

Full Length Research Paper

## **In vivo estrogenic activity of *Nigella sativa* different extracts using vaginal cornification assay**

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***Nigella sativa* (Black seed) is an amazing herb with a rich historical and religious background. *N. sativa* increases milk production and promotes menstruation in female. The purpose of this study was to assess which extract of *N. sativa* could improve vaginal epithelial cell in experimentally induced menopause rats. Forty ovariectomized Sprague Dawley rats were used in the study and randomly allotted into one of five experimental groups: treated with supercritical fluid extraction (SFE), methanol extract and hexane extract of *N. sativa* (300 mg/kg/day) compared to control (Olive oil 1 ml) and estrogen group (0.2 mg/kg conjugated equine estrogen). All groups were treated for 21 consecutive days. Vaginal smears were taken daily to observe the estrogenic effect on vaginal epithelium of rats and serum estradiol was measured on 0, 11 and 21 days. The occurrence of vaginal cornification after treatment indicated estrogenic like activity of *N. sativa*, but this effect was not as much as conjugated equine estrogen (CEE). On the other hand, the most influence of *N. sativa* in vaginal cornification was observed in methanol extract of *N. sativa* that was in agreement with serum estradiol of this group. The percent of cornified cells was significantly different compare to control group ( $p < 0.05$ ). These findings suggest that *N. sativa* posses estrogenic function in the ovariectomized rat model which can be helpful in managing menopausal symptoms as an alternative for hormone replacement therapy.**

**Key words:** *Nigella sativa*, vaginal cytology assay, ovariectomized rats, estrogenic activity, supercritical fluid extraction, solvent extraction.

### INTRODUCTION

Menopause usually occurs at the age between 45 and 54 years when the ovarian function begins to decrease (Kaplan and Manuck, 2008). World Health organization (WHO) has estimated that, by the year 2030, the world population of menopausal and postmenopausal women will be 1.2 billion, with 47 million new entrants each year (Borrelli and Ernst, 2008). Menopausal estrogen deficiency is associated with physical and psychological effects of menopause (Bones, 2006). Hormone

replacement therapy (HRT) is an effective intervention for these complaints (Kaplan and Manuck, 2008; Lekander et al., 2009). However, findings of two recent large randomized clinical trials (RCTs) (Hulley et al., 1998; Rossouw et al., 2002; Fugh-Berman, 2010) implied that the risk associated with HRT may outweigh the benefits for women on continuous estrogen and progestin regimens. Many patients and clinicians are therefore hesitant to continue using HRT (MacLennan et al., 2004; Haas et al., 2004). Various alternative therapies for managing menopausal symptoms are on offer, particularly herbal alternatives (Nedrow et al., 2006).

*N. sativa* is one of the most studied herbal medicines for the treatment of various ailments as well as improve

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general health status (Ghamarnia et al., 2010; Parhizkar et al., 2011a). Furthermore, its beneficial effects in gynecologic disorders have been reported in Ayurvedic (Atul et al., 2005) and Unani medicine (Al-Jishi, 2002). Conversely, among a long list of examples which claimed usefulness of *N. sativa* in medicine; very few studies have reported its influence on the reproductive system and almost all of them have focused on the male reproductive system. However, effect of *N. sativa* seeds on female reproductive functions remains largely unknown. Thus, the purpose of current study was to elucidate probable estrogenic like effect of *N. sativa*.

## MATERIALS AND METHODS

### Plant materials and extractions

*N. sativa* seeds (imported from India) were purchased from a local herb store in Serdang, Malaysia. Voucher specimens of seeds were kept at the Cancer Research Laboratory of Institute of Biosciences and the seed was identified and authenticated by Professor Dr. Nordin Hj Lajis, Head of Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia. The seeds were grounded to a powder shape using an electric grinder (National, Model MX-915, Kadoma, Osaka, Japan) for 6 min. Homogenized and grounded samples (100 g) were soaked overnight with solvents at a ratio of 1:5 (w/v ratio). Two different solvents were used: n-hexane (Pu: 99%, Merk, Darmstadt, Germany) and methanol. The mixture of sample and solvent were covered with aluminum foil, and were shaken using a Shaking Incubator (Heidolph Unimax 1010, Germany) at 5 to 7 rpm for 90 min. Then solvents were filtered using Whatman paper number 1. The residues were re-soaked with fresh solvent two times to ensure the complete extraction of the oil. Solvents were completely evaporated using a rotatory evaporator (Heidolph Laborata, Germany) at 50°C and 90 rpm that yielded a blackish-brown and yellowish concentrates for methanol and hexane extract, respectively which was kept at -20°C prior to use.

The extractive values (w/w %) of the methanol and hexane extract were 29 and 33%, respectively. Extraction of essential oil from the seed of *N. sativa* was also done using the speed SFE instrument. The seed powder of *N. sativa* was measured to 150 gr with digital scale (Shimadzu Model, Japan) balance before placing in the extraction vessel. The oil extract was obtained at 60 MPa and 40°C by means of SFE set (SFE-1000F Thar US Technology, USA). SFE flow-rates were maintained at 20.00 ml/min using a variable flow restrictor. The yellowish-brown color yield was collected within 3 h and its value (w/w %) was 26% which was stored at -20°C prior to use. The collected pressure and temperature were 0.1 MPa and 25°C, respectively. The extraction was carried out with pure CO<sub>2</sub>.

### Chemicals

CEE 0.625 mg was purchased from Wyeth, Montreal, Canada. CEE (Wyeth Montreal, Canada), was used as a positive control for the purpose of comparison with the treated groups. Estradiol Radioimmunoassay (RIA) kit was purchased from diagnostic systems laboratories (DSL), USA.

### Animals

Forty female Sprague–Dawley rats weighing between 250 and 350 g aged 4 months old were used in this study. They were supplied

by animal house of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (Serdang, Selangor, Malaysia). Experiments were carried out according to the guidelines for the use of animals and approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia with UPM/FPSK/PADS/BR/UUH/F01-00220 reference number for notice of approval. They were fed with standard rat chow pellets and allowed to drink water *ad libitum*.

### Experimental design

In order to induce menopause and to investigate reproductive changes following the supplementation with *N. sativa*, the rats were ovariectomized under a combination of xylazine and ketamine (10 + 75 mg/kg, i.p, respectively) anesthesia. Bilateral ovariectomy was performed via a dorso-lateral approach with a small lateral vertical skin incision (Parhizkar et al., 2008). The ovariectomized animals were acclimatized at the Animal House of Faculty of Medicine and Health Sciences for one month prior to supplementation.

Five experimental rat groups were established with 8 rats per group. The groups were as follows: group 1, negative control (1 ml olive oil), group 2, positive control (0.2 mg/kg/day CEE diluted in distilled water), group 3 SFE extract treated group (300 mg/kg in olive oil), group 4, methanol extract treated group (300 mg/kg in olive oil) and group 5, hexane extract treated group (300 mg/kg in olive oil). Dosage of the extracts were selected based on the optimum estrogen like effect of *N. sativa* in the previous experiment (Parhizkar et al., 2011), which was at low dose (300 mg/kg BW/day) and were administered by intra-gastric gavage for 3 weeks.

### Vaginal smear

Vaginal smears were carried out to monitor cellular differentiation and to evaluate the presence of leukocytes, nucleated epithelial cells, or cornified cells. Vaginal smear samples were collected between 08.00 and 10.00 am daily. The vaginal smears were prepared by washing with 10 µl of normal saline (NaCl 0.9%) then thinly spread on a glass slide. They were allowed to dry at room temperature and then stained using methylene blue dripping. The slides were rinsed in distilled water after 30 min and allowed to dry. The smears were studied using the light microscope (40x) and the cell type and their relative numbers were recorded. Vaginal smear cell counts were performed on 100 cells randomly. The percentage of cornified cells was determined according to Terenius (1971) using the following formula:

$$\text{Percentage of Cornified Cells} = \frac{\text{Cornified Cells}}{\text{Cornified Cells} + \text{Nucleated Cells} + \text{Leucocytes}} \times 100$$

### Blood collection

Fasting blood samples were collected by cardiac puncture at baseline (pre-treatment), day 11 (during treatment) and day 21 (after treatment).

### Statistical analysis

Data were expressed as means ± standard deviation. The data were analysed using SPSS window program version 15 (SPSS Institute, Inc., Chicago, IL, USA) or Statistical Analysis System SAS (SAS Institute Inc., Cary, NC) statistical packages. The One-Way Analysis of Variance (ANOVA) and General linear Model (GLM) followed by Duncan Multiple Range Test (DMRT) were used to

**Table 1.** Means of serum estradiol (pg/ml) concentration of OVX rats supplemented with extract from various methods of extraction of *N. sativa* or conjugated equine estrogen.

Treatment	Day			Total
	0	11	21	
SFE	13.95± 18.71 <sup>b</sup>	7.92± 3.25 <sup>b</sup>	11.11± 3.15 <sup>b</sup>	10.99± 10.91 <sup>B</sup>
ME	11.55± 3.49 <sup>b</sup>	12.65 ± 3.16 <sup>b</sup>	14.68± 12.24 <sup>b</sup>	12.96± 7.36 <sup>B</sup>
HE	10.35± 4.42 <sup>b</sup>	12.67± 11.43 <sup>b</sup>	9.18± 4.65 <sup>b</sup>	10.73± 7.38 <sup>B</sup>
CEE	14.88± 12.11 <sup>b</sup>	15.89± 13.37 <sup>b</sup>	53.51± 34.77 <sup>a</sup>	28.09±28.36 <sup>A</sup>
C	11.72± 7.43 <sup>b</sup>	5.94± 4.15 <sup>b</sup>	6.26± 5.51 <sup>b</sup>	7.98± 6.22 <sup>B</sup>
Total	12.49± 10.37 <sup>X</sup>	11.01± 8.69 <sup>X</sup>	18.95± 23.85 <sup>Y</sup>	

Data are expressed as Mean ± SD. treatment SFE, supercritical fluid extract of *N. sativa* (300 mg/kg/day); ME, methanol extract of *N. sativa* (300 mg/kg/day); HE, hexane extract of *N. sativa* (300 mg/kg/day); CEE, conjugated equine estrogen (0.2 mg/kg/day); and C= control (1 ml olive oil/day) groups. AB: Comparison of the means between rows within column significant at  $p < 0.05$ . XY: Comparison of the means between columns within row significant at  $p < 0.05$ . ab: Comparison of the means between column and between row significant at  $p < 0.05$ .

**Table 2.** ANOVA for serum estradiol of ovariectomized rats supplemented with extract from various methods of extraction of *N. sativa* or conjugated equine estrogen.

Source	df	Mean square	F
Treatment	4	637.635	3.716*
time	2	1360.885	7.931***
Treatment * time	8	697.238	4.063***
Error	105	171.599	
Total	120		

determine which extract of *N. sativa* showed optimum effects. A  $p$ -value less than 0.05 ( $p < 0.05$ ) was considered to be significant.

## RESULTS

### Serum estradiol

Over the period of treatment, all groups showed reduction in the level of estradiol except positive control CEE and methanol extract of *N. sativa*. But the third measurement showed tended to estradiol elevation in SFE, methanol extract (ME) ( $p > 0.05$ ) and a rapid and significant elevation among CEE compare to other groups of first and second measurements ( $p < 0.05$ ). In general, the levels of estradiol among different extracts were much higher than control groups (Table 1). Two-way analysis indicated that there was a significant effect for treatments and duration of treatment ( $p < 0.05$ ) and interaction effect ( $p < 0.001$ ) (Table 2).

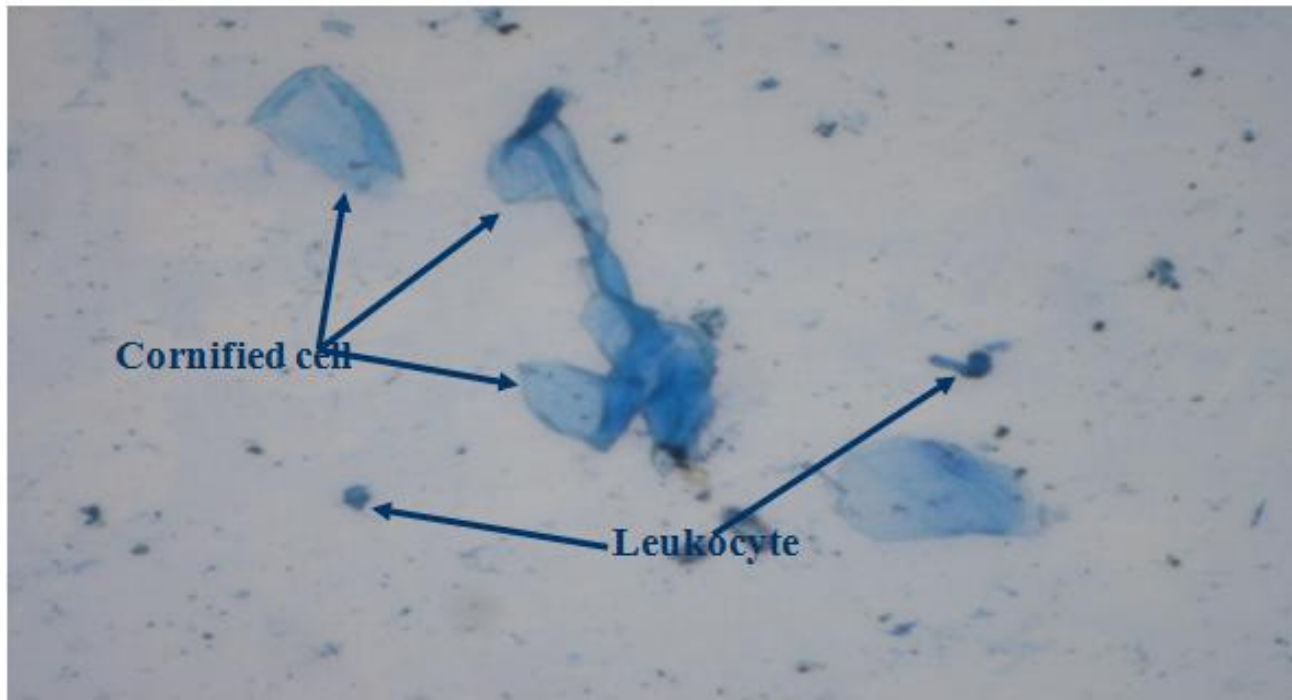
### Cornification of vaginal epithelial cells

The means of cornified cells percentage were not significantly different at baseline (day 0) in all groups

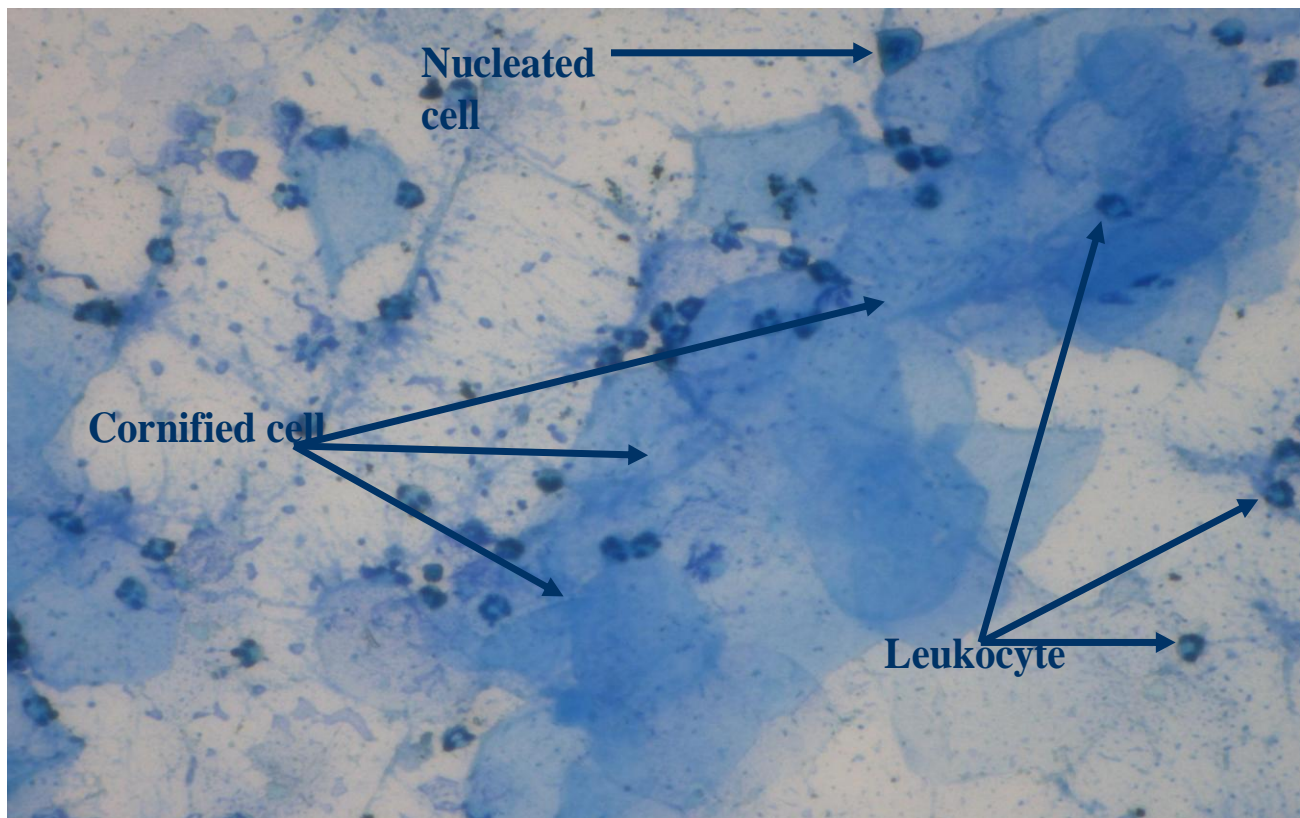
which confirmed a menopausal pattern of vaginal cells in ovariectomized rats. In ovariectomized rats, the vaginal smear showed an atrophic pattern consisting of leukocytes, mucus and scarce nucleated epithelial cells. When estrogens were administered to ovariectomized rats, the vaginal smear consisted mainly of exfoliated cornified cells. The percent of cornified cells did not display increase in control group and remained unchanged until the end of experiment (Figures 1, 2, 3, 4 and 5).

## DISCUSSION

At the first 10 days of treatment, all treatment groups including either methanol, hexane and SFE extracts or conjugated equine estrogen exerted cornification of vaginal cells and only CEE group showed significant increase ( $p < 0.05$ ) in cell's cornification. At the end of experiment, cornified cells percent increased significantly ( $p < 0.05$ ) in all treatment groups, while the rise of cornified cells in all extracts were lower than whole seeds supplementation in previous experiment and compare to that study estrogenic activity was less than crude powder (Parhizkar et al., 2011). This indicated that some components removed during extraction may possibly

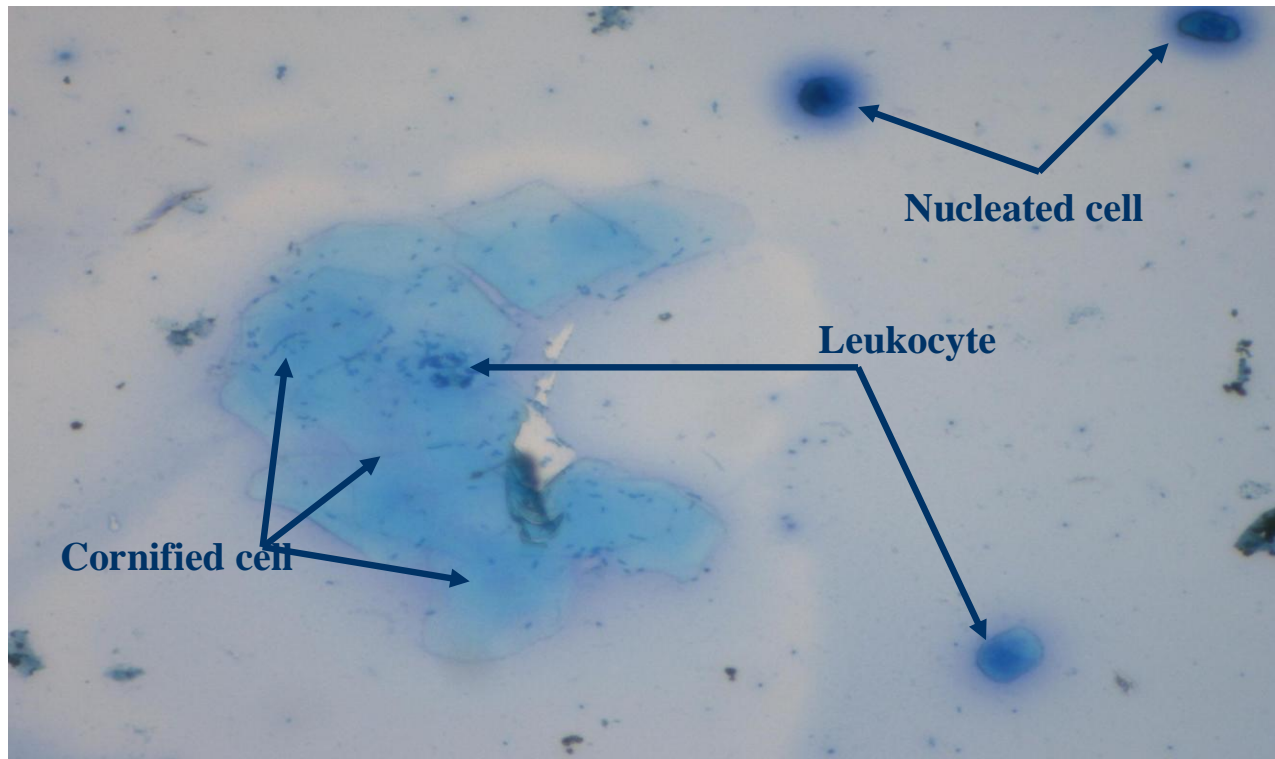


**Figure 1.** Vaginal smear of ovariectomized rat treated with supercritical fluid extraction of *N. sativa* (300 mg/kg) for 3 weeks. Cornified cells and leukocytes are observed (methylene blue staining, 40x).

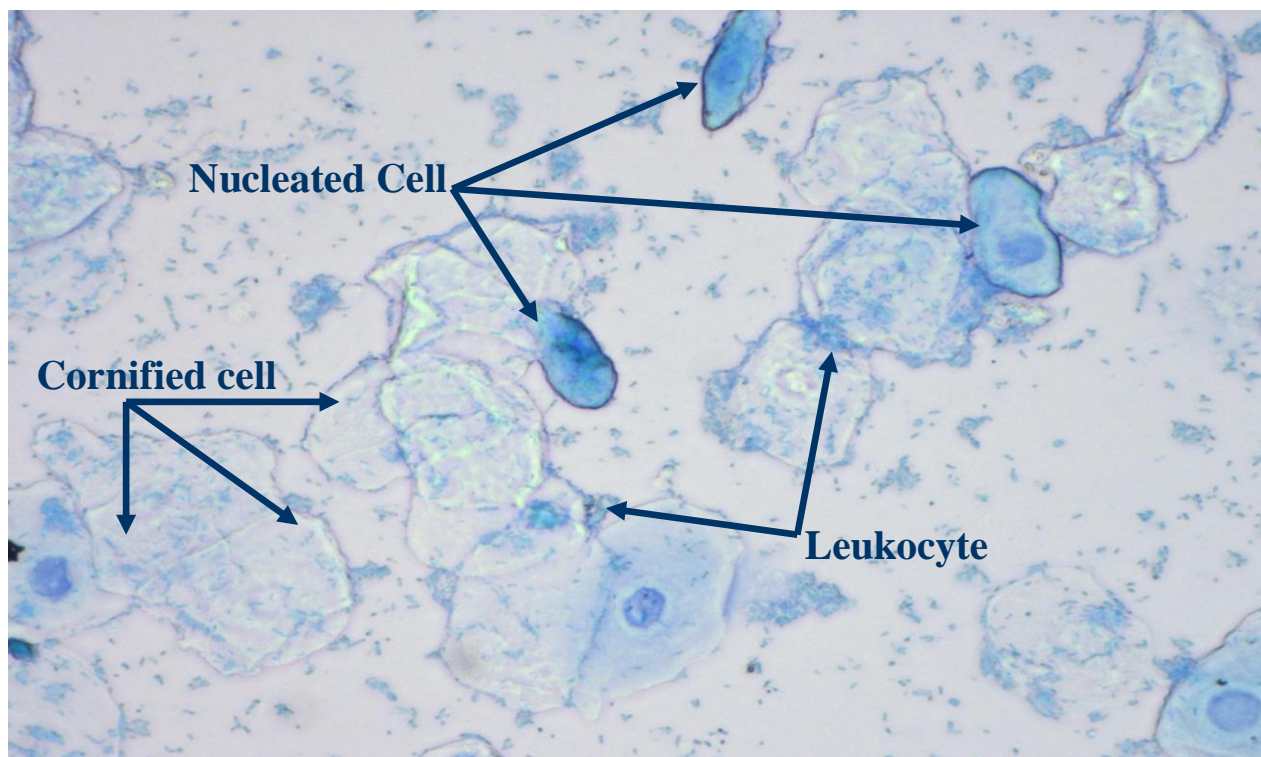


**Figure 2.** Vaginal smear of ovariectomized rat treated with methanol extract of *N. sativa* (300 mg/kg) for 3 weeks. Cornified cells and leukocytes are observed (methylene blue staining, 40x).

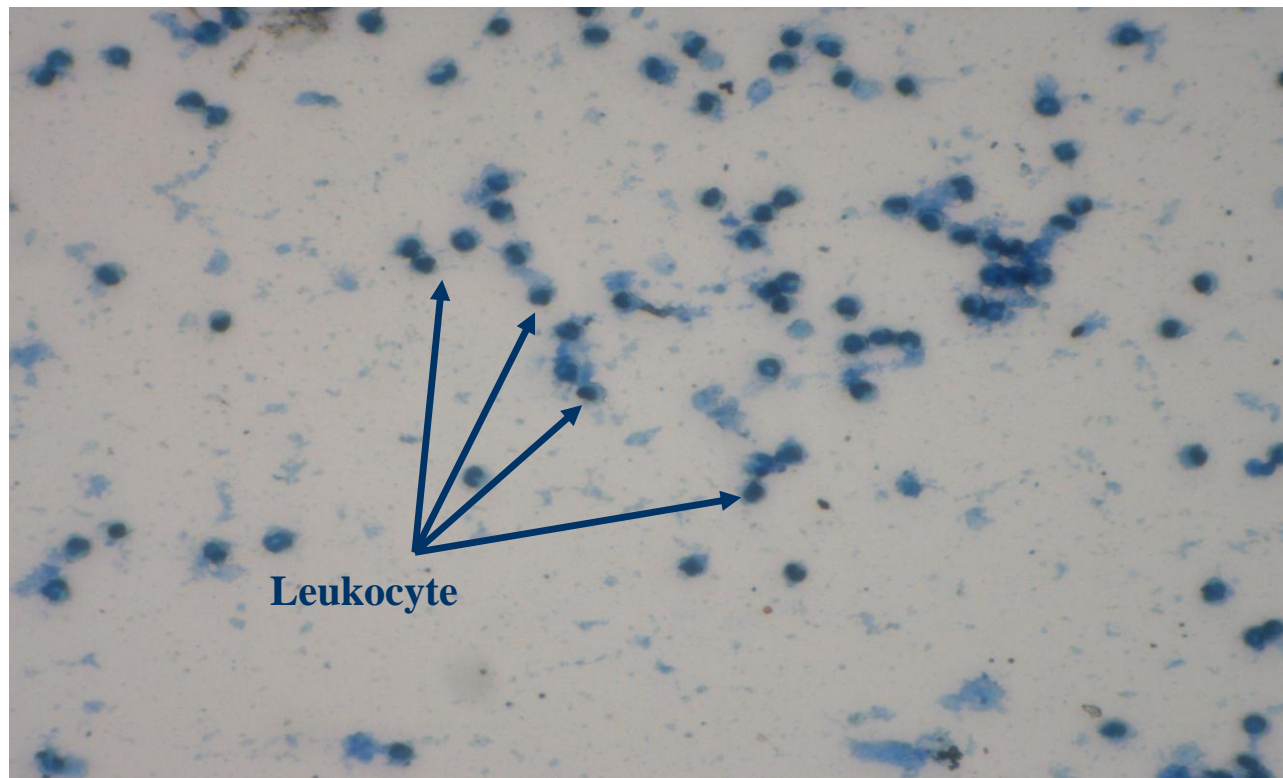




**Figure 3.** Vaginal smear of ovariectomized rat treated with hexane extract of *N. sativa* (300 mg/kg) for 3 weeks. Cornified cells, nucleated epithelial cells and leukocytes are observed (methylene blue staining, 40x).



**Figure 4.** Vaginal smear of ovariectomized rat treated with conjugated equine estrogen (0.2 mg/kg) for 3 weeks. A great number of cornified cells and also nucleated epithelial cells are observed (methylene blue staining, 40x).



**Figure 5.** Vaginal smear of ovariectomized rat from control group treated with olive oil for 3 weeks. A great number of leukocytes are observed (methylene blue staining, 40 $\times$ ).

have estrogenic action or could act as the active component. The finding showed higher percentage of cornified cells in methanol extract compare to other extracts.

These results are consistent with the trend for serum estradiol concentration which showed higher level in methanol extract of *N. sativa* in ovariectomized rats. Intan (2008) also reported an increase in the level of estradiol following 14 days *N. sativa* supplementation in ovariectomized rabbits. Bashandy (2007) and Saleh Mansi (2005) reported that *N. sativa* could improve reproductive performances such as increasing testosterone level in male rats. The ethanolic extract of *N. sativa* seeds showed anti-fertility effect in male rats that is probably due to the inherent estrogenic nature (Agarwal et al., 1990). In another study, the hexane extract of the seeds showed significant contraceptive activity in rats (Keshri et al., 1998). In a more recent study, *N. sativa* in combination with *Abramo augusta* in 1:2 ratio, was found to induce ovulation in buffaloes and two out of six buffaloes conceived (Kabir et al., 2001).

## Conclusion

This study concluded that *N. sativa* possesses estrogenic

function in the ovariectomized rat model which can be helpful in managing menopausal symptoms as an alternative to hormone replacement therapy.

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