Disruption of oocyte development and vitellogenesis in *Oryctes rhinoceros* treated with methanolic extract of *Eupatorium odoratum* leaves

K. B. Sreelatha and P. R.Geetha

ABSTRACT

Effect of methanolic extracts of *Eupatorium odoratum* (*Compositae*) leaves on the ovary of the coconut pest, *Oryctes rhinoceros* L.(Coleoptera: Scarabaeidae) was studied. Topical application of 10 and 20 µl extracts resulted in morphological, morphometric, gravimetric and histological aberrations in the ovary of the beetles. Ovariole of *O. rhinoceros* was telotrophic meroistic and consists of terminal filament, germarium and vitellarium. Terminal filaments of the ovarioles of the experiment are short and are not interconnected whereas in the control they are long and interconnected. Interfollicular tissue was distinct in the control and appears indistinct in the treated. Vitellarium of the treated insects shows fusion and abnormal orientation of follicles. Number of follicles and weight of ovary were reduced significantly ($p < 0.05$). Length of germarium was reduced in10µl treatment while it was increased in the 20 µl. Vitellarium and ovariole length was increased and reduced in 10 µl and 20 µl treated insects respectively. Topical application of the extract resulted in histological abnormalities including vacuolation of germarium, chromatin condensation and defective development of the follicular epithelium, lateral trophic, interfollicular and prefollicular tissues, vacuolation of ooplasm and abnormal /arrested vitellogenesis and choriogenesis. Vitellogenesis in *O.rhinoceros* can be disrupted by the application of *E. odoratum* extracts and this plant can be considered as a potential candidate for regulating this pest.

Key words: *Oryctes rhinoceros,* crop pest, *Eupatorium odoratum*, ovary, morphology, morphometry, histology, vitellogenesis

INTRODUCTION

Botanical insecticides have been identified as attractive alternatives to synthetic chemical insecticides for pest management. Many of them act as potent sterilants causing reproductive abnormalities including ovarian regression, abnormal/ arrested oocyte development and vitellogenesis (Singh, 2003; Sreelatha and Geetha, 2008). Anjali (2008) observed significant reduction in fecundity, hatchability and survival of eggs in *Epilachna dodecastigma* Weid exposed to sublethal concentration of neem leaf cake and oil. Application of *Melia azedarach* L.(Meliaceae) (Borges *et al.,* 2003) on *Boophilus microplus; Rumex dentatus* Hook.(Polygonaceae), *Portulaca oleracea* L.(Portulacaceae) and *Piper cubebae* L.(Piperaceae) (Khalaf, 2005) on *Fannia canicularis;*. *Pachypodanthium staudtii* (Annonaceae) (Koona and Koona, 2006) on the beetles *Acanthoscelides obtectus* and *Callosobruchus maculatus;* triterpenes from *Dysoxylum malabaricum* Bedd. (Meliaceae) (Nathan *et al.,* 2007) on *Anopheles stephensi* Liston and *Clausena dentata* (Rutaceae) (Malarvannan *et al.,* 2009) on *Helicoverpa armigera* Hubner resulted in reproductive abnormalities like reduced reproductive potential, inhibition of egg production, abnormal vitellogenesis and oocyte maturation and disturbance of ovarian proteins synthesis. *Azadirachta indica* A. Juss (Meliaceae) and *Hydnocarpus wightiana* Bl.(Bixaceae) induced significant reduction in the incidence of *O. rhinoceros* (Chandrika Mohan *et al;* 2000). *Annona squamosa* L.(Annonaceae) extract caused histomorphological derangements in the ovary of *O. rhinoceros* (Sreelatha and Geetha, 2008).

Eupatorium odoratum is a locally available plant which had proved its insecticidal property. Mixing of *E. odoratum* leaves with soil in sweet potato beds before planting, reduces weevil infestation (Rajamma, 1982). Inhibitory effects of phytochemicals on insect reproduction might be due to histological and biochemical alterations which lead to physiological impairments. Understanding of the malformations and structural deformities of the ovary could potentially be a foundation for devising strategies for safer pest control measures. The present study was undertaken to evaluate the effect of methanolic extracts of *E. odoratum* leaves in the ovary of the coconut rhinoceros beetle, *O. rhinoceros.*

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MATERIALS AND METHODS

Test animal

Adult *O. rhinoceros* emerged from the larvae and pupae collected from local dung pits and maintained in sterilized cow dung, were kept in the laboratory at an ambient temperature of 23-33^{0}C, 75-90% relative humidity and a photoperiod of about 12:12 LD. Larvae were kept individually in glass jars (18cm high and 9 cm diameter) with perforated lid. Adult eclosion date was noted on glass jar in order to identify the age of the beetle. One day old beetles having uniform length (4.5 cm) and weight (4 - 4.5 g) were used for the treatment. Both experiment and control groups consisted of 6 insects each. Experiment and control beetles were maintained in similar laboratory conditions and fed on ripe banana slices (40g/insect) at 24 hours interval and were checked twice a day to monitor their survival.

Preparation and application of the plant extract

Leaves of *E.odoratum* were dried in shade, powdered and extracted in Soxhlet apparatus using methanol in 1:4 (1gm leaf powder dissolved in 4 ml methanol) ratio. After 1 hour it was transferred to the oven and kept for 44- 48 hours for drying. 0.1% extract was prepared by dissolving 100mg solute in 100ml methanol. Using Hamilton's microlitre syringe, single dose of 10µl (10µg) extract was topically applied at the inter segmental membrane between the 6th and 7th abdominal segments to the first group of experimental insects. The respective control category insects were applied with 10µl methanol. 20µl (20µg) extract was applied topically to a second category of experimental insects while the corresponding controls were applied with 20 µl methanol.

Dissection of insects and morphological studies

Treated and control insects were ether anaesthetized for 5 minutes and dissected in cold insect Ringer solution (IRS) under binocular dissection microscope. Ovaries from control and experimental beetles were examined under the dissection microscope for morphological variations and photographs were taken using a digital camera.

Morphometric and gravimetric studies

For measurements, freshly dissected insects were used. Length of the ovariole was taken after straightening of the ovariole. It includes the length of germarium and vitellarium; Length and breadth of proximal and penultimate oocytes were also measured. Number of follicles in the vitellarium was counted. Gravimetric studies were made with an electronic balance. Weight of the beetles was taken just before dissections. Ovaries were dissected out and the ovarioles up to the lateral oviduct were isolated. Adhering fat body were completely removed and repeatedly rinsed in IRS and dried by placing on Whatman No.1 filter paper and wet weight of the ovaries were recorded. The data was analyzed statistically by Student's't' test.

Histological studies

Ovarioles were fixed in aqueous Bouins fixative for 48 hours. Following standard histological procedures (Gurr, 1962), 5µm thick paraffin sections were stained in Ehrlich's Haematoxylin and Eosin, mounted in DPX and examined under the light microscope and microphotographs were taken using digital camera.

RESULTS AND DISCUSSION

Morphological, morphometric and gravimetric studies

Each ovary of adult *O.rhinoceros* is formed of six ovarioles. Each ovariole consists of anterior terminal filament, middle germarium and posterior vitellarium (Fig 1a). The terminal filaments are long and those of each ovary are interconnected at their terminal ends forming a bundle in the control while in the experiments they are either very short or absent. According to Buning (1994), terminal filament is important in maintaining the integrity of the ovariole and direct contact with the somatic cells at the apical tip of the ovariole determines the asymmetric division of the oogonial stem cells in the germarium. In *Drosophila* ovariole morphogenesis starts with the formation of terminal filament and for ovariole morphogenesis, terminal filament cell cluster is essential (Sahut-Barnola *et al.*, 1995). Hence the abnormalities of

Table 1. Morphometric (mm) and gravimetric (mg) analyses of *O. rhinoceros* ovary

Treatment	LGM	LVM	LOV	NFO	LPX	LPN	BPX	BPN	WB	WO
Control (10µl)	5.17 ± 0.26	10.33 ±0.52	15.50 ±0.77	6.33 ± 0.52	3.17 ± 0.26	2.25 ± 0.27	2.08 ± 0.38	1.83 ± 0.26	4301.67±444.05	164.67± 5.57
EOE (10µl)	4.75 ± 0.27	12.08 ±0.38	16.83 ± 0.41	5.00 ± 0.00	4.42 ± 0.38	3.28 ± 0.25	3.00 ± 0.45	2.33 ± 0.26	2355.00±246.07	84.62±8.27
Control (20µl)	5.02 ± 0.10	10.58 ± 0.38	15.60 ± 0.33	6.17 ±0.41	3.17 ± 0.26	2.17± 0.26	1.93 ±0.10	1.88 ± 0.19	4093.33±549.61	166.17±4.67
EOE(20µl)	5.92± 0.38	7.50 ± 0.45	13.42 ± 0.58	1.50± 0.55	3.50 ± 0.32ns	2.00 ± 0.45ns	2.00 ± 0.32ns	1.58 ±0.20	2141.67±441.20	43.78±4.20

LGM-Length of germarium, LVM -Length of vitellarium, LPX -Length of proximal oocyte , LOV - Length of ovariole, LPN- Length of penultimate oocyte, BPX-Breadth of proximal oocyte, BPNBreadth of penultimate oocyte, NFO -Number of follicles/ovariole, WB- Weight of body, WO-Weight of ovary, EOE-*Eupatorium odoratum* extract, ns- not significant, all other values are statistically significant ($p < 0.05$)

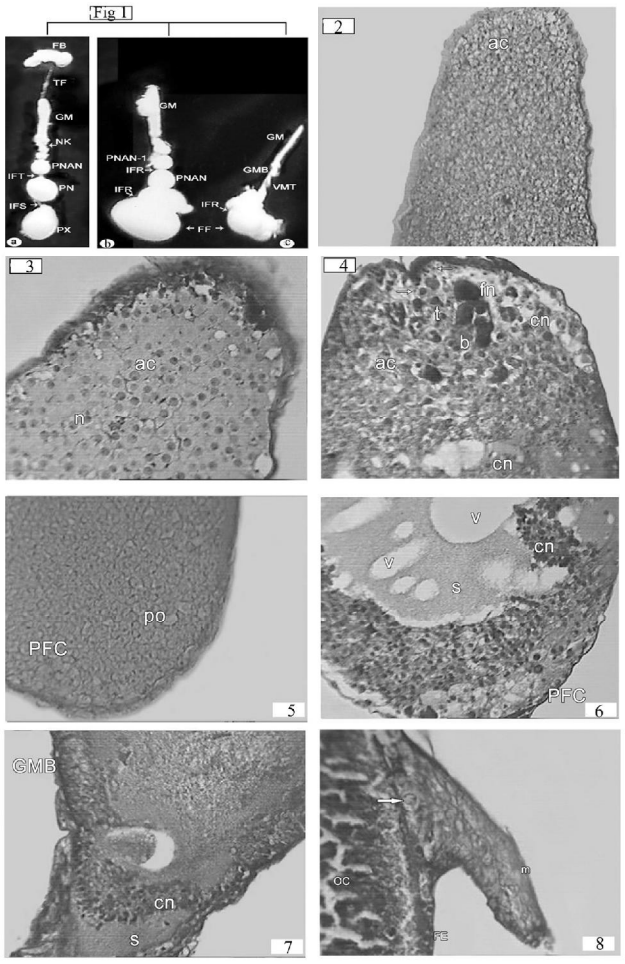

Fig 1. Morphology of mature ovariole, a - Control, b -10µ l *E. odoratum* extract (EOE), c - 20 µ l E OE, Fig.2-4. L.S of ovariole showing the anterior part of germarium x 400, Fig. 2.Control, Fig. 3. 10 µl EOE, Fig 4. 20 µl EOE, Fig 5-7. L.S of the ovariole showing posterior part of germarium x 400, Fig.5. Control, Fig 6. 10 µl EOE, Fig.7. 20 µl EOE, Fig.8. Oocyte in the control x 400, Fig.4 → Fragmented nuclei, Fig. 8→Follicle cells transforming into exochorion

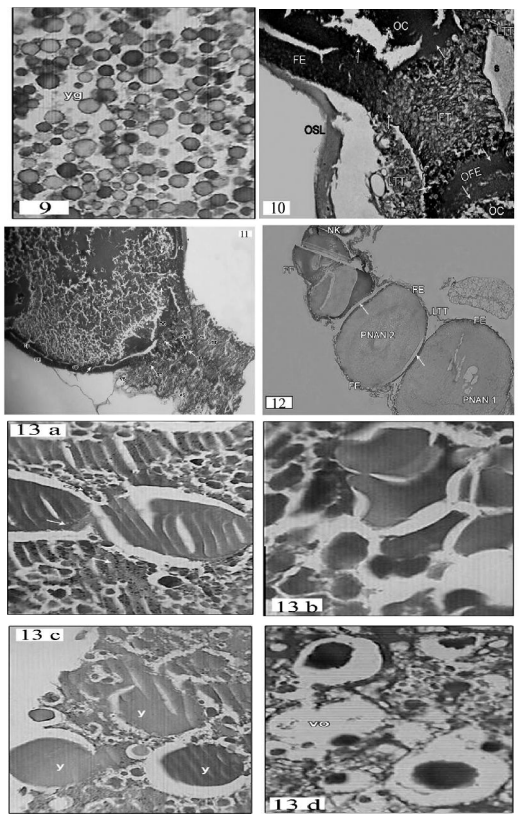

Fig 9. L.S of oocyte in the control showing yolk globules x 400 , Fig 10. L. S of Inter follicular region Control x 400, Fig 11. L.S of a vitellogenic oocyte, Control x 200, Fig 12. L. S of Inter follicular region, 10 µl EOE x 200, Fig13. L.S of oocyte in10 µl EOE x 400, Fig 10&11→Movement of materials from LTT, IFT and FE towards the oocyte, Fig 12. →Defective inter follicular regions, Fig13a.→ Small globules near the abnormal yolk

terminal filaments seen in the present study are competent to generate abnormalities in ovarian integrity. At the junction of the germarium and vitellarium there is a short constriction called the 'neck' in the controls which is indistinct or absent in both experiments. In the higher dose of the extract, a bulbular expansion is developed at the base of the germarium. Vitellarium of the control consists of a linear array of 5-7 follicles in various stages of maturation in which the proximal one is the oldest and most developed. Follicles are separated by interfollicular tissue (IFT); between vitellogenic follicles the tissue appears as stalks in the control (Fig1a). Vitellarium of the treated beetles shows abnormalities. In the lower dose, some of the anterior follicles are highly transparent and the vitellarium looks bulky, expanded and disproportionate mainly due to the expansion of the proximal and penultimate follicles (Fig 1b). The proximal follicles are expanded greatly that a slight pressure could rupture them resulting in the extrusion of the inner materials. Orientation of the follicles is not straight but slanting. Higher dose of the extract resulted in a short vitellarium with collapsed tubular region anteriorly and one or two abnormal shaped follicles posteriorly (Fig 1c). Both doses caused follicular fusion, indistinct 'neck' and IFT.

Length of ovariole is increased in the lower dose and decreased in the higher dose. Length and breadth of proximal and penultimate oocytes were significantly

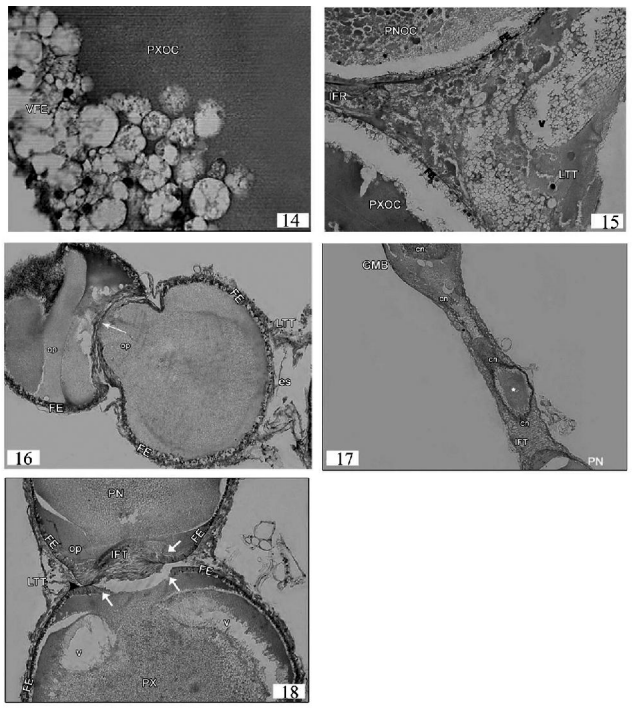

Fig 14. L. S of oocyte in10 µl EOE x 400, Fig 15-18. L.S of the ovariole, Fig 15. 10 µl EOE x 400, Fig 16. 10 µl EOE x 400, Fig 17. 20 µl EOE x 100, Fig.18. 20 µl EOE x 400, Fig 16, 18. → Defective inter follicular regions, Fig17. star → Oocyte like body, Fig18. Arrow Lead - Continuity of the FE.

Abbreviations

FB- Fat body, FE- Follicular epithelium, FF- Fused follicles, GM- Germarium, GMB- Bulb at the base of the germarium, IFR-Inter follicular region, IFS-Inter follicular stalk, IFT-Interfollicular tissue, LTT- Lateral tophic tissue, NK-Neck, OC- Oocyte, OFE- Open part of follicular epithelium, OSL-Outer layer of ovariole sheath, PFC- Prefollicular chamber, PNOC- Penultimate oocyte, PNAN1&2-Oocytes anterior to penultimate oocyte, PXOC-Proximal oocyte, TF-Terminal filament, VFE- Vacuolated follicular epithelium, VMT- Tubular part of the vitellarium, ac- anterior cells, b-binucleated cell, cn- condensed nuclei, cp- contact points, es- empty space, fn- fused nuclei, m- probable micropylar apparatus, n- nucleus, op- ooplasmic protrusion, po- pro oocyte, s- secretion, t- trinucleated cell, v-vacuole, vo-vacuolated ooplasm, vs- vesicles, y- yolk, yg- yolk globule.

increased ($P < 0.05$) in 10 µl treated ones. In the higher dose, length of vitellarium and beadth of penultimate oocytes revealed significant decrease. Number of follicles, mean body weight and ovary weight were decreased in all treated beetles (Table 1), hence interruption of metabolism is suggested. Morphological, morphometric and gravimetric variations as seen in the present study have been reported from other insects. Larvae of the teak defoliator *Hyblaea puera* that were chronically exposed to *Melia azedarach* extract recorded a reduction in weight (Nathan and Sehoon, 2006). Ovariole size, length, and oocyte number were reduced in D*ysdercus cingulatus* treated with extracts of *Vitex negundo* and *Eupatorium odoratum* (Prameela, 1997) and in *Corcyra cephalonica* emerged from neem fed larvae (Chanda and Chakravorty, 2000). Reduced size of ovary was reported in *Bactrocera cucurbitae* reared on food treated with methanolic extract of *Acorus calamus* (Nair and Thomas, 2001).

Histological studies

Ovariole of *O. rhinoceros* is meroistic telotrophic type since the trophic tissue is situated in the germarium. Germarium of the control is almost uniformly quiescent (Fig.2). In the 10µl group, anterior part of the germarium is formed of large cells with big nuclei looking like those in mitotic prophase (Fig 3) while in 20 µl treated insects, the anterior region of the germarium is occupied by condensed, fragmented and fused nuclei and cells with two or three nuclei (Fig 4). In the control, basal part of the germarium consists of the pre follicular chamber formed of the pre follicular cells and pro oocytes (Fig 5) but in the experimental insects the pre follicular chamber shows vacuoles, condensed chromatin and secretions (Fig 6, 7). Condensed trophic nuclei from the germarium of 20 µl treated ovarioles migrate downwards and aggregate in the bulbular part which continues to the vitellarium (Fig 7). Chromatin condensation and vacuolation in the germarium of treated insects can be an indication of cell death. According to Patricio and Cruz- Landim (2007) chromatin condensation in the somatic and germinal cell nuclei is the initial sign of cell death.

In the control, the vitellarium comprises follicles in progressive stages of maturation. A follicle is formed of an oocyte and a layer of follicular epithelium (FE) surrounding it. In the proximal oocytes vitellogenesis and choriogenesis are completed (Fig 8). Yolk consists of fine granules and globules of varying size (Fig 9). Follicular epithelial cells of the vitellogenic follicles are big and are engaged in the transfer of vitellogenic materials into the oocyte. Follicle cells maintain close contact with the oocyte at many points. Follicles are connected by interfollicular stalks formed of the IFT. On either side of the IFT, the inner layer of ovariole sheath referred to as the lateral trophic tissue (LTT) is expanded and modified for vitellogenic transfer. IFT is continuous with the FE of succeeding and preceding oocytes. The FE is open

towards the IFT to facilitate vitellogenic transfer (Fig 10, 11). In *O. rhinoceros* LTT and IFT have a significant role in yolk transfer.

Oocyte maturation and vitellogenesis were disrupted in the treated beetles. In 10 µl lot, vitellarium shows many abnormalities (Fig 12). Abnormal and disrupted vitellogenesis had taken place in the proximal and penultimate oocytes indicated by the presence of yolk of abnormal shape and size (Figures 13 a, b, c, d). Considerable fusion of yolk into patches and shrinkage of ooplasm were noticed. A major part of the FE is vacuolated and many vacuoles extend far into the oocyte (Fig 14). Due to heavy vacuolation, the follicular epithelium fails to remain as an integral layer. The LTT near the proximal and penultimate oocytes is filled with secretions and vacuoles; along many points the FE is very thin (Fig 15). No yolk transfer is observed from the follicle cells, IFT and LTT. Vitellogenesis is completely arrested in the anterior fused follicles and their FE is deformed and disrupted. Follicle cells are small and loosely arranged. Ooplasm of the fused follicles appear as condensed patches and protrusions. The IFT is absent or degenerated showing empty spaces. LTT at many points appear as membranes (Figures 12, 16). Yolk bodies of abnormal shape and size indicate altered integration and processing of yolk directing to defective and asynchronous vitellogenesis. Large vacuoles amidst the yolk and ooplasm might have resulted from ooplasmic shrinkage. Shrinkage of ooplasm, irregular shape of eggs, loosely arranged follicular cells, arrested/ partial vitellogenesis, compound egg chambers and vacuolated follicular epithelium were observed in *Corcyra cephalonica* emerged from larvae fed on neem mixed food (Chanda and Chakravorty, 2000). Ghazawi *et al*. (2007) reported that topical treatment of azadirachtin in *Heteracris littoralis* resulted in the shrinkage of ovaries with abolished oocyte growth and disintegration and destruction of follicle cells. Some of the present results like follicular fusion, defective vitellogenesis, abnormal FE and IFT resemble to the changes induced by JH analogue S-methoprene in *O. rhinoceros* (Leenamma, 1990) and in *Callosobruchus maculatus* (Sareen *et al*., 1992). Abnormal hormone modulations can be one of the reasons for the aberrations observed in the current study.

In 20 µl group, anterior half of the vitellarium is narrow and stalk like with condensed nuclei; there being no follicles (Fig 17). Proximal and penultimate oocytes are developed posteriorly in the vitellarium but they are abnormal; yolk deposition is totally arrested. Ooplasm is granular, shrunken, and vacuolated. The oocytes are closely pressed to each other as the IFT is absent or not properly oriented. The FE is broken and discontinuous. In the penultimate oocyte, the ooplasm is protruded. LTT is membraneous (Fig 18). Chorion is not formed in the experimental beetles. Chorion is a product of the follicle cells. Structural and functional anomalies induced by the phytochemicals in the follicle cells are possibly the reason for the failure of chorion deposition. Lucantoni *et al*. (2006) recorded a delay in oocyte development affecting vitellogenesis as well as choriogenesis in *Anopheles stephensi* treated with a commercial formulation from neem.

Impairment of follicle cell functioning remains a significant reason for arrested/ abnormal vitellogenesis observed in the treated insects. Fusion of follicles observed in the study is due to the absence of IFT. Abnormal shape and size of the follicles is due to the defective orientation of follicle cells. In all treated beetles, the ovarian somatic tissues (FE, LTT and IFT) show pronounced abnormalities competent to mess up vitellogenic transport. According to Nagoshi (2004) direct physical inter action between the follicle cells and the oocyte is necessary to define the shape and polarity of the egg as well as to control the differentiation and migration of follicle cells. Histomorphological derangements reported in the present study direct towards the potential of *E. odoratum* in inducing reproductive abnormalities in adult *O. rhinoceros*. Hence it is suggested that this plant can further be evaluated in pest management programmes**.**

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