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Histopathological effects of leaf extracts of *Clerodendrum infortunatum* and *Eupatorium odoratum* on the midgut tissue of sixth instar larvae of *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae)

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Abstract
The histomorphological changes due to the effect of leaf extracts of *Clerodendrum infortunatum* and *Eupatorium odoratum* on the midgut tissue of sixth instar larvae of *Orthaga exvinacea* was studied under laboratory conditions. The caterpillars were fed with five different concentrations (1%, 2%, 3%, 4%, and 5%) of botanicals treated mango leaves for 48 hours. The effect of botanicals on the morphometric and histological changes in the midgut tissue shows that they can cause degenerative effects in the midgut epithelium. The changes occurred in the structure of the epithelium mainly consist of elongation and vacuolation of columnar cells, increased secretory activity of columnar cells, sloughing off of the apical region of the columnar cells into the lumen and lysis of the sloughed off portion of the epithelium in the lumen. Since these botanicals cause disruptive changes they can be employed for the management of this pest.

Keywords: *Orthaga exvinacea, Clerodendrum infortunatum, Eupatorium odoratum, midgut tissue, histopathology.*

1. Introduction
In recent years the excessive use of synthetic chemical insecticides in agriculture as a pest control method in order to increase crop yields, has led to serious health hazards in other living organisms. These insecticides are often associated with residues that are dangerous for the consumer and the environment [1]. Application of chemical insecticides cause adverse effects like toxicity to non-target organisms, development of insecticide resistance, pest resurgence, environmental pollution and health hazards. Emphasis is now being given on integrated pest management which lays stress on minimal use of insecticides and their integration with other control techniques [2]. Botanical insecticides have been identified as attractive alternatives to synthetic chemical insecticides for pest management. Biopesticides are considered to be safe to natural enemies and free from residue problem on the crop and in the environment [3]. Many workers reported that plants are considered as one of the richest sources that can be used as pest control agents [4]. Many of them act as potent sterilants causing reproductive abnormalities including ovarian regression, abnormal, arrested oocyte development and vitellogenesis [5, 6, 7]. Effects of neem are anti-feedant, repellent, metamorphosis disruption, growth disruption, oviposition deterrent and anti-reproduction in insects [8].
The mango leaf webber, *O. exvinacea* is one of the major pests of mango tree. In caterpillar stage, it causes defoliation and reduction in crop yield. Heavy infestation by this pest adversely affects the flowering as well as the growth of new flush [9].
C. infortunatum and *E. odoratum* are locally available plants which had proved its insecticidal property. Pesticidal effect of *C. infortunatum* on the fat body of *Oryctes rhinoceros* causes drastic changes like reduction in the lobes of fat body and their impairment with the disintegration of cell membrane, shrunken and scattered nucleus [10]. *E. odoratum* leaves mixed with soil in sweet potato beds before planting reduces weevil infestation [11]. Methanolic extracts of *E. odoratum* leaves caused disruption of oocyte development and vitellogenesis in *Oryctes rhinoceros* [7].
The present study was undertaken to evaluate the effect of methanolic extracts of *C. infortunarum* and *E. odoratum* leaves in the midgut tissue of sixth instar larvae of *O. exvinacea*.

2. Materials and Methods

2.1 Rearing of *O. exvinacea*

The pupae and larvae of *O. exvinacea* were collected from the field, reared and maintained in laboratory conditions. The larvae were reared in plastic troughs covered with muslin clothes and kept inside rearing cages. Fresh mango leaves were given till the pupation of the larvae. Adult moths emerged were sorted out for their sexes and placed in plastic jars in the ratio of 1:1 and fed with 50% honey. When the eggs hatched, young larvae were fed with fresh tender leaves. Laboratory reared sixth instar larvae were used for the experiment.

2.2 Preparation of leaf extracts

Fresh leaves of both plants, *C. infortunatum* and *E. odoratum* 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 gm of this fine leaf powder was extracted using 500 ml methanol in Soxhlet apparatus at 70-80ºC temperature. The extract was allowed to evaporate in a pre-weighed Petri dish in a hot air oven at 50-60ºC. After complete evaporation of solvent, 10% stock solution was prepared from the weighed extract using methanol. From this stock different desirable concentrations of botanicals (1%, 2%, 3%, 4% and 5%) were prepared.

2.3 Histological and Morphometric studies

Newly moulted sixth instar larvae were used for the experiment. Fresh mango leaves were treated with different concentrations of plant extracts and allowed to air dry for a few minutes. This treated leaves were supplied to pre-starved experimental larvae for 48 hours. The control set was maintained with feeding larvae with methanol treated leaves. After 48 hours larvae were sacrificed to collect midgut tissue and fixed in Bouin's fixative for 24 hrs. Following standard histological procedures [12] 5 µm thick paraffin sections were stained in Delafield's Haematoxylin and Eosin, mounted in DPX and examined under light microscope and microphotographs were taken using digital camera.

Microphotographs taken were analysed for the morphometric studies. Height and width of columnar cells and diameter of nucleus were measured from five different region of same section by using Olympus Magnus-pro software. Data were statistically analysed by ANOVA using SPSS and expressed as Mean ± Standard Deviation. Level of significance of each experiment was highly significant ($p<0.01$).

3. Results and Discussion

3.1 Histological studies

General histomorphology of the midgut consist of two layers of muscle which includes inner circular and outer longitudinal and the epithelial layer consisting of columnar cells, goblet cells and regenerative cells and peritrophic membrane (Fig. 1).The histological effect of *C. infortunatum* shows that at 1% concentration, there occurs elongation of columnar cells, vacuolation of cytoplasm, enlargement of columnar nucleus and some secretory activity (Fig. 2). At 2% concentration, there occurs folding of the epithelium and elongation of the nucleus (Fig. 3). The effect of 3% concentration shows that the region of alternative folds and infolds of epithelial layer gets overlapped and there occurs detachment of epithelium from basement membrane (Fig. 4). In higher concentration (4%), excessive elongation and increased secretory activity of columnar cells and vacuolation were observed (Fig. 5). At 5%, due to excessive elongation and vacuolation, the apical region of the columnar cells were sloughed off and moved into the lumen and the size of columnar nucleus were decreased (Fig. 6).

The effects of different concentrations of *E. odaratum* on the midgut tissue epithelium show that in addition to increase in size of the goblet cells, there occurs cytoplasmic vacuolation of the columnar cells and thinning of muscle layers. In the case of 1% and 2% concentrations, there occurs increase in size of goblet cells and vacuolation of cytoplasm (Fig. 7 and 8). There was also increased secretory activity of the columnar cells in 1% concentration which was not observed in the case of 2%. In the case of 3% concentration, vacuolation of cytoplasm of columnar cells and pinching off apical tips of the cells were observed (Fig. 9). Thinning of muscle layer, enlargement of columnar nucleus and destruction of brush border of epithelial layer were found in 4% concentration (Fig. 10). In the case of 5% concentration, elongation of columnar cells, vacuolation of cytoplasm and sloughing off of apical region of epithelium into lumen were observed (Fig. 11).

3.2 Morphometric studies

Significant morphometric changes were observed in height, width and diameter of nucleus of columnar cells of midgut tissue treated with both botanicals. The normal height, width and diameter of nucleus of columnar cells of untreated tissue were 690.10 µm, 55.63 µm and 85.19 µm respectively (Table 1). The effects of *C. infortunatum* at concentration of 1%, height of columnar cells were increased to 732.70 µm and width and diameter of nucleus were decreased to 35 µm and 43 µm respectively but in 2%, height, width and nuclear diameter of columnar cells were decreased (Table 1). In the case of 3% and 4%, due to elongation of cells, height and width of columnar cells were increased and the diameters of nucleus were decreased. At 5%, excessive elongation caused sloughing off apical parts of columnar cells and thereby reduced height and width of the cell and diameter of nucleus also decreased (Table 1).

The effects of *E. odoratum* at 1% were that the length and nuclear diameter of columnar cells increased, but columnar width decreased (Table 1). When compared to 1%, columnar length were decreased in 2% and disruption of apical region caused the rapid reduction in length of columnar cells treated with 3% but the cell width and diameter of nucleus were increased. In higher concentration 4%, length and width of columnar cells were increased and enormous increase in nuclear diameter was noticed as a result of swelling of nucleus (Table 1). At 5%, destruction of brush border caused the decrease in length of columnar cells and decreased width and diameter of nucleus were also noticed.

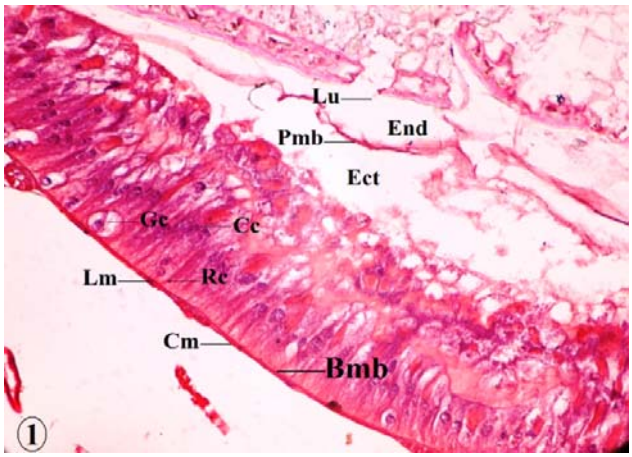

Fig 1: Histomorphology of the midgut tissue of untreated larvae: Lumen (Lu); Inner most Peritrophic membrane (Pmb); Endoperitrophic (End) and Ectoperitrophic (Ect) spaces; Columnar epithelial cells (Cc); Goblet cells (Gc); Regenerative cells (Rc); Basement membrane (Bmb); Circular muscle layer (Cm); Longitudinal muscle layer (Lm)(400x).

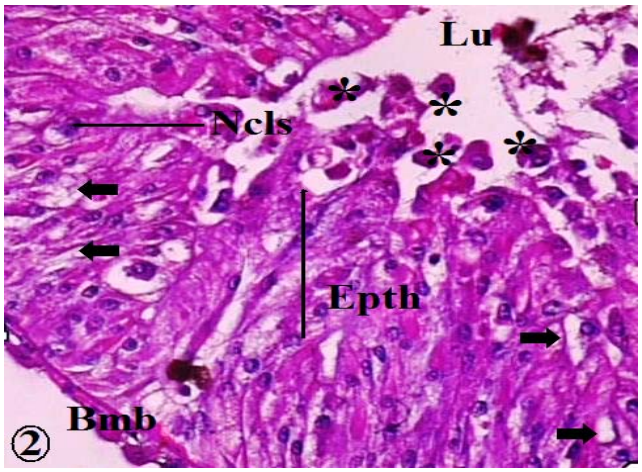

Fig 2: Cross section of the midgut tissue of *O. exvinace* larvae treated with 1% *C. infortunatum* showing elongation of epithelial cells (Long arrow); Vacuolation of cytoplasm (Thick arrows); enlargement of nucleus (Ncls); Secretory vesicles (*)(400x).

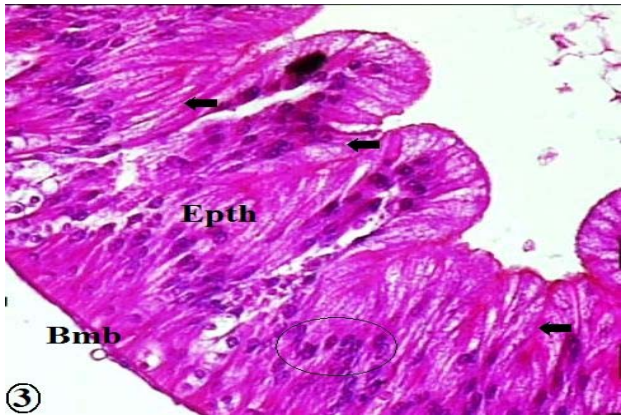

Fig 3: Larvae treated with 2% *C. infortunatum* showing the region of alternative folds and infolds in epithelial layer of migdut tissue overlap (arrows); due to overlapping columnar nucleus were elongated and congested (in circle)(400x).

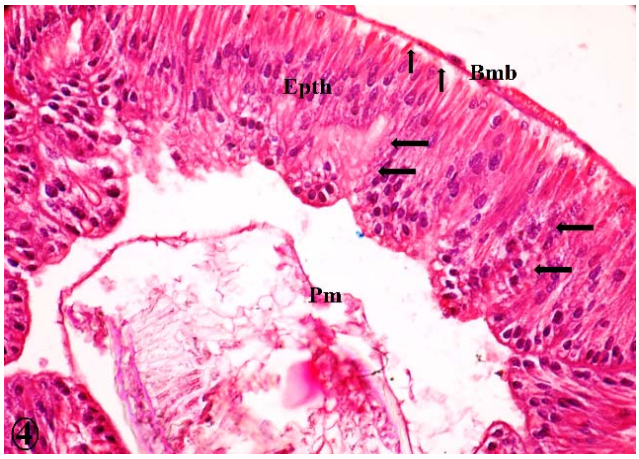

Fig 4: Cross section of the midgut tissue of larvae treated with 3% *C. infortunatum* showing the detachment of epithelial layer from the basement membrane (upward arrows); completely overlapped folded region (thick arrows)(400x).

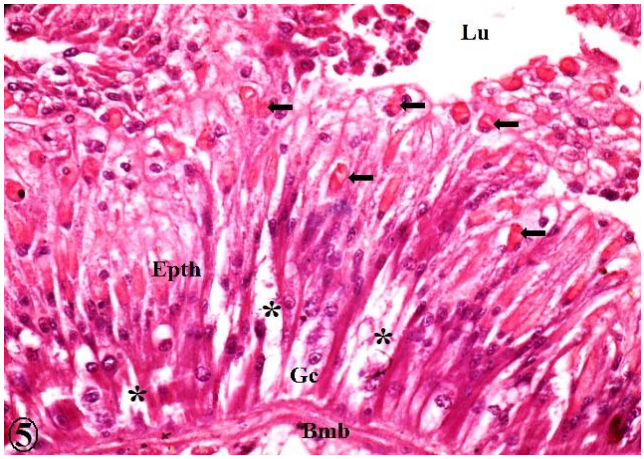

Fig 5: Larvae treated with 4% showing the secretory vesicles (arrows); Vacuoles due to excessive elongation (*)(400x).

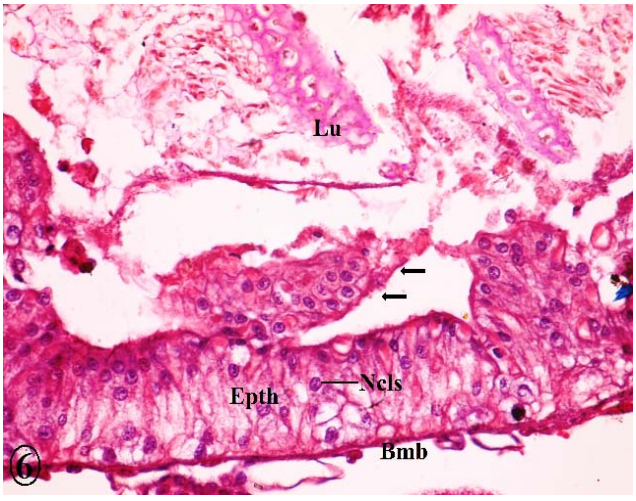

Fig 6: Cross section of the midgut tissue of larvae treated with 5% *C. infortunatum* showing that due to excessive elongation, the apical region of the columnar cells were sloughed off and moved into the lumen (arrows); Size of nucleus decreased (Ncls)(400x).

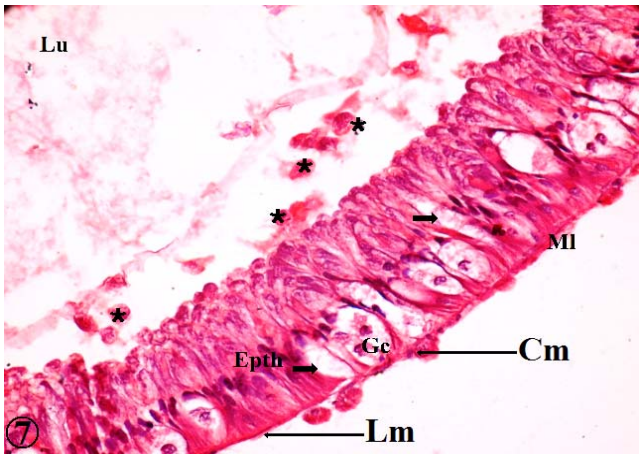

Fig 7: Cross section of the midgut tissue of *O. exvinace* larvae treated with 1% *E. odoratum* showing the vacuolation (arrows); Longitudinal muscle layer (Lm); Circular muscle layer (Cm); Secretory vesicles (*); Size of goblet cells were increased (Gc)(400x).

Fig 9: Cross section of the midgut of *O. exvinace* larvae treated with 3% *E. odoratum* showing the excessive vacuolation and destruction of the apical region of epithelial cells (arrows); Secretory vesicles (*) (400x).

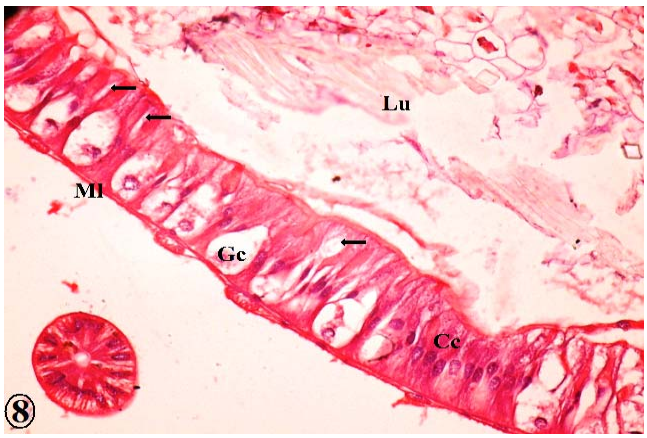

Fig 8: Larvae treated with 2% showing vacuolation (arrows); Size of goblet cells increased (Gc) as in 1% (400x).

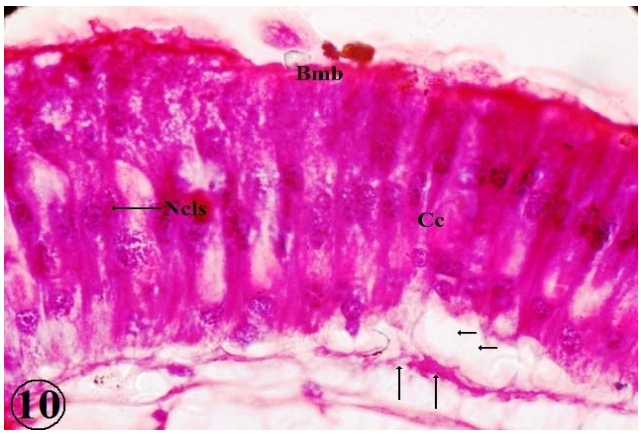

Fig 10: Larvae treated with 4% *E. odoratum* showing the destruction of brush border of epithelial layer (arrows); Nucleus size increased (Ncls)(1000x).

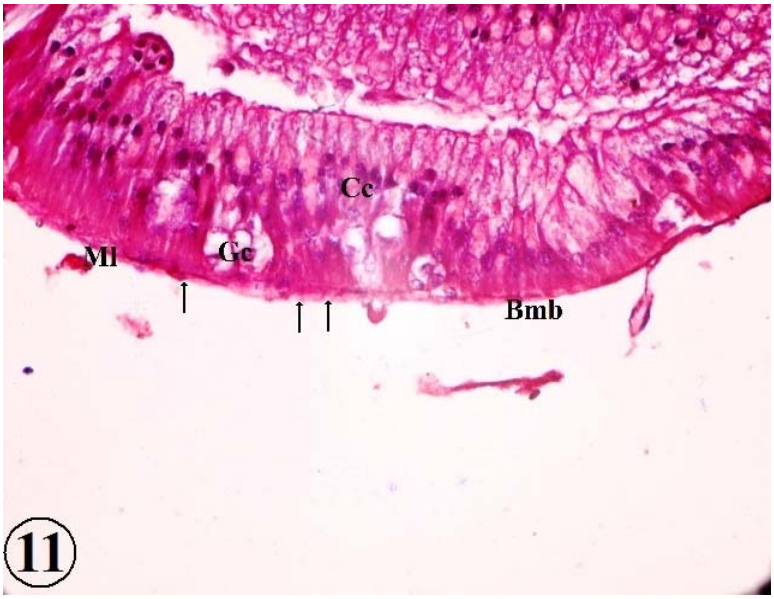

Fig 11: Cross section of the midgut tissue of *O. exvinace* larvae treated with 5% *E. odoratum* showing thinning of muscle layer (arrows) (400x).

Table 1: Showing the morphometric changes in columnar cells due to the effect both botanicals.

Treatment of botanicals	Parameters	Measurements in different concentrations (in micron)						F value
		Control	1%	2%	3%	4%	5%	
Clerodendrum infortunatum	Height of Columnar cells	690.10± 47.80	732.70± 34.87	508.66± 37.49	1156.85± 47.20	2399.25± 144.23	1033.10± 54.84	455.11
	Width of columnar cells	55.63± 7.95	35.16± 1.43	32.16± 3.77	53.35± 6.07	84.21± 11.80	83.77± 6.11	52.55
	Diameter of Columnar nucleus	85.19± 10.73	43.97± 4.17	36.88± 4.52	150.38± 11.28	109.17± 5.67	130.66± 6.69	177.86
Eupatorium odoratum	Height of Columnar cells	690.10± 47.80	1305.47± 24.80	1102.22± 70.48	371.88± 20.84	1991.38± 53.88	918.88± 88.43	491.92
	Width of columnar cells	55.63± 7.95	40.10± 4.19	57.36± 7.74	52.72± 2.44	182.90± 6.37	82.85± 2.57	434.11
	Diameter of Columnar nucleus	85.19± 10.73	162.88± 13.09	183.01± 6.08	48.67± 2.89	363.28± 19.59	151.26± 4.07	489.71

Each value represents- Mean±SD. Significant level- $p<0.01$ = highly significant.

The histopathological changes that occurred in the larval midgut epithelium of *O. exvinacea* treated with both botanicals *C. infortunatum* and *E. odoratum* mainly consist vacuolation and elongation of epithelial columnar cells, destruction of brush border of columnar epithelial cells and secretory activity and sloughing off of the apical region of the epithelial layer. Sayed *et al* [13]. reported that in the larval midgut of *Spodoptera littoralis* treated with *Azadirachta indica* and *Citrullus colocynthis* extracts there occurred vacuolation and necrosis of the epithelial cells and destruction of epithelial cells and their boundaries. Vacuoles may occur as result of cell elongation or as result of excessive fat droplets which dissolves during fixation and dehydration process [14]. Epithelial elongation, excessive vacuolation and enlargement of nucleus were observed as common effects of both botanicals in larval midgut tissue and the degeneration of epithelial cells were increased with concentration of both botanicals increased. According to Humbert and Desportes [15] the degeneration process allows for the removal of toxic elements from the alimentary canal. Plant flavonoids are group of allelochemiclas with widely noted prooxidant effects [16]. Effect of plant cyclotides on the larval midgut of *Helicoverpa armigera* showed that the cyclotides disrupt the plasma membrane of the epithelial cells forming holes or pores that lead to cell swelling and lysis [17].

The presence of active ingredients in both botanicals caused the histological and morphometric variations in epithelial layer which may lead to digestive and food absorptive disorders. Studies on the midgut tissue of *Hofmannophila pseudospretella* showed that there were differences in the size and activity of middle midgut columnar and goblet cells depending on food quality [18]. Significant difference was noticed in size of columnar and goblet cells in gypsy moth larvae fed with suitable *Quercus cerris* leaves compared with larvae fed on *Robinia pseudoacacia* leaves which is associated with higher digestive activity [19]. Terra *et al.* [20] observed that the cellular components that fill the apical region of the columnar cells were those involved in the elaboration of the digestive enzymes, so any morphometric changes or destruction of the apical region of the columnar cells may be the cause for digestive disorders.

Jing *et al*. [21] reported the histopathological changes in midgut epithelial cells, induced by ingestion of phorbol-type plant isolated compound Jatropherol-I, reveal that it caused disintegration of the epithelial cells and it lead to severe turbulence in insect metabolism, especially in protein metabolism with alterations in activities of various midgut enzymes. In the present study, the effect of active ingredients in the botanicals may be the cause for the histological and morphometric changes in the midgut tissue of *O. exvinacea* and may thereby affect digestion, absorption and various other physiological processes.

In conclusion, the disruptive effect of both botanicals proves that the emulsifiable concentrate of *C. infortunatum* and *E. odoratum* has potency for the management of *O. exvinacea*.

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