilled water respectively. Both distilled water and extract were given by gavage daily for eight weeks. At the end of the experiment, the animals were weighed and were sacrificed under ether anesthesia, and one testis from each animal was randomly chosen for study.

The testis index was calculated by dividing testis weight by body weight and then multiplying it by 100 (left testis weight/bodyweight $\times$ 100).

For stereological study, the selected testes were weighed then, fixed in fixative solution (formalin 10%) for six days at room temperature. The orientator method was used to obtain isotropic uniform random (IUR) sections [13]. Briefly, the testes were placed separately in a uniformly divided  $\Phi$  clock that was divided into 10 equal parts. A number was selected randomly between 0 and 9 for example here 6, was selected and the testis was cut into two pieces in this number. One of the two pieces of testis that has been generated by previous step was chosen at random, the cut surface of this piece of testis was placed vertically on the cosine-weighted  $\theta$  clock with unequal divisions. Finally a second uniform random number between 0 and 9 is generated. In the example 4 has been selected. The piece is then cut perpendicular to the plane of the clock and along the random direction selected (i.e. 4-4). Thus, a set of systematic cuts perpendicular to the plane of the clock was then generated along the random direction selected. The cut surface of the other piece of the testis was placed parallel to the 0-0 line of cosine-weighted  $\theta$  clock and the second cut done by selecting a random number (here 5). Then, this piece was completely cut as first piece. Finally, the entire testis was cut into slabs. All the slabs were embedded in blocks of paraffin. The first section (5 mm thick) of each block was stained with haematoxylin and eosin and used for the stereological and structural study.

For volume estimation, a microprojector (Ken-a-Vision, Inc., USA) were used to project images of the testes sections onto a desktop at a magnification of 20X. Projections were done in a dark room. Then, the volume testis was estimated by superimposing a transparent test point system on images and counting the points that fell on the images. Volume was estimated using the following formula and was calculated mm<sup>3</sup>:

$$V = \frac{\sum P \cdot a/p \cdot t}{M^2}$$

$\sum P$  denote the points counts, (a/p) represent the area associated with each test point, t is the mean slab thickness and M, is the linear magnification [14].

To estimate of the volume fraction of the interstitial and seminiferous tubule, point-counting was used [15, 16]. The stereological probe (composed of points) was superimposed on the pictures of the tissue sections that was produced by a microprojector on the desktop. All points hitting the interstitial tissue and seminiferous tubule were counted and divided by the points that had metg with the reference space (testis). The fraction volume (Vv) of each part at final magnification was determined using the following formula:

$$\text{estVv} = \frac{P(\text{part})}{P(\text{ref})}$$

where P (part) indicates the number of points hitting the interstitial tissue or seminiferous tubule and P(ref) the number of points hitting the testis. The absolute volume of each part was estimated by multiplying the fractional volume by the volume of the testis [17].

$$V(\text{part}) = V_v \times V(\text{testis})$$

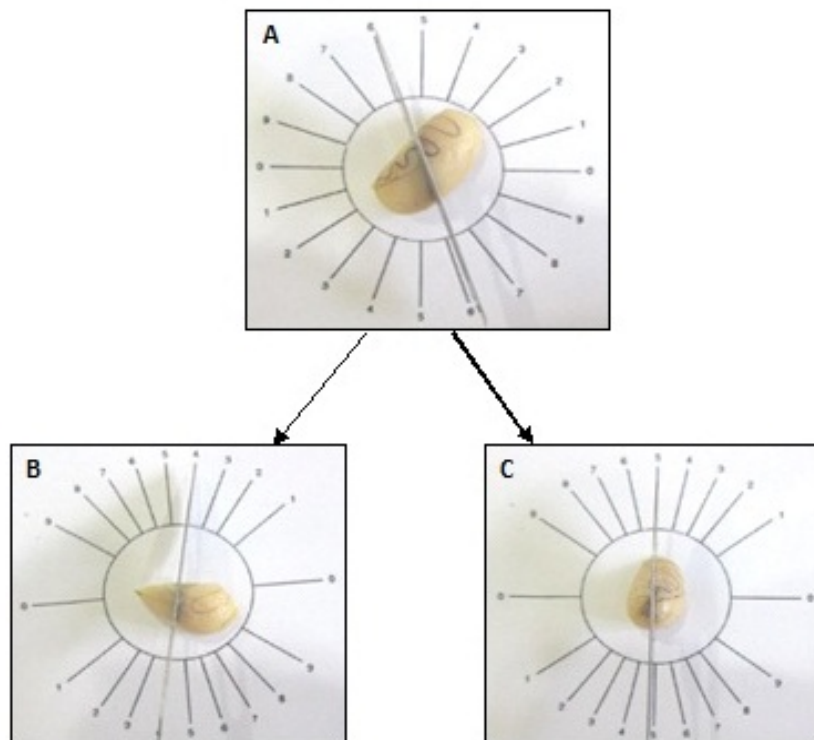
To estimation of total length 10-15 microscopic fields were examined in each testis. At first, tissue images by a microscope (Olympus, BH2, Japan) equipped with a camera and video-microscopy (BMZ.04-DZ, Behin Pajohesh, Enc, Iran) that connected to computer were thrown on monitor then, the length density of the seminiferous tubule was estimated by randomly overlaying an unbiased counting frame on pictures in the monitor at final magnification of 200. The tubule profiles completely inside the counting frame or partly inside the counting frame but only hitting the acceptance line (top and right lines) were counted. The tubule profiles hitting the forbidden line (bottom and left lines) and its extensions were ignored. The length density (Lv) of the tubule was estimated using the following formula:

$$\text{estLv (tub/test)} = 2 \times \frac{\Sigma Q}{a(\text{frame}) \times \Sigma \text{ frames}}$$

where  $\Sigma Q$  is the total number of the tubule profiles counted, a (frame) equals the area associated with a frame ( $600\mu\text{m} \times 600\mu\text{m}$ ), and  $\Sigma \text{ frames}$  is the total number of frames counted. Finally, to determine total length of tubules, was used the following formula:

$$L_{\text{Total}} = L_V \cdot V_{\text{testis}}$$

Data are expressed as the mean $\pm$ SD. One-way ANOVA by SPSS for Windows (version 15) was used for statistical analysis followed by Tukey's-test.  $P < 0.05$  was assumed as statistically significant



**Figure 1.** Isotropic Uniform Random (IUR) sections.

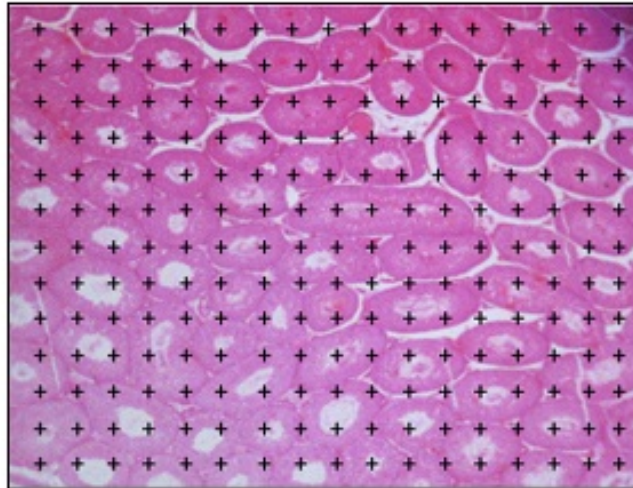


Figure 2. Transparent test point system on images (frame equals).

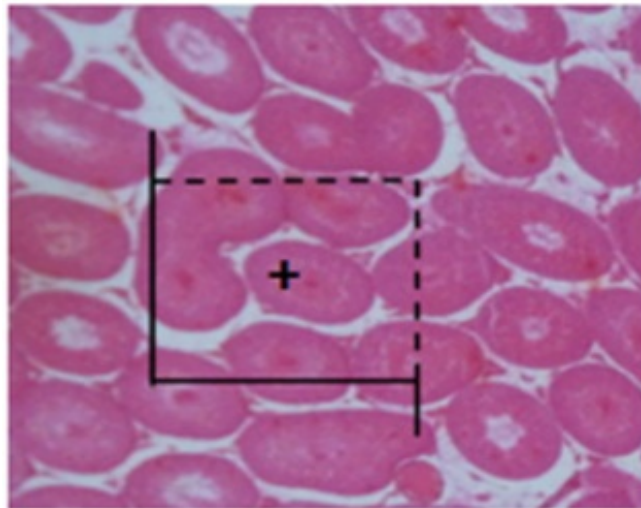


Figure 3. Transparent test point system on images.

### Results and discussion

Table 1 showed that administration of pomegranate hydro-alcoholic extract in doses of 250 and 500 mg/kg significantly increased testis weight and testis index of animals when compared to control group ( $p < 0.05$ ).

**Table I.** Comparison of testis index and testis weight of rats between groups after 8 weeks administration of pomegranate hydro-alcoholic extracts. I, control; II, received extract (250 mg/kg) and III, received extract (500 mg/kg)

Groups	Testis index	Testis weight (gr)
I	$.36 \pm .02$	$1.01 \pm 0.09$
II	$.48 \pm .07^*$	$1.33 \pm 0.1^*$
III	$.44 \pm .05^*$	$1.23 \pm 0.08^*$

Data are expressed as mean  $\pm$ SD, \*  $p < 0.05$  compared with the control group.

Stereological examination of the total volume of the testis and its components are shown in Table 2. Oral administration of pomegranate hydro-alcoholic extract at two doses given, significantly increased the total volume of the testis, seminiferous

tubules and interstitial tissue compared to ( $p < 0.05$ ) control. However, length of tubules did not show any significant difference in the rats which received pomegranate extract in comparison with the controls ( $p > 0.05$ ).

**Table II.** Comparison of testis, seminiferous tubules, interstitial volume and total length of testicular tubules between groups after 8 weeks administration of pomegranate hydro-alcoholic extracts. I, control; II, received extract (250 mg/kg) and III, received extract (500 mg/kg)

Groups	Testis volume (mm <sup>3</sup> )	Seminiferous tubule volume (mm <sup>3</sup> )	Interstitial volume (mm <sup>3</sup> )	Total length of testicular tubules (m)
I	523.66±28.62	351.5±32.41	75.16±9.06	18.71±1.32
II	706.50±34.42*	554.83±40.56*	117.66±7.17*	19.96±1.55
III	696.60±25.23*	507.66±45.87*	106.5±13.24*	17.89±2.23

Data are expressed as mean ±SD. \*  $p < 0.05$  compared with the control group.

The phytochemicals detected in pomegranate are mainly polyphenols, such as; tannins, anthocyanins, flavonoids, ellagitannins, ellagic tannins, gallic, ellagic acids and catechin [18]. Also the Pomegranate consists three estrogen compounds kaempferol, luteolin and quercetin [19]. Pomegranate is known to contain estrogens (estradiol, estrone, estriol) and indicates estrogenic activities in animals [20]. This means that pomegranate rind extract consists polyphenolic compounds that operate similar estrogens. In this study, gavage administration of pomegranate hydro-alcoholic extract increased testes weight and testes index. The body and organ weight can be affected by a prescribed amount of polyphenols. A diet with low levels of flavonoid or proanthocyanidin no significant effect on body weight, but it reported which some of compounds such as tannins interact with proteins and prevent digestion of endogenous protein probably cause weight loss [21]. Researchers also reported it was observed that the pomegranate had no significant effect on body weight [12]. But the increase of testicular weight in the groups that received extracts might be the result of a physiological response to exposure to some doses. Our results are similar to previous study [22]. The data show that testicular volume and length of seminiferous tubules in the different groups did not differ. These results support the report of [23]. However an increase in volume of seminiferous tubules and reduction in volume of interstitial tissue was observed. Increase of tubules volume may be caused by improvements of spermatogenic cell density. Because pomegranate prevent excessive generation of free radicals, produced by spermatogenic cells due to its antioxidant property. Thus reduce of free radicals decrease apoptosis in the germinal epithelium and increased density of the germinal epithelium cells [12]. Moreover, it was suggested that pomegranate increases testosterone, FSH and LH levels in blood. The increase in testosterone should enhance androgen-dependent parameters such as maintenance of spermatogenesis. Also, FSH stimulates spermatogenesis and increases the sertoli cells in tubules. Moreover, LH stimulates synthesis and release of testosterone in the leydig cells which these cells are the main cells in the interstitial tissue. These factors also lead to increased cell density in the seminiferous tubules [24]. Previous studies have confirmed our results [12, 25]. In conclusion, results of current study revealed that pomegranate hydro-alcoholic extract not only had no adverse effects on the testes but also increased testicular weight, cell density of the tubules and interstitial tissue in testis of rats.

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