Original Article

Effects of The Black cumin seeds (Nigella sativa Linn.) on Endometrial Mucins of Mature Rats; Histochemical Study

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Summary:

Background: *Nigella sativa Linn is a well known herb that is used by different societies, as food additive and as a medicinal herb; it used as a galactagouge for lactating women, and has long been described as an abortifacient-emmenagouge.*

Aim of the work: *is to investigate the effect of crude black seeds on the endometrium and the pattern of it's mucins, during the different phases of the Estrus cycle, making use of some special stains for the histochemical demonstration of mucins.*

Materials & Methods: *Crude Nigella sativa Linn. seeds were administrated for ten days, by an orogastric tube, on single regular daily dosage to properly selected mature Norway albino female rats. Animals were subdivided into subgroups, according to phases of the estrus cycle. Utera of these animals were routinely processed for carbohydrate histochemical (d-PAS, d AB2.5-PAS & AB1.0) studies using carnoy's fixative, paraffin embedded sections. Experimental specimens were compared with that of control subgroups.*

Results: *Results showed marked production of diastase fast-PAS reactive non-alcianophilic neutral mucins, with inhibited production of sulphated highly acidic mucins among the surface lining and the glandular epithelial cells, at Estrus. Effects of such changes in the pattern of endometrial mucins on the state of fertility, was discussed.*

Keywords: *Nigella Sativa, Linn., Metrial gland, Endometrial Histochemistry. Sialomucins, Fucomucins, Sulphated mucins. d-PAS, AB2.5PAS, AB1.0.*

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Introduction

Nigella sativa Linn is a well known herb that is used by different societies, It is used, both as food additive and as a medicinal herb; used in treating various diseases and disorders[1]. It was also used as a galactagouge for lactating women, and has long been described as an abortifacient-emmenagouge[1].

In 1995; a group of Indian workers[2], showed that it prevent pregnancy in al experimental rats, when used in a dose of 0.2g/100g B.wt; administrated as a single oral daily dose, on day 1-10 post coitum. However , at the above mentioned contraceptive dose, the authors reported a mild uterotrophic effect of such an extract , comparable almost to the ovulation dose of 17α-estradiol, in ovariectomized immature rat bioassay [2] .

According to these finding, and in consideration of reported lack antiovulatory activity of the black cumin seeds[3], authors[2] suggested the possibility towards the generation of a noval contraceptive agent of plant origin, that is devoided of a significant estrogenic activity.

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Based on the earlier mentioned reports, and as there was no earlier published work on the effects of the crude Nigella sativa Linn. on the mature rat endometrium (Medline and Extramed search 1972-2004); we designed this study to investigate the effect of crude black seeds on the endometrium and the pattern of it's mucins, during the different phases of the Estrus cycle, making use of some special stains for the histochemical demonstration of mucins.

Material and Methods :

Fifty young mature female Norway albino rats, having regular Estrus cycle, were employed in this work. Animals were not mixed with male for last two weeks before experiment. Rats were grouped according to their physiological states and the substance they received (Table – 1).

Experimental subgroups (P, E and D), received crude Nigella sativa Linn. at a dose of 0.2g/100 g B.wt[2].Seeds powder was mixed with four ml D.W.[4 ,5] and given thought an orogastric tube once daily for ten days[2] , starting at the Proestrus , Estrus and Diestrus phase respectively (Table - 1) Control animals were divided into two subgroups : (1) Non-treated controls (C_n), which were used as standard reference models ; (2) Treated subgroup

(C_R) received four ml D.W. only, through the orogastric tube, once daily for ten days (Table – 1). All Animals were sacrificed; each at a required phase of the vaginal cycle. Exposure of the peritoneal cavity, and removal of the body of the uterus was done under open ether anesthesia. Uterine specimens were then fixed for a period of two hours in Carnoy's fluid, the processed for routine paraffin wax-embedding. Serial sections, each of 5µ thickness were cut and processed for special histochemical stains (d-PAS [6, 7], AB2.5-PAS [8], AB1.0 [7])

Results:

1- d-PAS staining:

Table (1) : Animal groups and subgroups used. 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/100g B.wt.)
Controls	C_n	10	Non	.....	.....
	C_R	10	D.W. only	Irrespective	.....
Experimental	D	10	Crude Nigella sativa Linn.	Diestrus	0.2
	P	10	Crude Nigella sativa Linn.	Proestrus	0.2
	E	10	Crude Nigella sativa Linn.	Estrus	0.2

D.W.: Distilled water

Control subgroups: d-PAS sections, showed a diastase-fast PAS reactive magenta-to-red colored mucins, appeared as amorphous materials filling the uterine lumen (Fig. 2). Metestrus specimens showed the presence of such diastase-fast-PAS reactive complex to be limited to the apical portions of the luminal epithelial cells, as well as those lining neck parts of the metrial glands (Fig. 2). On the contrary; estrus specimens showed a much limited distribution of such complexes, being confined to the apical portions of the luminal epithelial cells (Fig. 1). A much fainter colored reaction was developed by the reticulin fibers of the endometrium stroma (Fig. 1). Experimental subgroups: A major change was found during the metestrus phase; this was represented by the enhanced production of diastase fast PAS reactive magenta-to-red colored mucins. Such complexes were mainly found in the apical portions of the luminal cells, as well as those of the glands (Fig. 4). Luminal secretions were similar in appearance to those of the control specimens, but were relatively deeper in color (Fig. 2 Vs Fig. 4). Such materials were also seen filling lumina of the glands (Fig. 4B). PAS-only stained comparable section showed that glycogen contributed little to the PAS-reactivity of the luminal secretions (Fig. 4B Vs C). However studying the endometrial glycogen is obviously out of the scope of this work. In contrary; estrus specimens showed a diffuse faint-red-colored reaction, to be confined to the apical parts of the luminal epithelial cells (Fig. 3). Luminal secretions were also reactive (Fig. 3B).

2- *AB2.5-PAS staining*:

Control subgroups: Stained section showed a generally similar pattern to d-PAS for the distribution of the distribution of the diastase fast PAS magnets-to-red colored mucins. During both estrus and metestrus. The luminal secretions showed a reactive predominance of magenta-to-red colored complexes over the deep-blue-stained cyanophilic mucins (Fig. 5 & 6). Moreover, the metestrus specimens showed a conjoint existence of the two colored complexes, to occur at the neck parts of the glands, with a relative predominance of the deep-blue-stained mucins (Fig. 6 A & B). At the estrus phase, such materials have a reticular pattern (Fig. 5), while changing into tricot-like considered and clumpy at the metestrus (Fig. 6).

Experimental subgroups: Blue stained complexes were mainly present at the brush-borders of the lumina, as well as glandular epithelial cells (Fig. 7 & 8). No cytoplasmic staining reactions could be detected amongst these cells. Both estrus and metestrus specimens, showed a generally similar pattern of reactivity (Fig. 7 & 8). However, during the metestrus, the deeply located glands showed increased production of diastase fast –PAS reactive apical cytoplasmic granules, that were unaffected by the application of AB2.5 staining in advanced. Thus attributing non alcianophilia to the chemical nature of such granules (Fig. 8 B).

3- AB1.0 staining:

Control subgroups: light blue stained sulphated highly acidic mucins, were seen to be confined to the estrus specimens (Fig. 9 Vs Fig 10 A & B). Their distribution was, in turn, limited to the cells of the luminal epithelium; their presence amongst these cells was in the form of light-blue stained cytoplasmic vacuoles, occurring at the apical portions of the cells. Brush-border lines of these cells were also reactive (Fig. 9).

Experimental subgroups: A marked reduction of the staining properties of the estrus specimens, for the sulphated-highly acidic mucins was observed; a

part from a faint staining reaction at the brush-border of the luminal epithelial cells, the sections were almost deficient of such sulphomucins. (Fig. 11).

Discussion:

Studying results of AB1.0 stain, there was marked reduction in AB1.0 staining for the sulfated highly acidic mucins, during the Estrus phase. This was demonstrated more profoundly in the apical portions of the luminal, as well as the metrial glands epithelium. This finding might suggest that crude Nigella sativa Linn. seeds, might have an inhibitory effect on the endometrial production of sulfated highly acidic mucins, at the Estrus phase.

In keeping with results of Hester et al[11], the above mentioned finding, seemed out of keeping with the other results mentioned in our previous work[12]. However, in view of another studies[13], the latter finding might suggest an unduly estrogen over stimulation, and/or lack of a significant direct estrogen-like action of crude Nigella sativa Linn. seeds.

Studying the results of the d-PAS and the d-AB2.5-PAS stains, there was marked production of diastase fast-neutral (fuco-) mucins, by both the luminal and the metrial glands epithelium at the metestrus phase, and to a lesser extent during the estrus phase. Diffuse red-to-magenta colored reactions were predominant staining reactions found amongst such cells. These findings might suggest that crude Nigella sativa Linn. seeds, might have stimulatory effect(s) on the luminal and the metrial glands epithelial-production of such complexes, during both the Estrus and the Metestrus phases.

Dische[14] showed that predominance of fucose molecules, attributed hydrophobic properties of these molecules, and resulted in "dehydration" of the surface mucus coat. On the other hand, production of the blue-stained alcianophilic mucins (Sialomucins) were much affected by crude Nigella sativa Linn. seeds. This observed reduction in staining properties for the Sialomucins seemed to be associated with simultaneous marked production of the neutral complexes (i.e. fucomucins). This finding seemed to be in agreement with the reported reciprocal relationship between fucose and sialic acid content of the epithelial glycoproteins[14, 15], and in favor of the production of a generally "dehydrated" mucus. According to Odeblad[13], such a dehydrated mucus might be away-far favorable for sperm penetration, and that the so chemically judged reduced synthesis of Sialic mucins, might greatly affect the process of initiation of implantation[16].

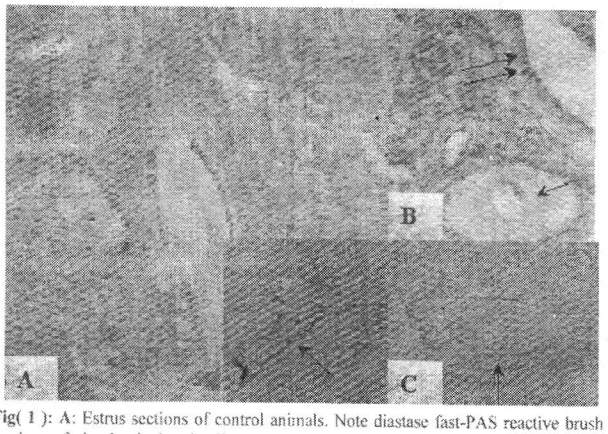
Crude Nigella sativa Linn. seemed to enhance production of diastase fast-magenta-to-red colored neutral apical cytoplasmic granules, by the epithelial lining of the deeply seated metrial glands, at the metestrus. In 1968, few workers[11], had reported the presence of such diastas fast-PAS reaction-non-alcianophilic apical cytoplasmic granules, amongst metrial glands of specimens obtained from donors treated with sequential oral contraceptives. They considered it of undetermined nature, and thought that it was not under hormonal influence. However, Friedrich[17] in his studies of the ultrastructure of the endometrium, had demonstrated the presence of such apical neutral cytoplasmic granules, and he suggested that they represent lysosomes. This proposal was agreed by Connell and associates[18], and suggested that their presence was associated with increased acid phosphates activity.

In this study, the luminal and the metrial glands cells, did not show any staining reactions with AB2.5. However, the apical borders of these cells were strongly reactive. This result is in agreement with those of two other studies[19 ,20]. Both studies showed that this apical staining reaction is quite normal feature, and is accounted for by the presence of cilia amongst such cell lines. However, Lurie and his associates[20] showed the development of intra-cytoplasmic alcianophilia, at pH 2.5 amongst such cells, was only limited to premalignant, and/or carcinomatus lesions of the endometrium. This shows that 10-days treatment with crude Nigella sativa Linn. seeds had not produced significant premalignant, and/or carcinomatus changes amongst the studied endometrial specimens.

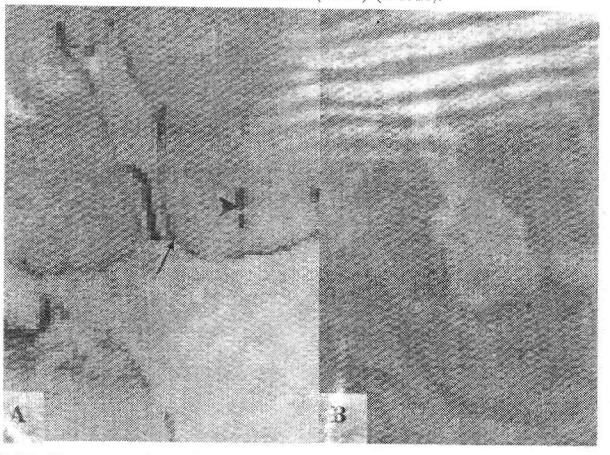
In general, results obtained in this work, and in agreement with those of Keshri and his associates[2], it seems likely that crude Nigella sativa Linn. seeds might have the potential of being a significant antifertility agent. The chemical analysis of the crude seeds of Nigella sativa Linn., indicated that this herb is a potential source for aromatic compounds such as Thymoquinone and Nigellone (Fig.12), and Stigmasterols (Fig. 13) .ARE EASILY ABSORBED, AND ARE MORE READILY CONVERTED INTO THE PREGNANE-TYPE STEROID HORMONES .Thymoquinone and its polymeric form Nigellone , and by virtue of their chemical resembelness to the proposed active site(s) of the estrane and the progestane molecular structure; the hydroxy, and/or the oxy-methyl substituted polyphenols[21], might in away be responsible for the observed effect of the crude seeds, at the administrated dose (0.2/100g B.wt.) on the endometrium and vaginal epithelium of the mature rat. Stigmasterol (Fig. 13) , on the other hand, was shown to be easily absorbed after oral administration, and is more readily converted into

Pregnenolone (a pregnane-type steroid hormones precursor) and this is more likely to add to the observed estrogen and /or progesterone like actions of the crude Black cumin seeds. However, the demonstrated histochemical changes, associated with such an administration of the Crude seeds of Nigella sativa Linn. might well be the result of a modified cellular function under such conditions, rather than to represent a specific estrogen, and/or progesterone like action.

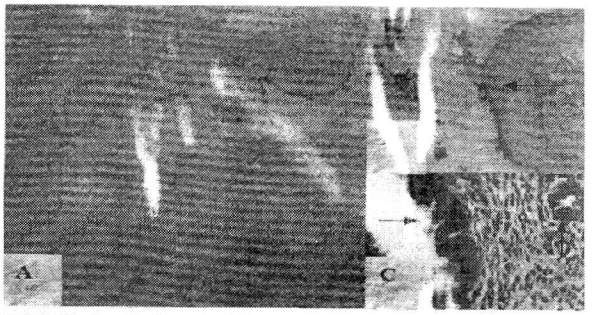
Figures(1- 4): d-PAS stained sections.



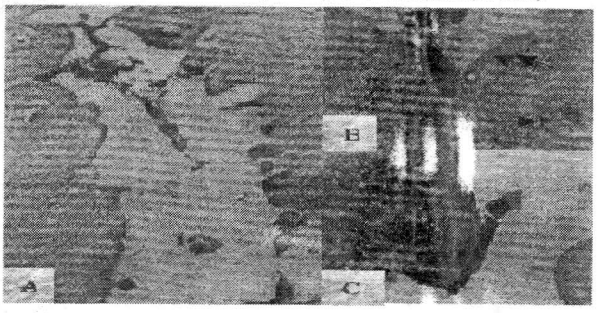
Fig(1): A: Estrus sections of control animals. Note diastase fast-PAS reactive brush borders of the luminal epithelium [inset arrow (X 1320)]. **B**: PAS-only stained comparable section. Showing contribution of glycogen to the reactivity of the luminal (A, inset arrow), and that of the glands (C, arrow). (X 330). **C**: Deep glands. Showing absence of diastase fast- PAS reactive mucins (arrow). (X 1320).



Fig(2): Metestrus sections of control animals. A: Showing production of diastase fast – PAS reactive magenta-to-red coloured mucins, by the luminal (thin arrow), and the metrial gland (head arrow). (X 330). **B**: Deep glands. Note absence of diastase fast – PAS reactive a..cal granules, cf. Fig (4). (X 1320).

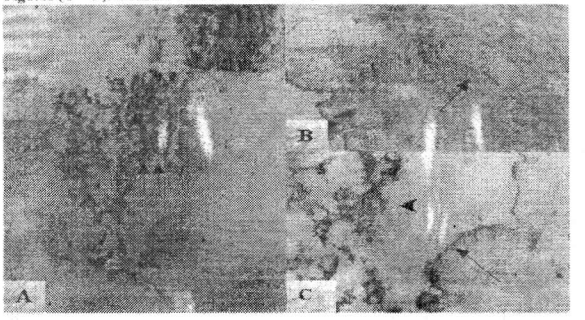


Fig(3): Nigella sativa linn. treated Estrus sections. **A:** Whole e.. on..trial section (X 22). Showing absence of diastase fast–PAS reactive, amongst glandu..r epithelium [inset (X 330)]. **B**: Luminal epithelium. Note the production of diastase fast–PAS reactive mucins, amongst such cells (arrow). (X 1320). **C**: Effect of Harris's haematoxylin-nuclear differentiation, on the final PAS reactivity of the epithelial mucins (arrows). (X 1320).



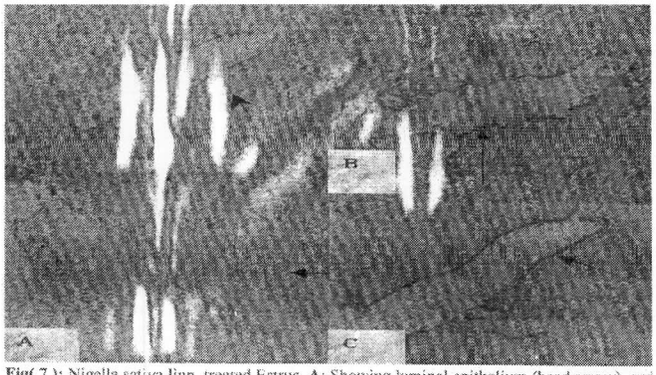
Fig(4): Nigella sati.. linn. treated Metestrus sections. **A:** Me.. im ..ower view (X 330). Showing general distr..ution o..f the diastase fast-PAS reactive mu..ns. B: Higher view of A , showing diastase fast ..AS re..ctive mucins, at the apical portio.. of the luminal (arrows), and glandular epithel..l cells arrow). (X 1320). **C**: PAS-only s..ained comparable section. Note contribution of glycogen to the final reactivity of mucins, cf. B. (X 1320).

Figures (5 – 8): d-AB2.5-PAS stained sections.

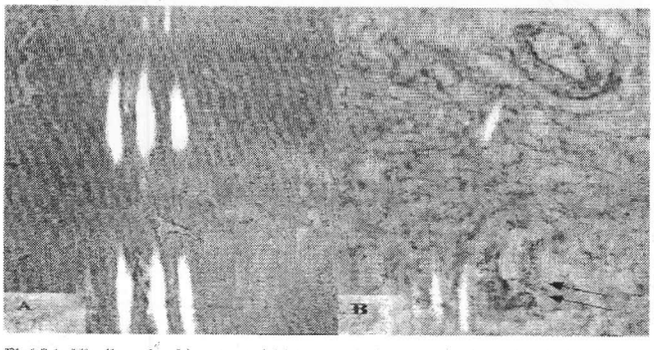


Fig(5): Estrus sections of ..ontrol animals. A: ..showing mixtu.. of diastase fast-PAS reactiv and alcianophilic mucins. N..e the reticular pattern of such com..plexes (arrows). (X 22). **B**: Dec glands. Showing absence of diastase fast-PAS reactive, and/or alcianophilic mucins (arrow). (330). **C**: Luminal epithelium, and secretion. Note the predominance of the d-PAS reactiv mucins, over the alcianophilic ones (head arrow), and the luminal cells-brush border d-PA reactivity (arrow). (X 1320).

Fig(6): Metestrus sections of control animals. A: Showing the associated presence of diastase fast-PAS reactive and/or alcianophilic mucins. (X 22). **B**: Higher view of A . Showing th associated presence of such complexes amongst the neck portions of the metrial glands (arrow Note the tricot-like luminal secretions (arrows). (X 1320).



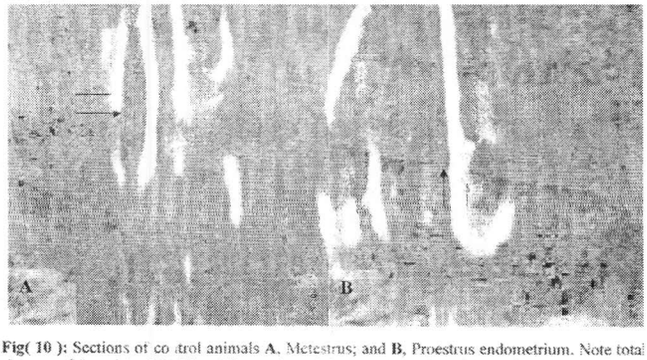
Fig(7): Nigella sativa linn. treated Estrus. **A**: Showing luminal epithelium (head arrow), and a glandular section (arrow). (X 330). **B**: Meurial glands, and **C**: Luminal epithelium. Note the alcianophilic staining of the brush border (arrow), and absence of intracytoplasmic alcianophilia. (X 1320)



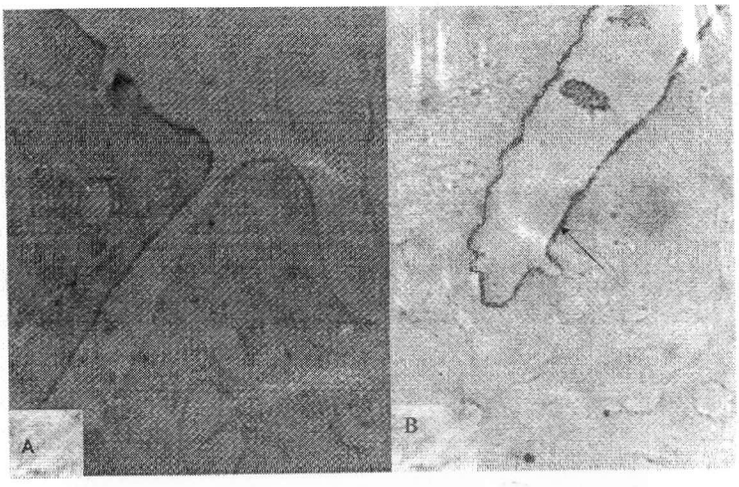
Fig(8): Nigella sativa Linn. treated Metestrus. **A**: Low power view of an isolated uterine horn, cervical canal at bottom. Note profound endometrial hypertrophy. (X 22). **B**: Higher view of A . showing deep glands. Note the non alcianophilic nature of the diastase fast-PAS reactive magenta colored apical granules (arrows); the alcianophilic brush border and the absence of intracytoplasmic alcianophilia. (X 1320).

Figures(9- 11): Alcian Blue 1.0 stained sections

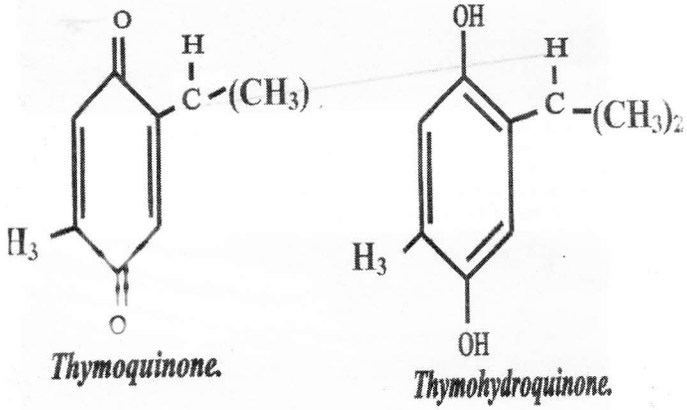
Fig(9): Estrus sections of control animals **A**: Showing alcianophilic staining of the luminal (arrow), and those lining neck portions of the glands (head arrow). (X 330). **B**: Higher view of A. showing the intracytoplasmic staining for sulfated highly acidic mu ns. (X 1320).



Fig(10): Sections of co trol animals **A**. Metestrus; and **B**, Proestrus endometrium. Note total absence of the sulfated-highly acidic mucin staining reaction, cf. Fig (9). (X 1320).



Fig(11): Nigella sativa linn. treated Estrus. **A:** Showing profound reduction in sulfated highly acidic mucin reaction. (X 330). **B**: Higher view of A. note restricted staining reaction to the luminal cells brush borders (arrow), cf. Fig (9). (X 1320).



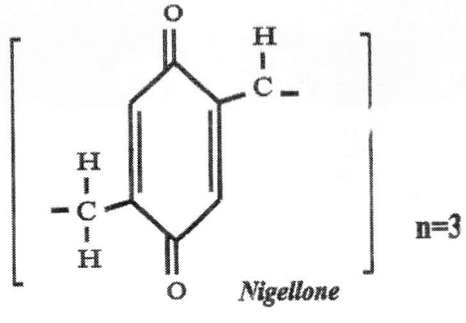


FIG (12): The chemical structure of Thymoquinone, [illegible] Thymohydroquinone ", and that of it's polymeric form [illegible] compounds found in The Black cumin seed[illegible].

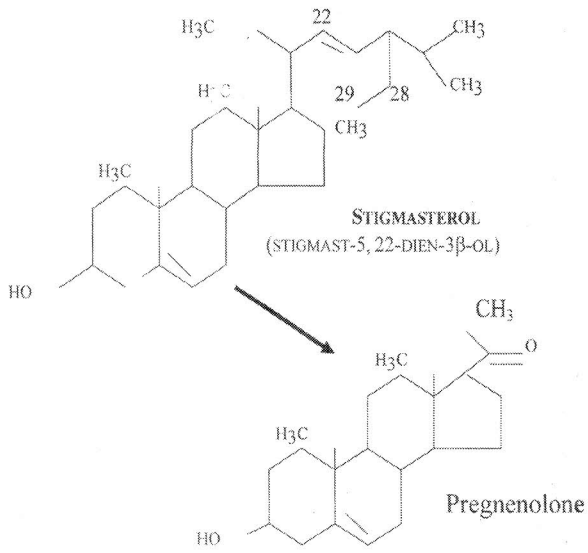


Fig (13) Structural formula of stigmasterol and that of pregnenolone (a pregnane type precursor). Notice the close structural resembelness of stigmasterol and Pregnenolone, a functional significance is attributable to the double bond at C22 position: Biochemical transformation of stigmasterol into pregnenolone in mammalian cells was found to occur [24].

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