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A Morphological Study of the Pharmacological Effects of the *Nigella sativa* on the Reproductive System in Experimental Rats

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Abstract

Nigella sativa (Black seed or Black cumin or Habbat Albaraka), which belongs to the Ranunculacea family, is an annual herb used in food as a spice. In addition, *N. sativa* is an important medicinal herb used traditionally against a wide range of diseases. This herb has been well-studied for its pharmacological activities. However, there is limited information regarding its effects on the female reproductive system. This study describes the effects of the aqueous extract of the seeds of *N. sativa* on the endometrium in female rats. A single daily dose of 0.2g / 100g body weight (B\wt) of the crude aqueous extract of the seeds of *N. sativa* was administered orally to mature fertile female albino rats for ten days. The rats were subdivided into subgroups, according to the phase of the estrus cycle. The utera of these animals were routinely processed for general histological studies, using carboy's fixed paraffin embedded sections. Compared with the control subgroups, the results showed an increase in the uterine wet weight of all the experimental subgroups, with a profound and persistent diffuse endometrial hypertrophy, and enhanced glandulogenesis. In conclusion, the crude aqueous extract of the seeds of *N. sativa* seemed to have achieved its effects via stimulating the endogenous release of estrogen and/or progesterone. However, direct estrogen and/or progesterone-like actions of the seeds could not be excluded.

Keywords: Black seeds (*Nigella sativa* L), Uterine wet weight, Glandulogenesis, Subnuclear Zone, Metrail gland, Diffuse Endometrial hypertrophy.

1. Introduction

The black seed *Nigella sativa* is an indigenous herbaceous plant, more commonly known as the fennel flower plant, which belongs to the buttercup or Ranunculacea family. It is a native plant from the Mediterranean area, and found growing in some other regions in the world such as Saudi Arabia and North Africa. The herb is widely cultivated throughout South Europe, Asia, Turkey, Pakistan, and India. The plant is used especially in the Middle East and South Asia as a spice, condiment, carminative, and for aromatic and medical purposes. There are several names attributed to *N sativa* in various countries around the world (Gilani *et al*., 2004; El-Tahir *et al.,* 2006; Assi et al., 2016; Randhawa and Alenazi, 2016). In Arab countries, the seeds are called 'Alhabba Alswda', "Habbat Albaraka" or 'Alkamoun Alaswad'. In Urdu and Hindi, they are 'Kalonji', and in Sanskirt 'Krishnajirika'. Moreover, they are referred to as 'Kalajira' in Bangali, 'Shonaiz' or 'Siyah Daneh' in Persian, and 'cörek Out' in Turkish. In English, they are called Black cumin', 'Ajenue' in Europe, 'Schwarz' in Germany, and 'Black Caraway' in American English. The scientific name is a derivative of the Latin word 'niger' meaning 'black'.

The active constituents of the seeds include volatile oil. Pharmacologically active constituents of volatile oil are: thymoquinone, dithymoquinone, thyohydroquinone, and thymol (Ghosheh *et al.,* 1999). Among these active components, thymoquinone (TQ) is the most abundant constituent (57-78 %) of the volatile oil of the *N. sativa* seeds (Gilani *et al.,* 2004), and is the constituent to which most properties of this herb are attributed (Tavakkoli *et al.,* 2018).

During the last two decades, literature has been replete with the subject of the pharmacological activities of *Nigella sativa* seeds. These studies revealed that *N. sativa* seeds, its oil, various extracts, and its active components have several therapeutic activities including: Bronchodilatory, anti-allergic (anti-histaminic), anti-inflammatory, analgesic, antipyretic, hypoglycemic, antibacterial, antifungal, anti-parasitic, antivirus, anti-cancer, antihypertensive, antioxidant, anticonvulsant, anti-parkinsonism, antidepressant, and antianexiety effects. The seeds have also been reported to reduce the volume of gastric acid secretion and ulcer index, and modulate the lipid profile (Khan, 1999; Gelani *et al.,* 2004; Kanter *et al*., 2005; El-Tahir and Bakeet, 2006; Ahmad *et al.,* 2013; Sandhu and Rana, 2013; Latiff *et al.,* 2014; Heshmati *et al*., 2015; Randhawa and Alenazi, 2016; Zafar *et al*., 2016; Daryabeygi-Khotbehsara *et al.,* 2017; Tavakkoli *et al.,* 2018). However very few studies have discussed the influence of the *N. sativa* seeds on the reproductive system, yet most of these limited studies have focused on the male reproductive system only (Agarwal *et al*., 1990; Gilani *et al.,* 2004). Therefore, the effects of the seeds on

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the female reproductive system have remained largely unknown (Kabir *et al.,* 2001; Yildiz and Balikci, 2016).

Moreover, the various preparations of *N. sativa* and its constituents have relaxant effects on various types of smooth muscles (Chakma *et al.,* 2001; Keyhanmanesh *et al.,* 2014). Aqel and Shaheen (1996) stated that the volatile oil of the black seeds of *N. sativa* prevents the spontaneous contraction of the uterine smooth muscle of rats and guinea pigs, and those induced by oxytocin.

Agarwal *et al.* (1990) stated that *N. sativa* ethanolic extract showed antifertility effects in male rats, probably due to its inherent estrogenic nature. In another study, (Kolahadooz *et al.,* 2014) stated that *N. sativa* could enhance sperm parameters, including sperm count, motility and morphology, semen volumes and pH. Other beneficial effects of the seeds on leydig cells and sexual hormones in infertile men have also been confirmed (Mahdavi *et al.,* 2015). In Unani medicine, *N. sativa* is recommended for use in the oligomenorrhoea therapy, infertility, and for inducing menstruation (Al-Jishi and Hozaifa, 2000). Parhizkar *et al.* (2016) stated that *N. sativa* plays a beneficial role in the treatment of postmenopausal symptoms, as its extracts displayed estrogenic activities. Other studies (Saha *et al.,* 1961; Agarwala *et al.,* 1971and 1986, Al-Snafi *et al*., 2014) showed that the seeds have beneficial effects on breast milk production in lactating women.

In 1995, a group of Indian workers (Keshri *et al.*) used the hexane extract of the seeds as a post-coital contraceptive in rats, when given at a single oral daily dose of 0.2g/100 g B.wt. on day 1-10 post-coitum. They reported a mild uterotrophic effect of such an extract, comparable almost to the ovulation dose of 17-α-estuarial, in the ovariectomized immature rat bioassay.

Accordingly, this study was conducted to investigate, for the first time, the effect of the crude aqueous extract of the seeds of *Nigella sativa* on the endometrium of mature rats during different physiological states. Comparative histological procedures at the level of light microscopy are attempted for this purpose.

2. Materials and Methods

2.1. Animals Used

Fifty fertile Norway albino female rats, aged 12-16 weeks, and weighting 194-199 g, were used in this study. Each animal was carefully selected to match the following criteria: (1) Being a mature female rat of a proven fertility that is decided by the history of previous deliveries. The last delivery should not be less than four weeks. (2) Had not been mixed with male partner(s) for the last two weeks. (3) Having regular estrus cycles, as judged by their vaginal smears cytology, done on a regular daily basis.

The animals were grouped according to their physiological state and the substance they received (Table 1). All rats were kept in air-conditioned quarters (30 $\pm$ 2C°), under standard husbandry conditions, with alternated 12h /12h dark/light cycle (Parhizkar *et al*., 2016). Animals were fed with a protein-standard pellet diet.

2.2. Experimental Animals

Thirty animals were used in this experiment. They were divided into three subgroups: (1) Proestrus (P), (2) Estrus (E), and (3) Diestrus (D), as shown in Table 1. Each rat received the crude aqueous extract of the crushed *N. sativa* seeds at the dose of 0.2g/100g B. wt (Keshri *et al.,* 1995), starting at the P, E, and D phase, respectively (Table 1). The seeds' powder was mixed with 4 mL of distilled water (Al-Khateeb, 1996; Sakran, 1999), administered via an orogastric tube (5.5 X 0.13), and was given once a day for the duration of ten days (Keshri *et al.,* 1995).

2.3. Control Animals

Twenty animals were used as control animals. They were divided into two subgroups: (1) Non-treated controls (C_n), which were used as standard reference models, and (2) A treated subgroup (C_R) which received four mL of distilled water (D.W.) only, via the orogastric tube, once a day for the duration of ten days (Table 1).

2.4. Uterine Wet Weight Measurement and Tissue Processing

For each animal (experimental and control), the exposure of the peritoneal cavity, and the removal of the body of uterus were done under open ether anesthesia. The wet weight of each respected uterus was recorded. Immediately afterwards, the specimens were fixed for a period of two hours in Carnoy's fluid (6 volumes absolute ethanol: 3 volumes chloroform: 1 volume Glacial acetic acid). The fixed tissue-specimens were processed for routine paraffin wax-embedding. Serial sections, each of a 5µ thickness were stained with the progressive Hematoxylin and Eosin stain.

2.5. Counts (Metrial Gland and Eosinophils Count)

The point-counting technique (Weibel and Gomez, 1962) with a Zeiss point-counting eyepiece graticule had been attempted in counting metrial gland sections per unit area at estrus. This was achieved by counting the number of metrial gland sections on each of a total of sixteen power fields (each of 0.37 mm diameter), in corresponding areas of adjacent sections.

Table 1. Animal groups and subgroups used in this study. All animals, were sacrificed after ten days.

Groups	Subgroups	No. of Animals	Substance received	Specific Phase of the cycle during which treatment commenced.	Dosage (g/100 g B.wt.)
Control	C_n	10	None	_____	_____
	C_R	10	D.W. only	Irrespective	_____
Experimental	D	10	Crude aqueous extract of the *N. sativa* seeds	Diestrus	0.2
	P	10	Crude aqueous extract of the *N. sativa* seeds	Proestrus	0.2
	E	10	Crude aqueous extract of the *N, sativa* seeds.	Estrus	0.2

Counting the infiltrating eosinophils was achieved by virtue of the method described by Al-Hadithi, 1994: the mean count of five randomly selected high power fields; they were taken to represent the eosinophils count of an individual section. For individual animals, the mean of at least four serial sections was taken to represent its infiltrating eosinophils count.

2.6. Data Analysis

The statistically significant difference between two groups' mean values, with continuous variables was calculated, using the two-tailed student t-test, and $P<0.05$ was taken as significant. The Schiff's f-test was also used for multiple comparisons between pairs of means when appropriate (Daniel, 1983).

3. Results

Vaginal smears of rats treated with the crude aqueous extract of the seeds of *Nigella sativa* showed a shortening of the vaginal cycle with prolongation of both the estrus and the metestrus phases; the diestrus phase was undetectable.

The experimental rats treated with the aqueous extract of the *Nigella sativa* seeds showed a significant increase in their uterine weights (almost ten times elevation) compared to their control fellows ($P<0.05$). No significant differences among the individual experimental subgroups were found (Figure 1).

Control rats' uteri , under a low power objective (X 22), were seen as pear-shaped elongated, cylindrical organs.

The two uterine horns were diverging proximally; while their outlets, approaching each other at the cervix, and were directed distally.

The examination of sections of the control rats' uteri at the proestrus phase showed that the uterine lumen was seen as distended, and the luminal epithelium was low cuboidal (Figure 2 A, inset arrow). The endometrial surface was thrown into marked longitudinal folds, but with very narrow crypts (Figure 4 A). The endometrial stromal glands were relatively scarce (Figure 2 B head arrows), and are lined with a single layer of low cuboidal cells (Figure 2C, head arrow); the stromal cells were generally elongated or spindle in shape with darkly stained oval nuclei. Nucleoli were generally absent, and the chromatin granules were evenly distributed. (Figure 2 C, thin arrow). Eosinophilic granulocytes were seen moderately infiltrating the stroma (Figure 2C inset), and different types of blood vessels were seen throughout the stroma (Figure 2B small arrows).

Sections of the control rats' uteri at the estrus phase showed that the luminal epithelium was tall columnar in type with large vesicular nuclei occupying a parabasal position (Figure 3 A inset arrows). Luminal crypts were dilated, and its lumen was distended with fluid (Figure 3 A). At a low power, the endometrial stroma showed increased cellularity (Figure 3 B). The metrial glands were lined with darkly stained columnar cells (Figure 3C head arrow). Endometrial stroma was heavily infiltrated with eosinophil granulocyte (Figure 3 C double arrow), and stromal cells were more or less spindle-shaped with darkly stained elongated nuclei. Mitosis was quite frequent amongst these cells (Figure 3 C, thin arrow).

The examination of sections of the control rats' uteri at the metestrus, showed that the endometrium was found shrunk, with a general distorted appearance. The functional layer of the endometrium (superficial layer) was disintegrated, and sheets of stromal elements, as well as surface epithelium were seen casted-off the endometrium (Figure 4 A, B, and C). However, the basalis layer of the endometrium (containing the deep part of the endometrial glands as well as its blood vessels) were relatively intact. Eosinophil granulocytes were remarkably absent amongst the stromal section (Figure 4 C).

The uterine sections of rats treated with *N. sativa* at the proestrus phase, showed profound endometrial hyperatrophy, with enhanced glandulogenesis (Figure 5 A and B). The luminal epithelium consists of tall columnar cells, with a characteristic subnuclear zone (Figure 5 C, white arrows), and subepithelial decidualization.

The uterine sections of the rats treated with *N. sativa*, at the esterus phase, showed diffuse endometrial hyperatrophy, with enhanced glandulogenesis (Figure 6A and B). Eosinophil granulocytes were absence (Figure 6 A). The surface epithelium was high columnar in shape, with well-developed subnuclear zones (Figure 6 C, thin arrows).

Stromal cells showed marked nuclear changes; being large and round or oval with coarsely granular and unevenly distributed chromatin materials, and eccentric nucleoli (Figure 6 C). Cells with reniform nuclei were also seen scattered throughout the stroma (Figure 6C, open arrow). Vascularity of the endometrium was well-established (Figure 6 C, open arrow). Mitosis of many stromal cells was frequent.

During the metestrus phase, the uterine sections of rats treated with *N. sativa* showed the persistence of metrial glands (Figure 7 A, arrows), as well as well-developed endometrial stroma and subepithelial decidualization reaction (Figure 7B, arrows), with a pseudostratification of nuclei (Figure 7B, head arrow). The luminal epithelium showed subnuclear vacular spaces (Figure 7 C, head arrow). The stromal cells nuclei showed vesicular patterns (Figure 7C, long arrow), and those of the reniform type (Figure 7 C, small head arrow).

The statistical analysis of eosinophil count, as well as mean numbers of metrial gland sections, counted per unit area of the estrus specimens, for both the control and the experimental rats, are presented in Table 2, respectively. Statistically, these data showed: (1) A significant increase in the mean number of glandular sections per unit area amongst the estrus endometrial sections of experimental rats ($P < 0.05$). However, no significant differences could be elicited amongst individual subgroups of the same group (Table 2); (2) A significant decline in the number of infiltrating eosinophils granulocytes endometrial sections ($P < 0.005$) amongst all experimental rats, at the estrus (Table 2).

Table 2. Mean number of infiltrating eosinophils granulocytes per HPF, and that of metrial gland sections per unit area of estrus endometrial sections. Data expressed as means of at least forty sections (ten rats) ± SD.

Group	Subgroups	Eosinophils per HPF±SD	Metrial gland sections/unit area±SD
Control	Treated	111±4.5	1.3±0.47
	Non-treated	113±6.4	1.4±0.35
Experimental	D	4.3±3.8	5.2±0.49
	P	5.3±3.5	5.1±1.1
	E	5.6±3.5	4.7±1.2

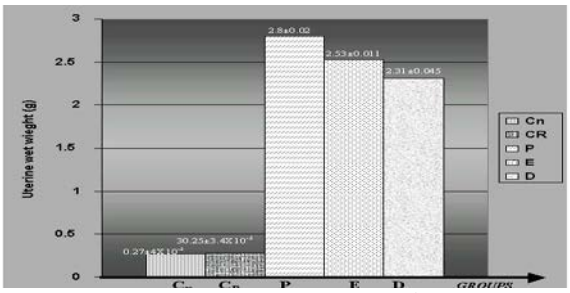

Figure 1. Frequency histogram showing values for uterine wet weight for the control and the experimental rats. Data expressed as mean ± SD of readings obtained from ten rats at the end of experiment.

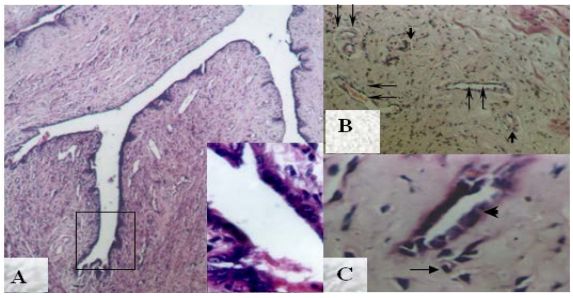

Figure 2. Proestrus section of control animals. A: Endometrial surface was thrown into marked longitudinal folds and narrow crypts (X 330); Note the low cuboidal lining of the crypts [inset arrow, (X 1320)]. B: General architecture of the endometrial stroma (X 330); Note the scarcity of the glands (head arrows), and relative abundance of the blood vessels (small arrows). C: Finer details of the stroma (X 1320), showing the low cuboidal lining of the glands (head arrow), the moderate eosinophils granulocytes infiltration [inset, (X 1320)], and the generally spindle shaped stromal cells with darkly stained oval nuclei (thin arrow).

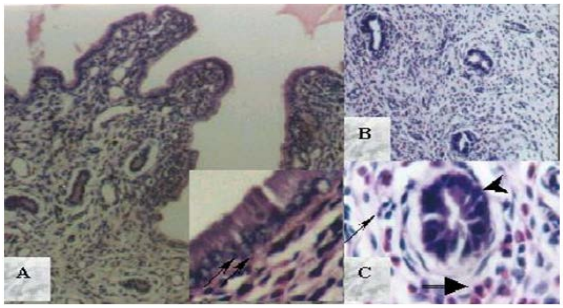

Figure 3. Estrus section of control animals**. A**: Dilatation and widening of the luminal crypts (X 330). Note the columnar type luminal epithelium, and the parabasal position of the nuclei [inset arrows (X 1320)]. B Endometrial stroma. showing increased stromal cellularity (X 1320**).** C: Finer stromal details. Note the increased eosinophils granulocytes infiltration (double arrow), frequent mitotic figures (thin arrow) and the columnar lining of the glands (head arrow), cf , Fig. (4C). (X 1320).

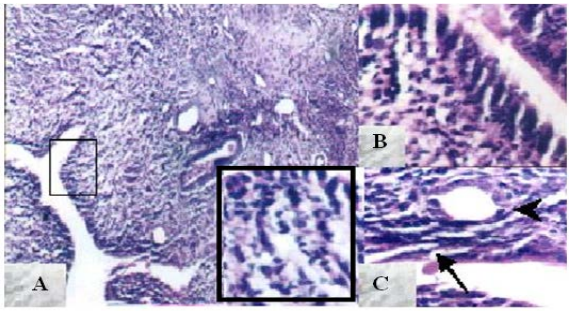

Figure 4. Metestrus section of control animals. A: General appearance of the sloughing endometrium (X 330). Note the absence of glands and loss of normal tissue architecture, of the superficial layers [inset, (X 1320)]. B: Degenerated luminal epithelium (X 1320). C: Loss of normal glands architecture (head arrow), and sheets of degenerated stromal elements (thin arrow). Note the absence of eosinophils granulocytes, cf . Fig. (5C) (X 1320).

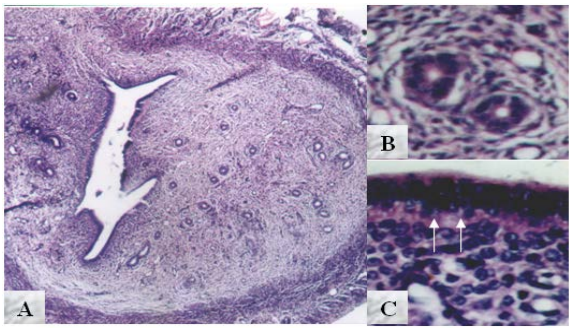

Figure 5. Habbat-Baraka (Nigella sativa Linn. seeds) treated proestrus. A & B: Showing profound endometrial hypertrophy, with enhanced glandulogenesis, cf. Fig (4A & B). [B (X 330)]. C: Luminal epithelium showing well-developed tall columnar cell, with characteristic subnuclear zones (white arrows), and subepithelial decidualization, cf. Fig (4 C). (X 1320).

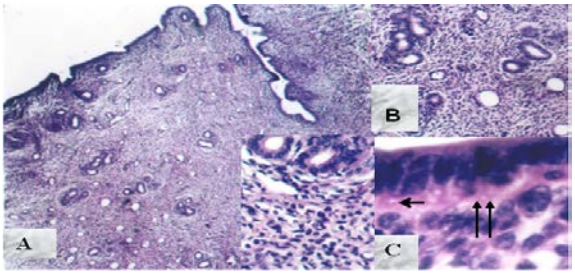

Figure 6. Habbat-Baraka (*Nigella sativa* Linn. seeds) treated Estrus. A & B: showing diffuse endometrial hyperatrophy, with enhanced glandulogenesis [B (X 330)]. Note the absence of eosinophils granulocytes infiltration of the stroma, cf. Fig.(5 C). [A, inset (X 1320)]. **C**: luminal epithelium showing well-developed sub-nuclear zones (thin arrows). Note the vesicular pattern of stromal cells nuclei, and those of reniform type (open arrow). (X 1320).

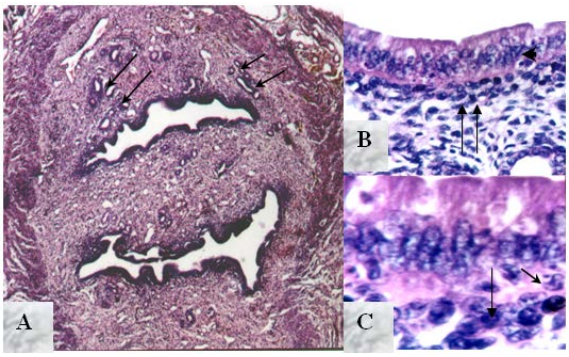

Figure 7. Habbat-Baraka (*Nigella sativa* Linn. Seeds) treated metestrus. A: showing the persistence of metrial glands (arrows), and well-maintained endometrial stroma, cf. Fig.(6 A). (X 330). B: Subepithelial decidualization reaction (arrows). Note the pseudostratification of nuclei (head arrow). (X 1320). C: Luminal epithelium showing subnuclear vacuolar spaces (head arrow). Note the vesicular pattern of the stromal cells nuclei (long arrow), and those of the reniform type (small head arrow). (X 1320)

4. Discussion

Vaginal smears of rats treated with the crude aqueous extract of *Nigella sativa* seeds showed a shortening of the vaginal cycles with the prolongation of both the estrus and the metestrus phases; the diestrus was undetectable.

The shortening of a five-day vaginal cycle reported earlier (Vander-Schoot and Uilenbroek, 1983), suggested mechanism of a prolonged exposure to progesterone after the metestrus, a consecutive effect to the inhibition of luteolysis. *Nigella sativa* was shown to prolong the life-span expectancy of the ovulatory corpus luteum, amongst locally bred hybrid albino female rats (Hassen, 2000). This provides supplementary evidence to the observed post-ovulatory, progesterone-like action of the administrated crude aqueous extract of the *N. sativa* seeds as shown from the vaginal smears of treated rats. Thus, this provides a clue that whole *N. sativa* might have achieved its effects by stimulating the endogenous release of estrogen and/or progesterone. However, direct estrogen and /or progesterone-like actions of the Black seeds, could not be excluded.

The results of this study showed that the crude aqueous extract of the *N. sativa* L. seeds at the dose of 0.2g/100g B. wt, leads to a significant increase ($P<0.05$) in the uterine weights of rats (almost ten times elevation) compared to their control fellows (Figure 1). Similar results were obtained by Parhizkar *et al*., 2016. The number of the metrial glands was significantly elevated (Table 2), suggesting an increased rate of glandulogenesis. On the other hand, the vesicular pattern of the stromal cells nuclei, and the ostensibly increased nucleo-cytoplasmic ratio among such cells, can be a suggestive histological feature indicating an increase in the metabolic activities (Robertson, 1981; Junqueira and Carnneiro, 2005). Berene and Levy, 1988; Ganong, 2010 stated that estrogen has a rapid effect on cell replication, but has a slower effect on cytoplasmic differentiation.

Mitosis was frequently seen amongst the stromal cells, and cells with indented, bilobed and/or reniform nuclei, were also produced and were predominantly seen at the immediate subepithelial zones. Such types of stromal cells had been reported (Robertson, 1981). These cells were implicated in the process of decidualization of the rodent endometrial, and are shown to be responsible for the endometrial production of the polypeptide hormone Relaxin (Robertson, 1981). Relaxin has been found to stimulate the uterine edema via the activation of estrogen receptors (Pillai *et al.,* 1999). Such endometrial edematous changes might be responsible for some of the observed increment in the uterine wet weight values obtained in this study.

The luminal epithelial cells were persistently high columnar in type. Throughout the phases of the endometrial cycle, these cells developed the characteristic sub-nuclear vacuolar spaces. Since estrogen supports the growth of the luminal epithelial cells (Berene and Levy, 1988), the height of these cells was taken as a sensitive parameter in assessing the estrogen site of a given compound (Fuhrman *et al.,* 1998), and in accordance with the fact that the development of the sub-nuclear spaces amongst the luminal epithelium was shown to be functionally associated with ovulation (Lesson *et al.,* 1988), and is brought out by progesterone actions on the estrogen-primed luminal epithelium (Stenchever, 1987; Lesson *et al.,* 1988). The results obtained from this study indicate that the crude aqueous extract of the *Nigella sativa* seeds might possibly have an estrogen and/or progesterone-like action on mature rats' endometrium. Such indication is supported by other authors (Liu *et al.,* 2004; Parhizkar *et al.,* 2011, 2012, and 2016).

The failure to detect diestrus on the vaginal cytology (Hassen, 2000) of the experimental rats is supported by their endometrial histology: the absence of degenerative changes amongst metestrus endometrial sections was quite evident when compared with those of their controls (Figure 6) since the mechanisms of endometrial sloughing involved estrogen and/or progesterone withdrawal (Ganong, 2010) because blood estrogen concentrations should be above a critical level together with progesterone for both the maintenance and the proliferation of the secretory phase of the endometrium of the uterus (Laurence *et al.,* 1997). These findings provide further supporting evidence that the crude aqueous extract of the *N. sativa* seeds has an estrogen and/or progesterone-like action(s).

Stromal infiltration with eosinophils granulocytes is a unique feature of rodents' Estrus, which coincides with the periovulatory peak of estrogen (Hebel and Stromberg, 1986), and is considered as one of the histological parameters in assessing the uterotrophic effect of a given compound (Patriarca *et al.,* 1996). In this study, eosinophils granulocytes infiltration of the endometrial stroma, at Estrus, was significantly reduced after the administration of the aqueous extract of the *N. sativa* seeds. A low eosinophils count, at the Estrus of rats, had been reported earlier, \and showed that anti-estrogens can induce it with intrinsic estrogen-agonist activity (Patriarca *et al.,* 1996).

However, inhibited eosinophils granulocytes infiltration of the decidualized endometrium is a well-known fact amongst rodents, and this suggests a possible role of these cells in the initiation of implantation (Hebel and Stromberg, 1986).

The current study reported that the inhibition of eosinophils granulocytes infiltration of the endometrial stroma at Estrus, suggests the presence of anti-estrogens

with intrinsic estrogen-agonist activity (Patriarca *et al*., 1996); or this could be a consecutive effect of the observed utilization of the promoting effects of the herb on rats' ovaries (Hassen, 2000; Parhizkar *et al*., 2011). However, direct decidualization promoting effect(s) of the crude aqueous extract of the seeds on the endometrial-stromal elements might not be ruled out by the present work.

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