



## Toxic impacts of Leaf extract of *Clerodendrum infortunatum* on the Ultrastructure of the Midgut epithelium of sixth instar larvae of *Orthaga exvinacea* Hampson

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### Abstract

The effect of methanolic leaf extract of *Clerodendrum infortunatum* on the ultrastructure of the midgut of sixth instar larvae of *Orthaga exvinacea* were evaluated under laboratory condition. Among five different concentrations of botanicals tested, one was selected for ultrastructural study. The effect of botanical showed drastic changes in ultrastructure of the midgut epithelium, mainly consisting of separation of myofibrils in muscle layer, vacuolization and elongation of columnar cells, detachment of epithelial cells from the basement membrane, loss of microvilli in both inside the goblet cells and apical portion of columnar cells and the changes in the shape of mitochondria and columnar cell nucleus. Since these changes may lead to serious digestive and metabolic disorders, thereby affecting the growth of the larvae, this botanical can be used for the management of this pest.

**Keywords:** Methanolic leaf extract, Ultrastructure, *Clerodendrum infortunatum*, *Orthaga exvinacea*.

### Introduction

The mango leaf webber *O. exvinacea* is one of the major pests responsible for the low productivity in mango fruit crop. The heavily infested trees presented a burnt look and severe infestation resulted in complete failure of flowering<sup>1</sup>. *O. exvinacea* has gained the status of serious pest due to its widely distribution in different agro-climatic zones of India<sup>2</sup>. Heavy infestation by this pest adversely affects the flowering as well as the growth of new flush<sup>3</sup>. Life cycle consists of egg, larval stage comprising of six instars, a prepupal stage, pupal and adult stage. The entire life cycle of this species might take 45 to 52 days. During larval stage, they are initially gregarious and feed by scraping the leaf surface but in late instar it feed individually on the whole leaf lamina leaving only the midrib. Use of synthetic insecticides to control this pest in order to increase crop yields, might involve health problems for other organisms. Synthetic insecticides could leave potentially toxic residues in food products and could be deleterious to non-target organisms in the environment<sup>4</sup>. So there has to develop an alternate approach to manage this pest. Plants are regarded as rich sources of natural substances that could be utilized in the development of environmentally safe methods for insect control<sup>5,6</sup>. Botanicals are found to be easily degradable, environmentally safe, have broad spectrum in action, non-persistent and easily processed<sup>7,8</sup>. Certain purified phytochemicals have proved their detrimental effects on insects including toxicity, growth retardation, feeding inhibition, oviposition deterrence, suppression of calling behaviour and

reduction of fecundity and fertility<sup>9, 10</sup>. Such broad spectrum of insecticidal effects of botanicals provide them as potential alternatives to the use of synthetic chemical insecticides<sup>5</sup>.

Many workers have reported that the toxic impact of *C. infortunatum* in insect pest control. It was observed that *Clerodendrum* spp. have insecticidal activity against various pests<sup>11,12</sup>. Furthermore, the impact of *Clerodendrum infortunatum* against *Oryctes rhinoceros*<sup>13,14</sup> and *Helopeltis theivora* Waterhouse<sup>15</sup> were also reported. In the present study an attempt was made to evaluate the toxic effect of *C. infortunatum* on the ultrastructure of the midgut epithelium of sixth instar larvae of *O. exvinacea* and thereby to prove the potency of the botanical in managing this pest.

### Materials and Methods

**Culturing of *O. exvinacea*:** The pupae and larvae of *O. exvinacea* were collected from the field and maintained in laboratory conditions. The larvae were reared in plastic troughs closed with muslin clothes and kept inside rearing cages, the two sides of which were netted with wire gauze. Fresh mango leaves were given till the pupation of the larvae. After pupation of larvae, the pupae were collected from the rearing cages and transferred into adult emerging cage. Adult moths emerged were sorted out for their sexes and kept in plastic jars in the ratio of 1:1 and closed the jars with cotton clothes. The sorted out adults were fed with 50% honey. When the egg hatched, young larvae were fed with fresh tender mango leaves. Laboratory reared

sixth instar larvae were used for the experiment.

**Preparation of leaf extracts:** Fresh leaves of *C. infortunatum* were collected from the field, washed and shade dried. These dried leaves were ground into fine powder with an electric mixer grinder and sieved through a muslin cloth. 50 gms of leaf powder was extracted using 500 ml methanol in Soxhlet apparatus at 70-80°C temperature. The extract was allowed to evaporate in a pre-weighed petridish in an oven at 50-60°C. After complete evaporation of solvent, 10% stock solution was prepared from the weighed extract using methanol. From this stock different desirable concentrations of botanicals were prepared.

**Transmission Electron Microscopy:** Sixth instar larvae of *O. exvinacea* were used for the experiment. From the observations on light microscopic studies, on the effect of different concentrations of botanicals (1%, 2%, 3%, 4%, 5%), the highly effective concentration-5% was selected for the ultrastructural studies. The pre-starved experimental larvae were fed with 5% of *C. infortunatum* treated mango leaves and the control larvae were fed with methanol treated leaves. After feeding for 48 hours, the larvae were sacrificed to collect midgut tissue and fixed in 3% glutaraldehyde in 0.1M phosphate buffer fixative. Washed and post fixed tissues were dehydrated in the series of ethanol (70%, 90%, 95% and 100%) and propylene oxide and embedded in araldite. Semi and ultrathin sections were cut using ultra microtome (Leica, Germany). Semithin sections were stained with 1% Toluidine blue and observed and photographed with an Olympus light microscope. Ultrathin sections were stained with lead citrate and examined with a Hitachi H500 TEM.

## Results and Discussion

Ultrastructural aspects of the midgut tissue of the normal (control) sixth instar larvae of *O. exvinacea* comprised of a basement membrane with connective tissue and inner to it a layer of epithelial cells and outer to it a muscle layer consisting of inner circular and outer longitudinal muscles which are formed of actin and myosin filaments (Figure-1.a,b). The epithelial cells consist of columnar cells, regenerative cells, goblet cells and endocrine cells which are not distinctly observed (Figure-1.f). General structure of muscle layers consisted of discontinuous network of circular muscle layer and longitudinal muscle layer (Figure-1.b,c). Circular muscle layer was found in inner side of muscle layer (Figure-1.e) and longitudinal muscle layer was in outer region (Figure-1.d). Thick arrangements of actin and myosin filaments, rich in mitochondria were observed (Figure-1.d,e). The basement membrane was found in basal region of epithelial cells in association with circular muscle layer (Figure-1.a). Muscle layer and basement membrane together separate the epithelial cells from haemolymph (Figure-1.a). The basement membrane has structural specialisations that facilitate elasticity exhibited by midgut that helps to change in volume.

**Columnar cells:** In ultrastructural studies, columnar cells were observed as the most commonly found cells in epithelium. (Figure-2a). The basal wall of columnar cells was folded along with invaginations of basement membrane (Figure-1f). The nucleus of columnar cells was located in mid-basal part of the cell with condensed chromatin granules (Figure-2a). Abundant secretory vesicles and rough endoplasmic reticulum were concentrated around the nucleus (Figure-2b,c). Secretory granules were present in variable density in the cytoplasm representing enzymes or their precursor proteins (Figure-2d). Numerous mitochondria and secretory vesicles were observed in cytoplasm (Figure-2 e,f). Apical region of columnar cells were rich in microvilli and presence of smooth endoplasmic reticulum were found (Figure-2g,h). Peritrophic membrane was observed in control larvae which was situated in between lumen and brush border of epithelial cells (Figure-2.i).

**Goblet cells:** Goblet cells were present in the basal region of the epithelium with flask shaped basal cavity. The cavity, goblet chamber was formed from the invaginations of the apical border of the cell. (Figure-3.a). In control, goblet cells have enormous microvilli rich in mitochondria (Figure-3.b,c). These microvilli were concentrated mainly at the basal region of the goblet cells or valve like packing was observed (Figure-3.b). Nucleus of the goblet cells was found in the basal part of the cell.

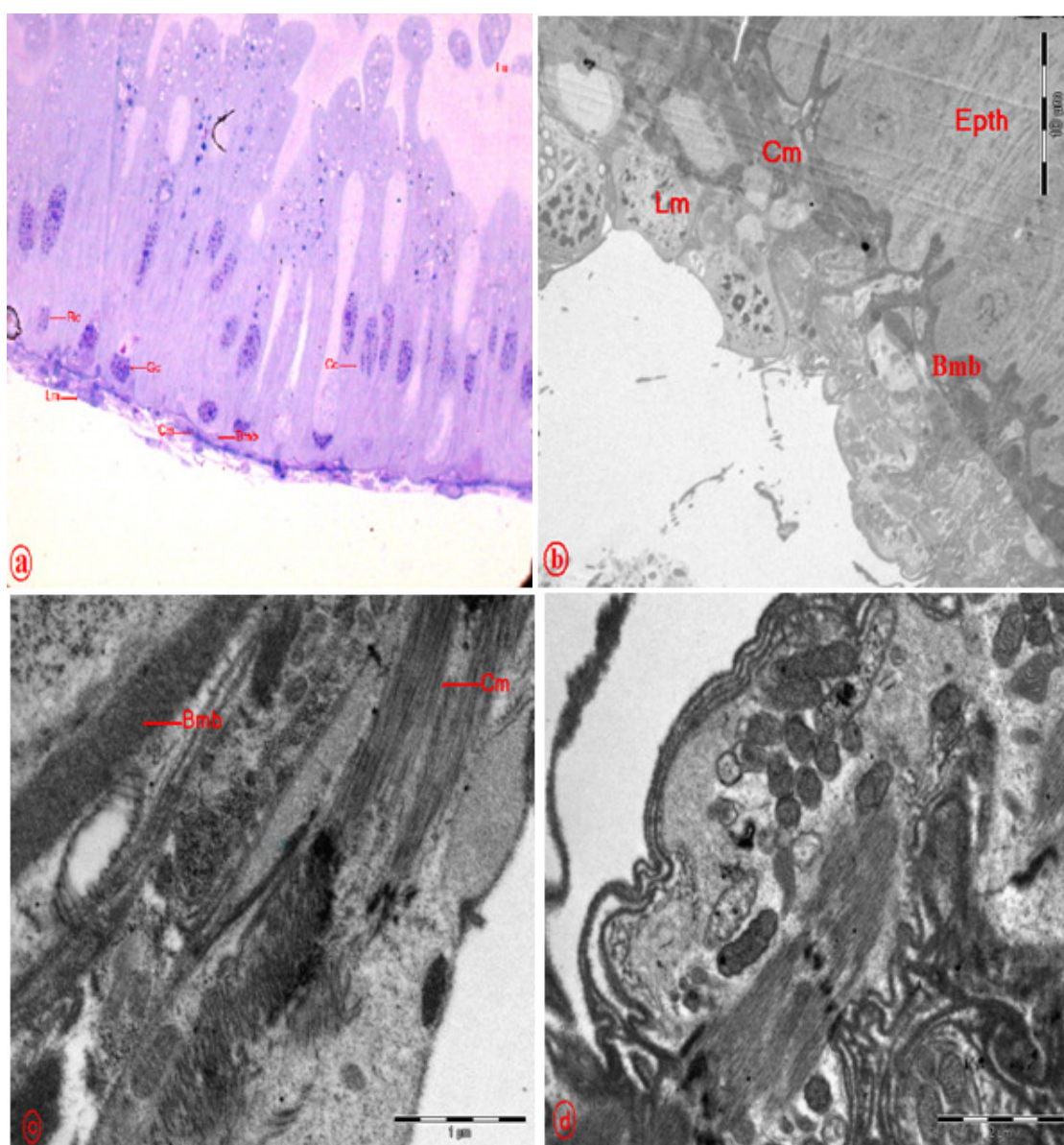
**Regenerative cells:** Regenerative or undifferentiated cells were found in the basal region of epithelium in between columnar cells and goblet cells (Figure-1.f). The cells were so called because of its undifferentiated state at the time of development. They were engaged in renewal or replacement of injured cells.

**Cytotoxicity of *C. infortunatum*:** The ultrastructural changes in the histomorphology of the midgut tissue of the sixth instar larvae treated with 5% of *C. infortunatum* were observed in both muscle and epithelial layer (Figure-4a,b). In circular muscle layer, the separation of myofibrils and vacuole like formation were noticed (Figure-4c). Excessive vacuolization in the basal region of the epithelial cells and detachment of epithelial cell layer from the basement membrane were observed (Figure-4b). Enormous elongation and cytoplasmic vacuolization were observed in columnar cells (Figure-4d). Nucleus of the columnar cells became shrunken and some vacuoles were found around the nucleus (Figure-4e). The rough endoplasmic reticulum was not distinctly seen in the columnar cells. The cytoplasm of columnar cells was vacuolated and the shape of mitochondria was changed (Figure-4f). In the case of goblet cells, the whole microvilli present in the cell were ruptured and became dense in colour (Figure-4g,h). Changes in the shape and reduction in the number of mitochondria were also noticed in the goblet cells (Figure-4h). Destruction of microvilli in the apical region of columnar cells was also observed (Figure-4.i) and the presence of smooth endoplasmic reticulum was very poor.

The midgut of insects is the main part of the digestive tract, in

which digestion and absorption occur; the wall comprises of a single layer of digestive epithelium and two muscle layers<sup>16</sup>. Active compounds present in *C. infortunatum* might be the cause for the ultrastructural variations found in both epithelial and muscle layers of the midgut tissue of the sixth instar larvae of *O. exvinacea*. It was reported that when *Spodoptera littoralis* was treated with the extract of both *Clerodendrum inerme* and *Conyzadio scorids* there occurred slight and severe disintegration of the epithelium, fading of the boundaries of epithelial cells and detachment of epithelial cells<sup>17</sup>. Cytoplasmic vacuolization and necrosis of the epithelial cells and destruction of epithelial cell boundaries were reported in the larval midgut of *S. littoralis* treated with *Azadiracta indica* and *Citrullus colocynthis* extracts<sup>18</sup>. Vacuoles might occur as result of cell

elongation or as result of excessive fat droplets which dissolves during fixation and dehydration process<sup>19</sup>. Destruction of microvilli and reduction in number and changes in the shape of mitochondria might affect the function of goblet cells concerned with excretion of potassium. It was reported that squamocin from *Annona squamosa* on *Aedes aegypti* larvae prevented the production of ATP by the electrons in the mitochondrial complex I and caused the death of the insect by affecting cellular respiration<sup>20,21</sup>. Histopathological changes in midgut epithelial cells induced by ingestion of phorbol-type compound Jatropherol-I, revealed that it caused disintegration of the epithelial cells and it led to severe turbulence in insect metabolism, especially in protein metabolism with alterations in activities of various midgut enzymes<sup>22</sup>.





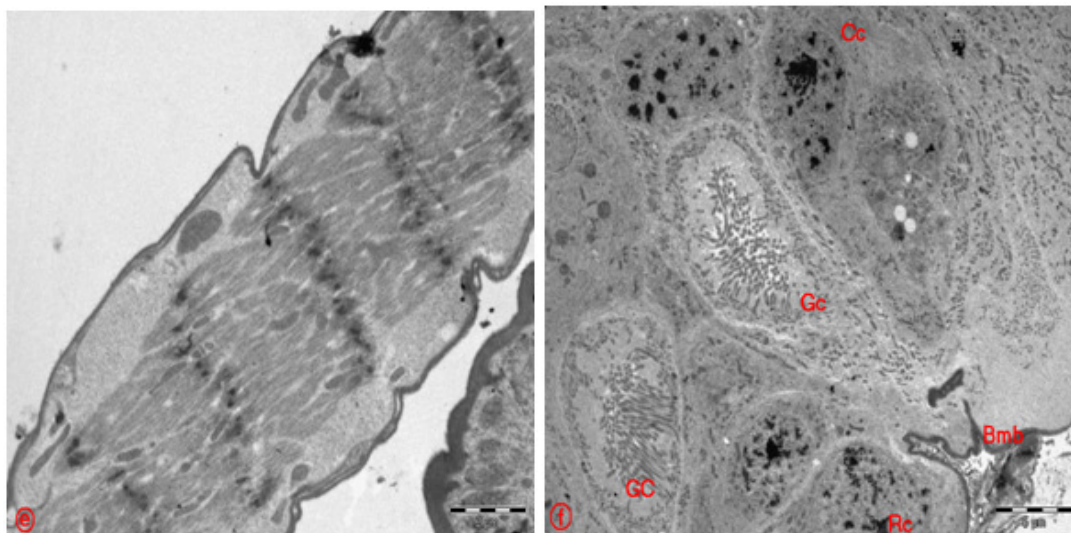
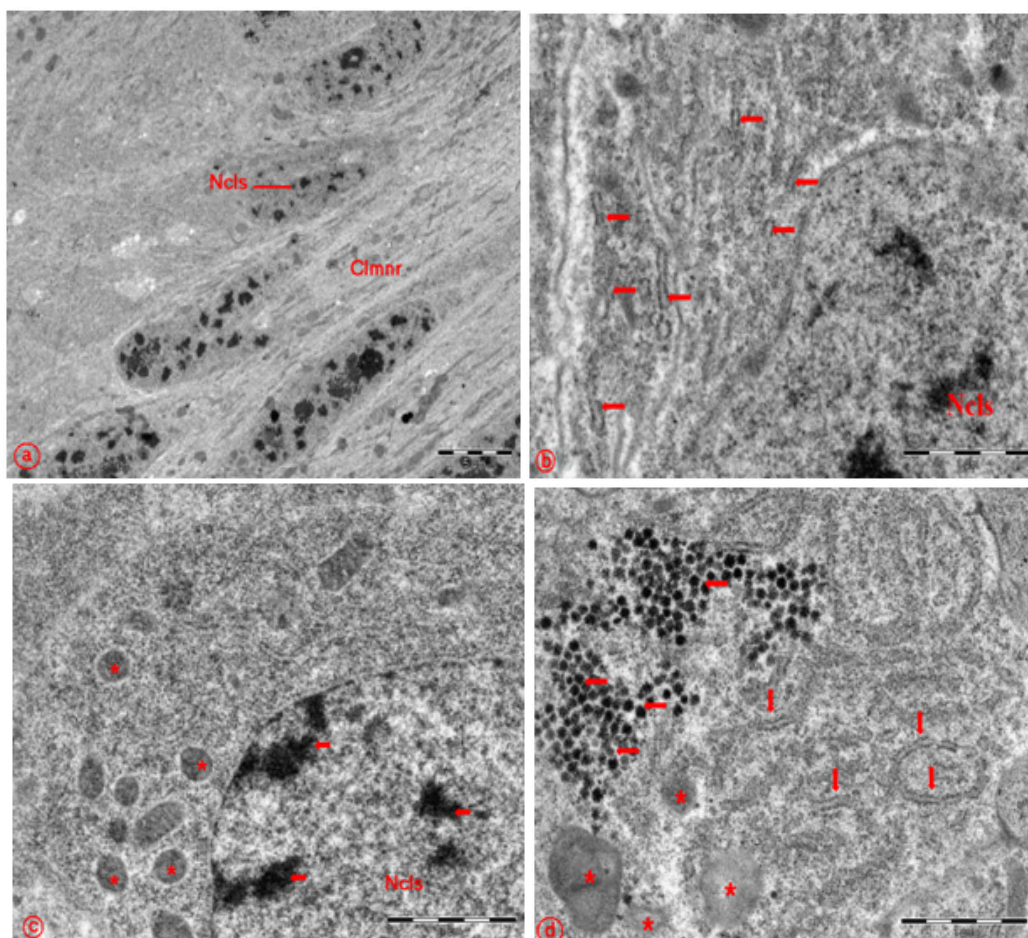
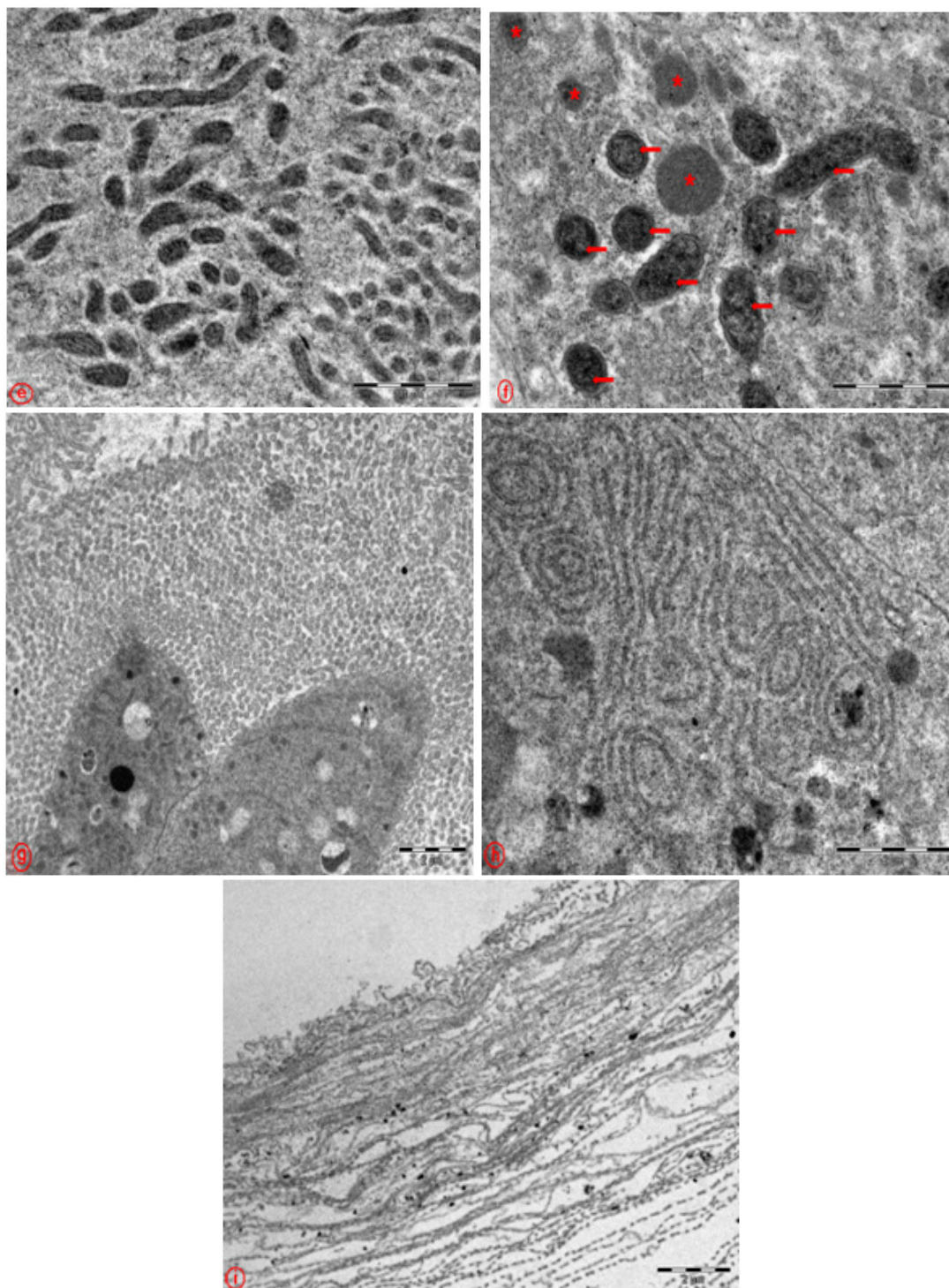


Figure-1

a. General aspects of the midgut tissue of the sixth instar larvae of *O. exvinacea* showing both muscle and epithelial layer (Semi thin section).(1000X). b. Ultrastructural aspects of longitudinal muscle layer (Lm); Circular muscle layer (Cm); basement membrane (Bmb). (1900X). c. Magnified portion of muscle layer and basement membrane (Bmb) (23000X). d. Longitudinal muscle layer. (11000X). e. Circular muscle layer. (6800X). f. Ultrastructural aspects of different epithelial cells. (2900X)

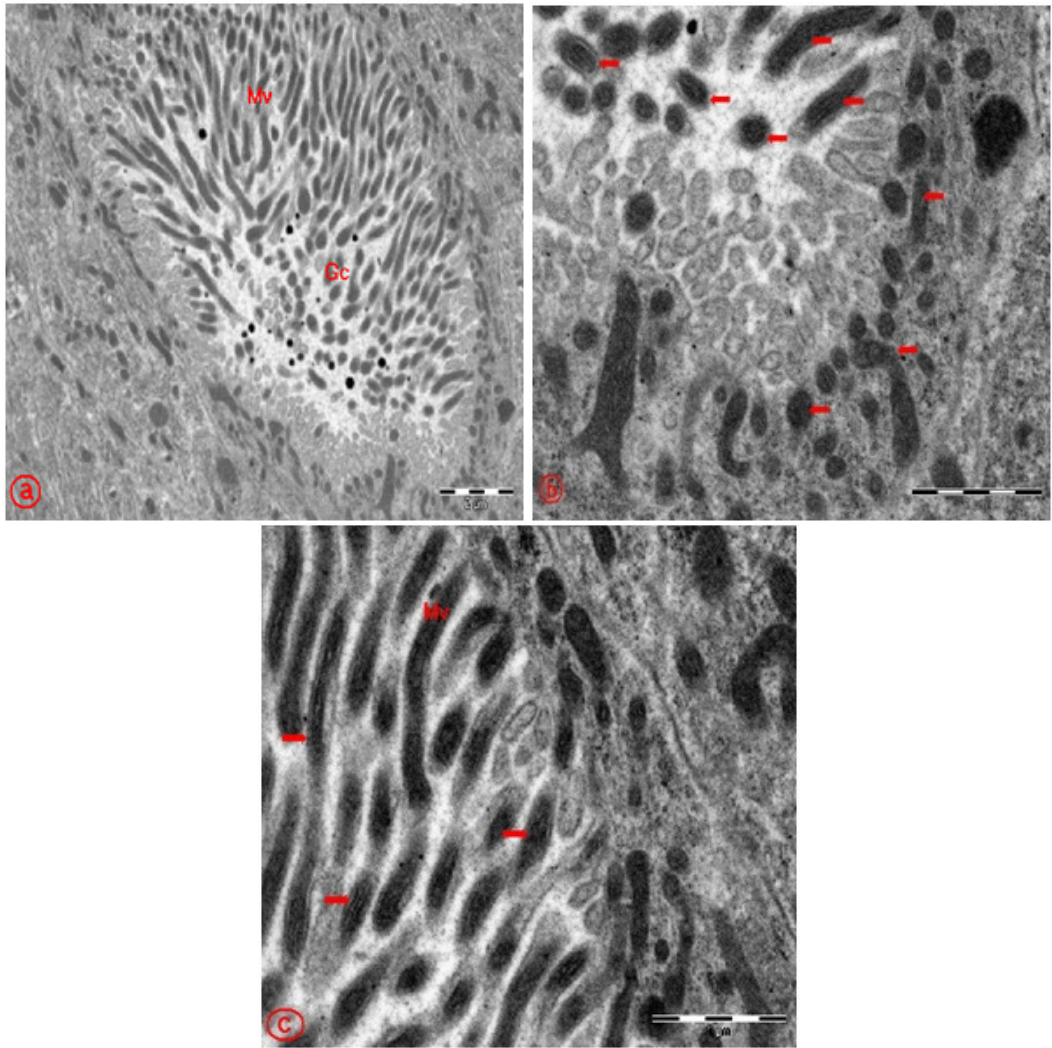




**Figure-2**

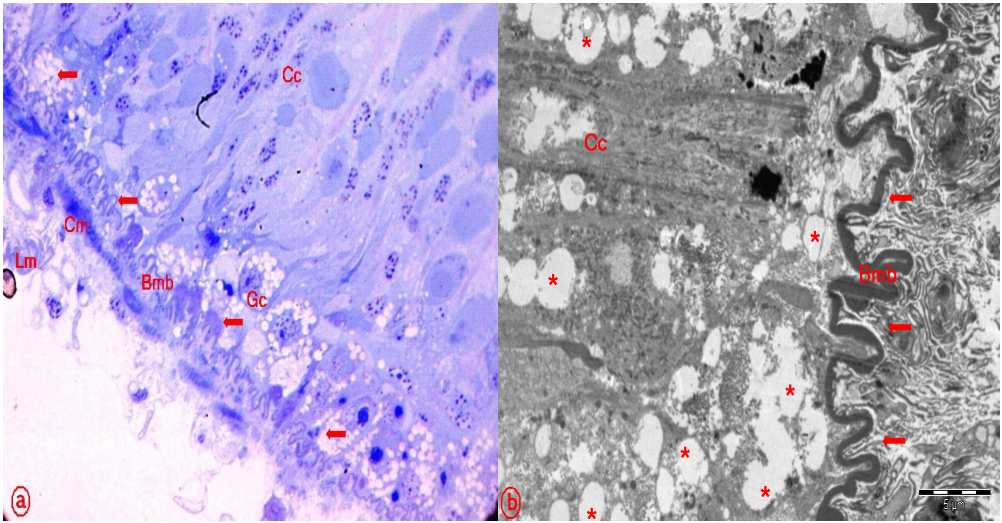
- a. Ultrastructural aspects of untreated Columnar cells. (2900X). b. Nucleus of columnar cells and rich in RER (arrows). (2900X). c. Secretory vesicles (asterisks) and nucleus having condensed chromatin granules (arrows). (23000X). d. Showing numerous secretory granules (arrows); secretory vesicles (asterisks); and RER (downward arrows) in columnar cytoplasm.(23000X). e. Rich in mitochondria. (23000X). f. Enlarged portion of secretory vesicles (asterisk) and mitochondria (arrows). (23000X). g. Showing apical region of columnar cells with numerous microvilli. (6800X). h. Abundant SER in apical region of columnar cells.(11000X). i. A magnified portion of peritrophic membrane.(6800X)



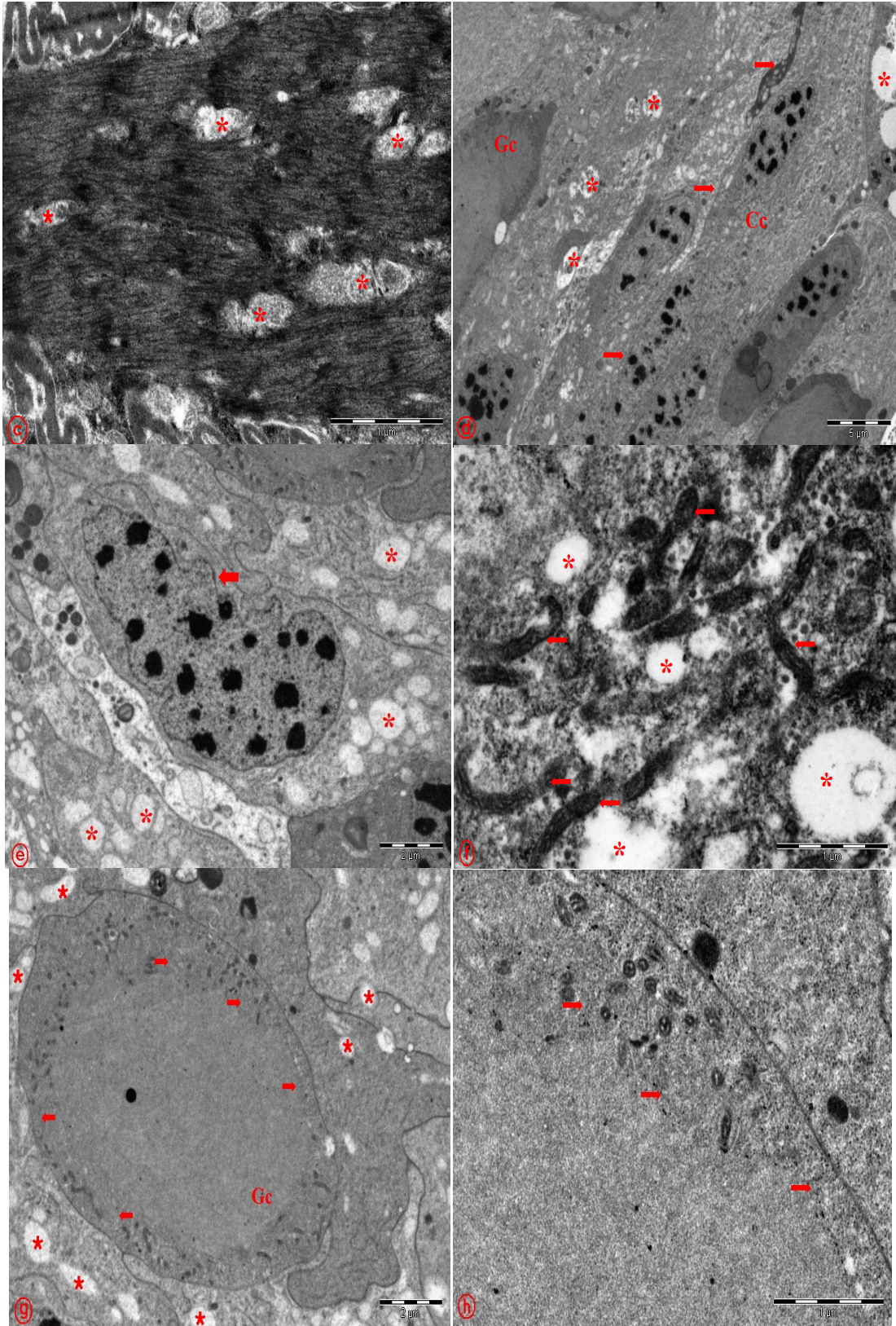


**Figure-3**

- a. Ultrastructural aspects of goblet cells (6800X). b. Showing numerous microvilli in inside goblet cells (arrows).(23000X).  
c. Magnified portion of microvilli rich in mitochondria (arrows). (23000X)**







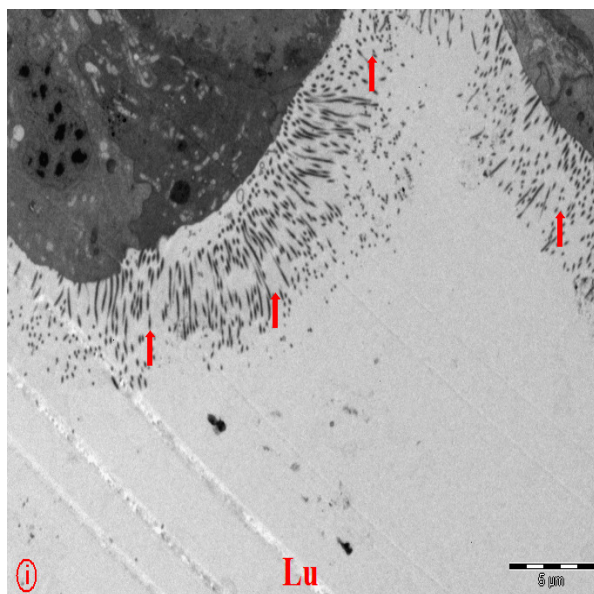


Figure-4

a. Semi thin section of larval midgut treated with 5% of *C. infortunatum* showing excessive epithelial vacuolization in the basal region (arrows). (1000X) . b. Ultrastructural view of vacuolization (asterisks) and detachment of epithelial cells from the muscle layer (arrows). (2900X) c. Separation of myofilaments and vacuole like formation in the circular muscle layer (asterisk).(23000X). d. Showing the excessive elongation of epithelial cells (arrows) and cytoplasmic vacuolization (asterisks). (2900X). e. Excessive vacuolization in columnar cytoplasm (asterisks) and shrunken nucleus (arrow). (6800X). f. Showing shape of mitochondria were changed (arrows) and vacuole formation (asterisk).(23000X). g. Showing the effect on goblet cells as ruptured microvilli inside the goblet cells (arrows) and vacuolization (asterisk).(6800X). h. Magnified view of ruptured microvilli with shrunken mitochondria inside the goblet cells (arrows).(23000X). i. Loss of microvilli in the apical region of columnar cells (arrows).(2900X)

## Conclusion

In conclusion, it is suggested that the effect of the active ingredients in the leaf extract of *C. infortunatum* might be the reason for the ultrastructural alterations in the epithelial cells, destruction of microvilli, large areas of cytoplasmic vacuolization, mitochondrial damage and shrunken nucleus. These deleterious changes might cause serious absorptive disorders and it might affect normal growth and reproduction of *O. exvinacea*, so this botanical can be used for the management of this pest.

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