

1 ORIGINAL ARTICLE

2 Aqueous Extract of *Nigella sativa* Seeds Suppresses
3 Testicular Steroidogenesis in Mice Leydig Cells in
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10 ABSTRACT

11 *Nigella sativa* (black seed) is an important medicinal herb with folkloric use in wide range of diseases. It is
12 well studied for its biological activities. However, there is limited information regarding its effect on the
13 male reproductive system. This study describes the effect of the aqueous extract of *N. sativa* (NSE) on
14 testicular steroidogenesis from mice Leydig cells *in vitro*. Mice testicular cells were incubated in a media
15 containing either no treatment or NSE or LH alone or combination of LH and NSE. Incubations were
16 carried out for three hours in a shaking water bath at 34°C. Testosterone was measured by
17 radioimmunoassay. At all doses, NSE significantly ($p < 0.05$) inhibited both basal and LH-stimulated *in*
18 *vitro* testosterone secretion. At a dose of 1000 µg, NSE inhibited 52% of basal testosterone and 97% of
19 LH-stimulated testosterone, compared to control (0.32 ± 0.008 ng/ml) and LH alone (0.33 ± 0.01 ng/ml)
20 respectively. Thus, it is concluded that that both the basal and the LH-stimulated secretion of testosterone
21 from Leydig cells are suppressed significantly in the presence of different doses of NSE *in vitro*. However,
22 further studies are needed to explore the effect of chronic treatment with NSE in male and its potential to
23 be used as a contraceptive in male.

24 **Keywords:** *Nigella sativa*, Black seed, Male reproductive system, aqueous extract, Leydig cell,
25 testosterone

26 The seeds of *Nigella sativa* Lin. (Ranunculaceae), 41 for its different biological activities which includes
27 commonly, known as black seed or black cumin and 42 antioxidant, hepatoprotective [3], nephroprotective,
28 locally as Kalonji have been used in folk (herbal) 43 antihypertensive [4], muscle relaxant, bronchodilator
29 medicine for centuries for treatment of many acute as 44 [5,6], CNS depressant effects [7], antilipidemic [8],
30 well as chronic conditions worldwide [1,2]. It has been 45 antidiabetic [9,10], anticancer [11,12], analgesic
31 used in the treatment of asthma, diarrhea, indigestion, 46 [13,14], anti-inflammatory [13,15], antiulcer [16] and
32 dizziness, influenza, dyslipidemia, many dermatological 47 neuroprotective effects [17,18].

33 conditions and as a diuretic and immune modulator. The 48 Much of the biological activities of the black seeds
34 seeds contain 36%-38% fixed oils, proteins, alkaloids, 49 have been shown to be due to the presence of
35 saponins, 0.4%-2.5% essential oil, crude fiber, minerals, 50 thymoquinone, which is the major component of the
36 vitamins, aliphatic alcohols and ketones [1]. 51 essential oil and fixed oil. Nigellone, is another
37 Many studies have been conducted on the 52 compound of *Nigella sativa*, which has been shown to
38 pharmacological action(s) of *Nigella sativa* seed extract 53 be very effective in inhibiting histamine release induced
39 or its active compound(s) on various body systems *in* 54 by the secretagogues: antigen in sensitized cells [19].
40 *vivo* or *in vitro*. The herb has been extensively studied 55 However, the herb is not well studied for its effect on

56 reproductive system. Moreover, the existing information
57 in this regard is quite scanty and rather contradictory.
58 Significant abortifacient activity of *N. sativa* seed
59 powder, ethanolic and hexane extracts was
60 demonstrated in rats [20]. However, Prakash et al [21]
61 did not find any anti-fertility activity in aqueous,
62 ethanolic and petroleum ether extracts of the seeds of *N.*
63 *sativa* when tested at a dose of 150-200 mg/kg daily in
64 rats on the days 1-7 post-coitum schedule.

65 There is a growing demand for men to share the
66 burden of responsibility and risks of contraception
67 because of growing population pressures and the
68 increasing dissatisfaction of women in assuming almost
69 all the risks of adequate contraception. A major
70 challenge in this field is that the most of the male
71 contraceptive agents currently in use offer little promise
72 and about 15% of the 200 most commonly prescribed
73 drugs can have adverse effects on male reproduction
74 either by influencing its hormonal profile or impairing
75 their sexual performance. The discovery of key
76 regulators of gonadal hormones and gametogenesis
77 from black seed may provide opportunities to alter our
78 approaches towards management of contraception.

79 Since, no data on the effect of *N sativa* on testicular
80 steroidogenesis is available, we designed this *in vitro*
81 study to investigate the direct effect of crude aqueous
82 extract on basal and LH-stimulated testicular
83 steroidogenesis by mice Leydig cells.

84 MATERIALS AND METHODS

85 Preparation of the crude extract

86 Dried black seeds of *Nigella sativa* were purchased
87 from the local market in Karachi. The plant seeds were
88 cleaned of any adulterant materials. NS seeds were
89 ground with an electric grinder into a coarse powder. A
90 measured quantity was soaked in 70% aqueous-
91 methanol (30:70) at room temperature by cold
92 maceration for a total of 3 days. Thereafter, the filtrate
93 was collected through Whatman's qualitative grade
94 filter papers and the plant material was again subjected
95 to the same treatment as the first macerate. The
96 combined filtrate was concentrated using a rotary
97 evaporator at 40°C under reduced pressure. Extract was
98 stored at -4°C until used for biological activity.

99 Leydig cells preparation

100 Three bulbee male mice (weight 36 ± 2) were used
101 for each experiment. Animals were obtained from the
102 AKU animal facility, where they were maintained under
103 standard conditions of 14-hour light and 10-hour dark
104 cycle.

105 Direct effect of aqueous extract of *Nigella sativa*
106 seeds (NSE) on testosterone secretion was studied by
107 the incubation of Leydig cells as described by Van-
108 Damme et al, 1974 [22], with minor modifications. Mice
109 were killed by cervical dislocation. Testes were

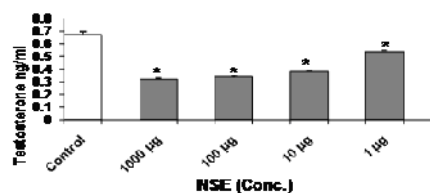


Fig 1. Effect of aqueous extract of *Nigella sativa* seeds (NSE) on basal testosterone by mice Leydig cells *in vitro*

*Significant difference between control and treated groups ($p < 0.05$)

110 dissected out immediately and de-capsulated. Leydig
111 cells were isolated as described earlier [22]. Leydig
112 cells (80,000/tube) were pre-incubated for 1 h to remove
113 the endogenous testosterone, the media were replaced
114 with either fresh medium or medium containing graded
115 doses of crude aqueous extract of NS seeds (1.0-1000
116 µg/tube).

117 Moreover, to test the ability of the extract to
118 modulate stimulated testosterone secretion, samples
119 were challenged with LH (500 µIU/tube) alone or with
120 different doses of NSE (1.0-1000µg). After 3 h, the
121 incubation reaction was stopped by dipping the tubes in
122 water bath at 60°C for 10 min. Samples were kept frozen
123 until testosterone was measured by highly-specific
124 radioimmunoassay.

125 Radioimmunoassay

126 Testosterone was measured in the incubation
127 medium directly by a highly-sensitive RIA according to
128 WHO protocol, using ^3H -labeled testosterone, as tracer.
129 Highly specific antiserum for testosterone was acquired
130 from Guildhay UK. RIA reagents were directly added to
131 tubes containing incubation medium. After addition of
132 all the reagents, tubes were incubated for 30 min. at 4°C.
133 The bound and unbound fractions were separated by the
134 addition of 0.1% activated charcoal. Radioactivity was
135 measured in a scintillation counter. Testosterone
136 concentration was calculated by logit-log
137 transformation [23].

138 The sensitivity of T assay was 0.0125 ng and the
139 intra-assay coefficient of variation was less than 10%.
140 The levels of testosterone in the media are expressed as
141 ng/ml.

142 Statistical analysis

143 Data are expressed as mean \pm S.E.M. Results were
144 analyzed for statistical significance using an
145 independent t test on SPSS. A p value < 0.05 was
146 considered significant.

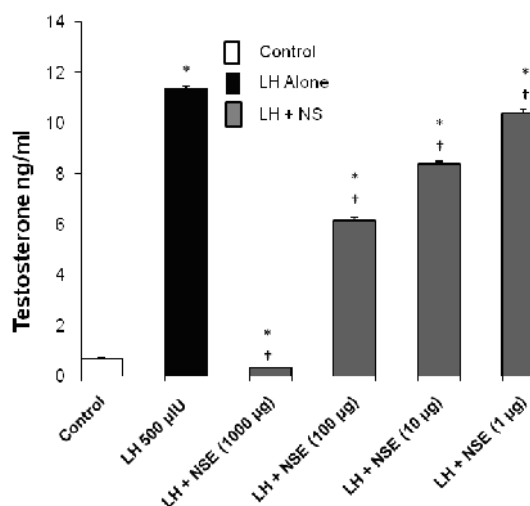


Fig 2. Effect of aqueous extract of *Nigella sativa* seeds (NSE) on LH-stimulated testosterone production by mice Leydig cells in vitro

*Significant difference between control and treated groups ($p < 0.05$)

†Significant difference between LH alone and treated groups ($p < 0.05$)

RESULTS

147

148 *Nigella sativa* seeds extract was able to inhibit significantly ($p < 0.05$) both basal and LH-stimulated testicular testosterone secretion *in vitro*. Moreover, the inhibitory effect of NS seed extract was more pronounced at the higher doses.

153 Effect on basal testicular steroidogenesis

154 As shown in the Fig 1, basal testosterone production in the cells treated with NS seed extract (1.0–1000 µg/tube) was significantly ($p < 0.05$) reduced compared with the control in a dose-dependent manner. The inhibition was more pronounced at the higher doses. Inhibitory effect of NSE was able to inhibit 52% of the basal testosterone production and this inhibition was still present at the lowest NS dose of 1.0 µg.

162 Effect on LH-stimulated testicular steroidogenesis

163 As shown in Fig 2, administration of different doses of NS seed extract (1.0–1000 µg) caused a significant ($p < 0.05$) and a dose-dependent inhibition of LH-stimulated (500 µIU) testosterone production. The inhibition was more pronounced at higher doses of NSE with maximum effect (97% inhibition) obtained at 1000 µg dose of NSE. LH (500 µIU) was used for maximal stimulation. This dose was selected from LH/testosterone dose–response curve to variable doses of LH (16–500 µIU) (data not shown). Treatment with NSE caused dose-dependent inhibition of the LH-stimulated testosterone production when compared to LH 500 µI response (Fig 2, with maximum effect (97% inhibition) obtained at 1000 µg dose).

77

DISCUSSION

78 This study provides the first evidence for a strong effect of *N. sativa* seed extract on testicular steroidogenesis indicating a potential contraceptive role. Our data suggest that *N sativa* extract inhibits both basal and LH-stimulated testosterone biosynthesis signaling pathways. The mechanism behind its effect is not clear and further studies are needed to elucidate its further role and mechanism of action. The crude extract of *N. sativa* seeds has been reported to possess calcium channel blocking activity [5] and there is evidence that calcium may be involved in the signaling mechanism [24]. Significant abortifacient activity of *N. sativa* seed powder, ethanolic and hexane extract, is demonstrated in women [20,25] and rats [21]. However, Prakash et al. [26] did not find any anti-fertility activity in aqueous, ethanolic and petroleum ether extracts of the seeds of *Nigella sativa* when tested at a dose of 150-200 mg/kg daily in rats on the days 1-7 post-coitum schedule. The volatile oil of *Nigella* seeds inhibits the spontaneous movements of rat and guinea pig uterine smooth muscle and also the oxytocin-induced contractions [27]. A single report in male rats has suggested that seed extract treatment not only causes a general reduction in the size of reproductive organs but also suppresses spermatogenesis at the spermatocyte stage. However, similar changes in the reproductive hormones of the treated animals was not observed [28].

The testis is a complex male reproductive organ that serves two crucial functions: synthesis and secretion of testosterone by Leydig cells and production of a sufficient number of competent spermatozoa supported by Sertoli cells, to attain fertility. It is well known that the essential prerequisite for normal testicular development and maintenance of spermatogenesis is the controlled secretion of Luteinizing hormone (LH), Follicle stimulating hormone (FSH), and testosterone during fetal and postnatal life [29]. A deficiency of these hormones leads to hypogonadism and sterility, a condition that can be treated with specific replacement therapies [30]. Testosterone biosynthesis in the Leydig cells is primarily regulated by LH [31]. Deficiency of these hormones leads to hypogonadism and sterility, a condition that can be treated with specific replacement therapies [30]. Reversible inhibition of these hormones by any external measure may be beneficial as it can be used as a contraceptive. Oral administration of crude ethanol extracts showed significant contraceptive effect in male rats [20]. Since, no data about the effect of NS seed extract on testicular steroidogenesis have yet been available; these results open new fronts in the exploration of possible effects of *Nigella sativa* on the reproductive functions. These data offer insights into potential contraceptive effects of *Nigella sativa* on the hormonal regulation of male reproductive axis. This study has provided us with important insight towards formulation of a new contraceptive pill that would temporarily stop spermatogenesis, thus producing reversible infertility.

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