

Effect of Methanolic and Phenolic Extracts of *Nigella sativa* Seeds on Testicular Expression Levels of inhibin alpha and beta Genes in Mature Male Wistar Rats

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ABSTRACT

To examine the effect of crude methanolic extract and phenolic compounds obtained from *Nigella sativa* seeds on testicular expression levels of inhibin- α , - β A, and - β B subunits of intact mature male rats, thirty males were randomly assigned to three equal groups (C, M, and P). Males were orally administered with distilled water, methanolic extract of *N. sativa* seed at the dose of 1.0 g/ kg b.w., and phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg b. w., daily for 18 days. At the end of treatment period, male rats were anesthetized by single injection of thiopental (100 mg/ kg, i.p.), sacrificed, and testes were removed. Samples from testicular tissues were quickly dipped in DEPC solution and frozen at -80 °C until gene expression levels assessment of inhibin subunits by using semi-quantitative RT-PCR analyses. Male rats that have been treated with phenolic compounds (P group) registered marked increase of all inhibin subunits compared with those treated with methanolic extract, which they were also higher than that of control males. It can be concluded that phenolic compounds of *N. sativa* seeds have the more efficient role in reproductive functions than methanolic extract of *N. sativa* seed.

INTRODUCTION

Nigella sativa, from the Ranunculacea family, is one of the most widely used herbal medicines for the treatment of various diseases. Its pharmacological properties are thought to be due to Nigellone and Thymoquinone contents which have been reported to include anti-inflammatory, anti-cancer, anti-diabetic, anti-microbial, anti-histaminic, anti-infertility and hypotensive effects (Mukhallad *et al.*, 2009; Alenzi *et al.*, 2010). Thymoquinone is the active compound of the essential oil with anti-oxidative effect that works as a scavenger of various radical oxygen species including superoxide radical anion and hydroxyl radicals through different mechanisms as well as to its ability to significantly boost the human immune system, if taken over time (Haq *et al.* 1999; Mansour *et al.*, 2002; Badary *et al.*, 2003; Mahgoub 2003; Salem, 2005). Numerous studies have shown that

the seed and oil of black seeds are characterized by a very low degree of toxicity (Gali-Muhtasib *et al.*, 2006). Hormones are chemicals that transfer information and instructions between cells in animals hence described as the body's chemical messengers, these regulate growth and development, control the function of various tissues, support reproductive functions, and regulate metabolism in a slow manner (Melmed and Conn, 2005).

FSH and LH mainly regulate inhibin production in the ovary; both gonadotropins stimulate its production in granulosa cells and in the corpus luteum, respectively (Zhang *et al.*, 1987; Tsonis *et al.*, 1987).

Inhibins and activins are members of the TGF- β superfamily. Inhibins are heterodimeric glycoproteins composed of a unique α -subunit linked with one of β -subunits (β A and β B) by disulphide bonds. Hetero-dimerization of the α -subunit with either β A- or β B-subunit generates dimeric inhibin A (α - β A) and inhibin B (α - β B), respectively. These factors are the strongly inhibitors of pituitary FSH secretion (Burger, 1993; Knight and Glister, 2001).

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The proportion of the α -subunit in the tissue where it is synthesized, as well as in circulation, is higher than that of the β -subunit (Knight *et al.*, 1989). Activins are group of growth and differentiation factors that have a variety of functions. Activins are synthesized in a large number of tissues and are considered paracrine rather endocrine factors (Welt and Crowley, 1998).

It has been found that daily oral administration of *N. sativa* oil improved reproductive efficiency of hyperlipidemic rats (Samir Bashandy, 2007). Mukhallad *et al* (2009) reported an increased spermatogenesis of male rats treated with the aqueous extracts of *N. sativa*. In addition, El-Tahomi *et al* (2010) registered an improvement of the semen characteristics and reduction of free radicals in the seminal plasma of male rats treated with a mixture of equal quantities from radish, rocket and black cumin (*N. sativa*) meals. Therefore, this study was conducted to examine the effect of crude methanolic extract and phenolic compounds obtained from *Nigella sativa* seeds on fertility potential through evaluation of testicular expression levels of inhibin- α , - β A, and - β B subunits of mature male rats.

MATERIALS AND METHODS

Experimental Animals

Adult male Wister rats (average weight was 245 ± 10 g.), were obtained from the National Laboratory Animal Center and reared under controlled conditions (12L:12D cycles at 20-22 C°) and fed standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water.

Preparation of methanolic and phenolic extracts

N. sativa seeds were purchased from the local market and classified by State Board for Seed Testing and Classification, Ministry of Agriculture, Iraq (SBSTC). Phenolic extract has been prepared according to Ribereau-Gayon (1972), whereas methanolic extract has been prepared according to Harborne (1984).

Experimental Design

Thirty mature male rats were randomly assigned to three equal groups. All males were orally administered, for 30 days, with distal water (control group), phenolic extract of *N. sativa* seed at the dose of 0.3 g/ kg bw (P group), and methanolic extract of *N. sativa* seed at the dose of 1 g/ kg bw (M group). Twenty four hours after the last treatment, male rats were anaesthetized with thiopental (100 mg/ kg, i.p.), sacrificed and testis were removed. Samples from testis of rats in all groups have been quickly dipped in DEPC solution, and frozen at -80 °C for determination of inhibin alpha and beta gene expression levels by semi-quantitative RT-PCR analyses.

RT-PCR analysis

RNA has been extracted from the testis tissues according to the protocol mentioned by Surzcki (2000). Master Mix for each sample was prepared according to the recommendations of the

manufacturers, and the same procedure followed in the determination of endogenous gene and target genes. PCR products have been transferred into gel electrophoresis apparatus for obtaining and determining the studied genes bands. This step has been done by mixing 2 μ l of loading dye with 10 μ l of each PCR product (each sample) and 2 μ l of ladder. The mixture was loaded into gel lanes.

Finally, gel electrophoresis apparatus was turned on at 90 mV for 40 minutes. Gel documentation step has been carried out according to the protocol mentioned by Surzcki (2000).

Statistical Analysis

The results were expressed as mean \pm standard deviation of the mean (SDM). Comparisons were performed using one way analysis of variance (ANOVA1) and newman- keuls to test all groups' unpaired values. Differences were considered to be significant at the level of $P < 0.05$. The statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA, 2011).

RESULTS

Concentration of RNA in the testis

The result of total RNA concentration in testis tissues clarified in figure (1) revealed significant ($P \leq 0.05$) increase in mRNA concentration of P extract treated males compared with M extract treated males, which they also showed significant ($P \leq 0.05$) increase in comparison with control males.

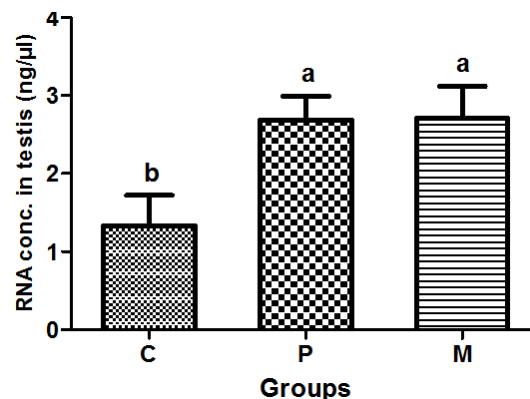


Fig 1: Effect of methanolic and phenolic extract of *N. sativa* seed on RNA concentration (ng/ μ l) in testis tissues of adult male Wistar rats.

The results represented as mean \pm SE.

Different small letters denotes the present of significant differences ($P < 0.05$) between groups.

C: control male rats orally administered with distal water for 30 days.

P: male rats treated with P extract of *N. sativa* seed (0.3 g/kg bw) for 30 days.

M: male rats treated with M extract of *N. sativa* seed (1 g/kg bw) for 30 days.

RNA normalization

The present findings showed that the optical density of each sample at 260 nm and 280 nm as well as the dilution folds needed to prepare the final concentration (100 ng/ μ l) necessary to complete the following steps of semi-quantitative RT-PCR, were within the normal range, where the ratio between the two optical densities were more than 1.8 and less than 2.2.

mRNA expression level of beta actin

Beta actin gene expression level has been used as a corresponding gene for normalization and quantification of inhibin- α , - β a, and - β b genes (figure-2).

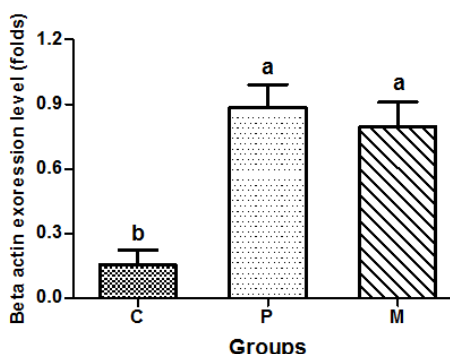


Fig 2: Effect of methanolic and phenolic extract of *N. sativa* seed on mRNA expression level of Beta actin (folds) in testis tissues of adult male Wistar rats. The results represented as mean \pm SE.

Different small letters denotes the present of significant differences ($P < 0.05$) between groups.

C: control male rats orally administered with distal water for 30 days.

P: male rats treated with P extract of *N. sativa* seed (0.3 g/kg bw) for 30 days.

M: male rats treated with M extract of *N. sativa* seed (1 g/kg bw) for 30 days.

mRNA Expression Level of inhibin alpha in testis tissues

mRNA expression level of inhibin- α subunit gene, illustrated in figures (3), of P extract treated males testes increased significantly ($P \leq 0.05$) in comparison with that recorded by M extract treated males testes, which was also significantly ($P \leq 0.05$) higher than that of control males throughout the experimental period.

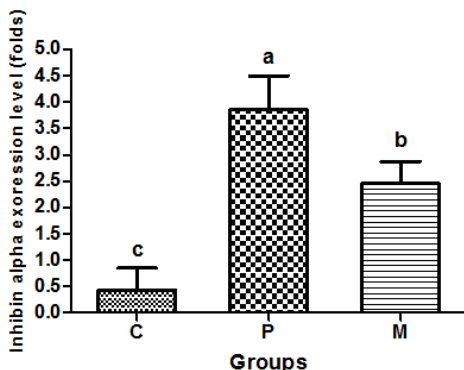


Fig 3: effect of methanolic and phenolic extract of *N. sativa* seed on mRNA expression level of inhibin alpha subunit (folds) in testis tissues of adult male Wistar rats.

The results represented as mean \pm SE.

Different small letters denotes the present of significant differences ($P < 0.05$) between groups.

C: control male rats orally administered with distal water for 30 days.

P: male rats treated with P extract of *N. sativa* seed (0.3 g/kg bw) for 30 days.

M: male rats treated with M extract of *N. sativa* seed (1 g/kg bw) for 30 days.

mRNA Expression Level of inhibin beta-A and beta-B in testis tissues

The results of mRNA expression level of inhibin β gene illustrated in figures (4) recorded insignificant ($P \geq 0.05$) changes between P and M groups but they were significantly ($P \leq 0.05$) higher than control. Figure (5) showed significant ($P \leq 0.05$)

increase of β b expression level in P group than that of control and M groups, whereas M group also recorded significant ($P \leq 0.05$) increase compared with control.

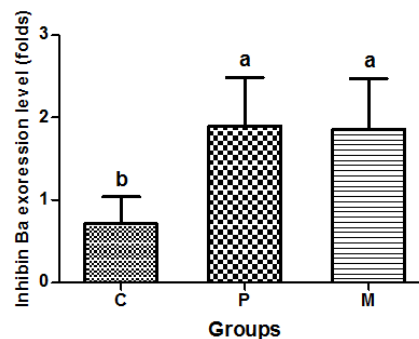


Fig 4: Effect of methanolic and phenolic extract of *N. sativa* seed on mRNA expression level of inhibin Beta-A subunit (folds) in testis tissues of adult male Wistar rats.

The results represented as mean \pm SE.

Different small letters denotes the present of significant differences ($P < 0.05$) between groups.

C: control male rats orally administered with distal water for 30 days.

P: male rats treated with P extract of *N. sativa* seed (0.3 g/kg bw) for 30 days.

M: male rats treated with M extract of *N. sativa* seed (1 g/kg bw) for 30 days.

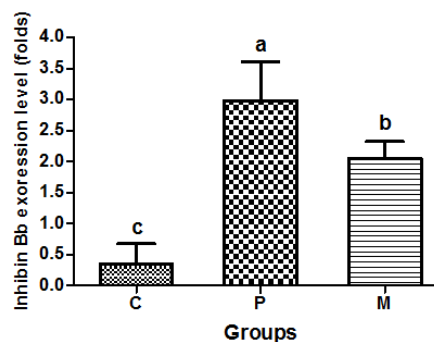


Fig 5: Effect of methanolic and phenolic extract of *N. sativa* seed on mRNA expression level of inhibin Beta-B subunit (folds) in testis tissues of adult male Wistar rats.

The results represented as mean \pm SE.

Different small letters denotes the present of significant differences ($P < 0.05$) between groups.

C: control male rats orally administered with distal water for 30 days.

P: male rats treated with P extract of *N. sativa* seed (0.3 g/kg bw) for 30 days.

M: male rats treated with M extract of *N. sativa* seed (1 g/kg bw) for 30 days.

DISCUSSION

The increase of testicular alpha subunit expression level in treated groups could reflect the high production of inhibins. On the other hand, the increase of expression levels of beta-B and unchanged beta-A unit expression reflect the high production of inhibin-B but not inhibin-A nor activins by Sertoli cells, since these cells are responsible for the production of inhibins and activins in response to high levels of pituitary gonadotropin secretion (Melmed and Conn, 2005). In the findings, treating male rats with M and P extracts of *Nigella sativa* revealed significant inhibin subunits genes expression levels elevations in comparison with control males, particularly male rats that treated with P extract. Most properties of the whole black seeds or their extracts are mainly attributed to the quinone constituents present in the

volatile oil, of which thymoquinone, about 27–57%, is the most abundant component (Ali and Blunden, 2003; Gali-Muhtasib *et al.*, 2006). Thymoquinone was reported to re-establish spermatogenesis after testicular injury caused by chronic toluene exposure in rats (Kanter, 2011). A nother study by Arak and Assi (2011) reported that adult rats exposed to 10 mg/kg of lead acetate caused a significant reduction on ovarian function and the treatment of 100 mg/kg of *Nigella sativa* caused significant enhancement on the reproductive function with a decrease in number and diameter of Graafian follicles in comparison to the normal structure. The increased inhibin- α subunit especially in *Nigella sativa* P extract treated male's testicular tissues could be a response to the high activity of Sertoli cells, since these cells activities were always under the influence of FSH, where it has been evidenced that *Nigella sativa* extract increased the circulatory levels of FSH in mature male rats (Al-Sa'aidi *et al.*, 2009). On the other hand, although FSH secretion is primarily regulated by GnRH and gonadal steroids, there are other paracrine and endocrine factors such as pituitary activin and follistatin and testicular inhibin β that only regulate FSH without affecting LH secretion and synthesis. Activin stimulates FSH β gene transcription through activation of the Smad family of proteins. The testicular protein inhibin β , secreted by Sertoli cells, competes with activin for binding to the activin receptor, preventing the initiation of signaling of Smads and thus decreasing FSH gene transcription. Sertoli cells thus appear to mediate the biologic actions of both circulating hormones and the paracrine regulatory factors. The presence of activins in spermatocytes at defined stages, in unfertilized and fertilized ova, and in various organs throughout embryogenesis suggests that they must be important to development (Mitrani *et al.*, 1990). The inhibin secretion by Sertoli cells in response to FSH stimulation is so important, since inhibin will furtherly suppresses pituitary FSH, and therefore can act as a tumor suppressor, and has paracrine functions in the gonads (Bernard *et al.*, 2001; Wood and Strauss, 2002).

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