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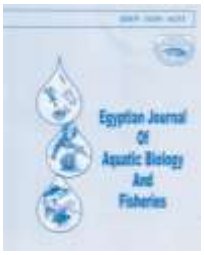

Histological and ultrastructural alternations in the digestive gland of the Egyptian slug, *Limax maximus* (Linnaeus, 1758) treated with botanic molluscicidal thymol, with reference to biological studies

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ABSTRACT

The present work included biological studies, morphological and anatomical features, beside histological and ultrastructural alternations due to effects of the botanical thymol, as poison bait, on the digestive gland of the Egyptian giant garden slug, *Limax maximus* (Family: Limacidae) after treating it with LC_{50} and LC_{90} for 48 hrs. Thymol is considered as a botanic molluscicidal mono-terpenoid, that is found in several plants. The estimated value of LC_{50} and LC_{90} of thymol are 269.77 and 362.79 ppm respectively. The effect of LC_{90} on the digestive gland caused severe histological changes and ultrastructural abnormalities; as: cytoplasmic vacuolation, scattered toxic agents, degeneration of some nuclei and cells, rupture of microvilli, increasing of calcium spherules inside secretory cells and wide-fused vacuoles. So, thymol may be of great value in the field to control the target slug, as safe and economic molluscicide, which no harm upon ecosystems instead of using chemical pesticides that could pollute the environment.

INTRODUCTION

The giant garden slug, *Limax maximus* (Family: Limacidae) has a natural Western Palearctic distribution, but had been introduced into North and South America, South Africa, some Pacific Islands, Australia and New Zealand and many places in the world (Herbert, 2010).

In Egypt, few studies had been achieved on the slug *L. maximus* by El-okda (1980) in Alexandria and Beheira, Azzam (1995) in several Egyptian governorates and Beltagi, *et al.* (2016) in Al-Galubeiah, Egypt.

The slugs considered a crop pest, where they destroy fruit trees, vegetables and ornamental plants. Thus, they cause serious reduction in the yield production of crops and fruits, beside destroying plant seedlings (El-okda, 1980). The mucus from the slug activity is also known to accelerate nutrient cycling and used for treatment of skin warts and wounds (Thomas, 2013). In addition, it is used as slug syrup to cure cough and bronchitis. (Mustafa, 2001).

In contrast, synthetic chemical molluscicides had a toxic effects on non-target organisms, where they contaminate soil and water and may consequently affect local populations of humans and other animals (Thiengo *et al.*, 2005).

The molluscicidal plants have many advantages as: low toxicity effect to non-target organisms, biodegradable, not expensive and more safely to the environment (Abdel-Haleem, 2013).

Thymol is mono-terpenoid found in a wide variety of plants: from which *Thymus vulgaris* L. and *Origanum vulgare* (Grodnitzky and Coast, 2002). It has been shown to possess remarkable molluscicidal activity against *Lymnaea acuminate* (Singh and Singh, 2000), the land snail *Helix aspersa* (El-Zemity, *et al.,* 2001); land snail *Subulina octona* (Ferreira, *et al.,* 2009) slugs *Arion lusitanicus* and *Deroceras reticulatum* (Kozlowski and Kozlowski, 2007) insecticidal activity (Pavela and Sedlák, 2018) and antibacterial activity (Sokolik, *et al.,* 2018).

The present studies aim to describe the external and internal features, through morphological and anatomical studies, on the slug. In addition, examination of the histological and ultrastructural effects of the thymol in cell-types of the digestive gland of the treated slug, after treatment with LC_{50} and LC_{90} of thymol compound.

MATERIALS AND METHODS

Biological studies:

Morphology:

Adult slug *Limax maximus* had been examined under binuclear to examine, photographed and diagrammatically draw details of morphological features of it.

Anatomy:

Adult slug *L. maximus* had been dissected under binuclear to examine, photographed and draw diagrammatic representation of the details of internal organs of it.

Experimental animals

The slugs *L. maximus* were acclimatized under laboratory conditions before being used in the experimental tests for 48 hrs. Adult slug had been collected from the garden of Faculty of Education, Ain Shams University, Cairo, Egypt. They kept in transparent plastic storage boxes containing humid soil, 3cm depth, in the laboratory and covered with muslin cloth. Slugs were daily fed with fresh lettuce leaves and water were added to provide suitable humidity for slug activity and the continuous cleaning of the boxes achieved.

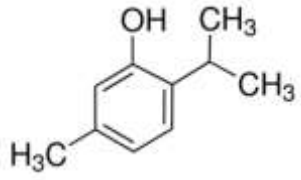
Thymol product:

The plant product used as molluscicide in the present work is thymol, that purchased from El Gomhoureya co. as following:

Molecular Formula: $C_{10}H_{14}O$.

Molecular Weight: 150.221 g/mol.

Chemical structure:



Experimental preparations:

Prepared concentrations, 200, 250, 300, 350, 400 ppm of thymol had been mixed with molasses and wheat-bran in 250 ml-plastic cups, to obtain poison bait. Controls (un-treated slugs) were prepared with the same procedure without adding poison baits. Then, slugs examined daily for 48 hrs. Dead individuals were counted and removed. Mortality percentages were recorded (48 hrs. post-treatment).

To estimate LC_{50} & LC_{90} of thymol, the experimental samples were divided into three groups (10 slugs/cage per each) as follows:
Group 1: Control (untreated) slugs.
Group 2: Treated slugs with LC_{50}.
Group 3: Treated slugs with LC_{90}.
Finally, this experiment had been repeated four times and the mean of the estimated data obtained.

Bioassay:

The values of LC_{50} and LC_{90} of thymol were calculated according to the method of Litchfield and Wilcoxon (1949). The percentage of the mortality were recorded after 48 hrs. LC_{50} and LC_{90} for thymol were determined by the probit analysis method according to Finney (1971).

Histological preparation:

Alive slugs from control and treated experiments were dissected and digestive glands were immediately excised and cut into small pieces. These specimens were fixed in aqueous bouin's fixative for 24 hrs, then kept in a mixture of 70% ethanol and glycerol (95:5). Then dehydration achieved through an ascending series of ethanol followed by clearing in terpineol for three days, washed in benzene and embedded in paraffin wax. Sections ,6µm thick, were prepared, mounted on clean glass-slides and stained with Ehrlich's acid alum haematoxylin and counter stained by Eosin. Finally, the slides were examined and photographed using light microscope (Olympus CX31) connected with digital camera (model No. E -330) at central lab., Faculty of Education, Ain Shams University.

Ultrastructural preparation:

Control and treated slugs were anesthetized with 30% ethyl alcohol/ chloroform and dissected to obtain the digestive gland. Dissected glands were cut into small pieces, fixed with 2.5% paraformaldehyde-3% glutaraldehyde (pH 6.7) at room temperature for four hours, post-fixed with (1%) phosphate buffered OsO_4 (15 minutes) and the specimens were rinsed in 0.2 M phosphate buffer (pH 7.3), finally specimens were dehydrated in ethyl alcohol and embedded in Epon 812 mixture. Then, semi-thin sections were obtained with LKB-V ultramicrotome and stained with uranyl acetate and lead citrate. Finally, prepared sections were examined under TEM (JEM 100CX-II, 80kV) at the Regional center of Mycology and Biotechnology (RCMB), Al-Azhar University.

RESULTS

Biological studies:

Morphology:

The slug *Limax maximus* is belonging to family Limacidae. Its measures are 10-20 cm in length and is generally light grey or grey-brown colored with darker spots and blotches and characterized by short keel. The head occupies the anterior region and has the mouth opening on its front surface and dorsally bears two pairs of sensory tentacles. The 1st anterior pair of tentacles is olfactory in function and relatively short, whereas the posterior 2nd pair of tentacles is optical in function, relatively longer than the 1st one and has terminal black eye-spot. The slug has internal short shell. Pneumostome is situated at the right side of the mantle. Foot is a muscular organ, creamy white in color and secretes colorless mucus (Fig. 1, a & b).

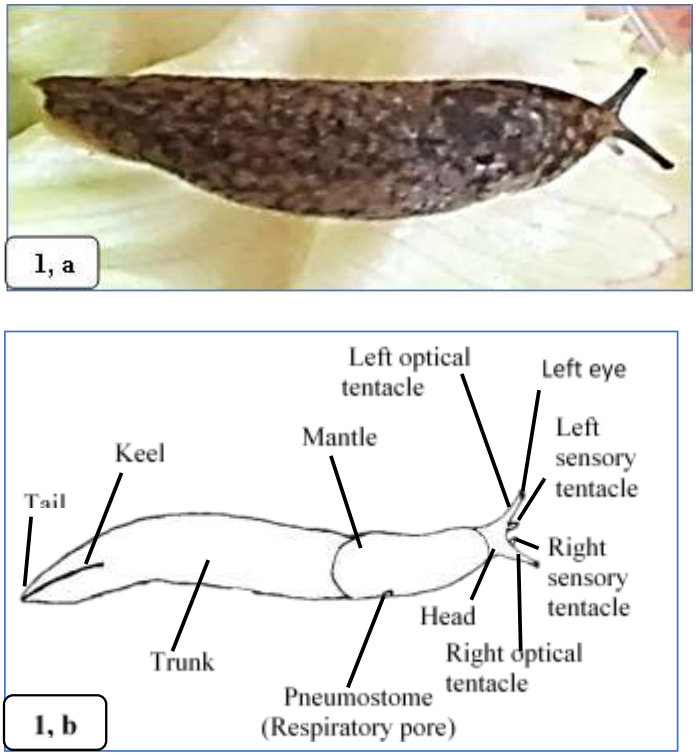


Fig. 1: A photograph of *L. maximus* showing external features (a) and a diagrammatic drawing of external feature of the slug (b).

Anatomy:

After being dissected, the slug *L. maximus* showed buccal mass leads to esophagus continuing through crop, stomach to three loops of intestinal canal leading into rectum and finally the anus.

There are right and left salivary glands, each has salivary duct. Digestive gland is formed of two large lobed-digestive caeca, darkly brown in color and connected with the stomach and intestine. The internal organs of the reproductive system include dark brownish ovotestis which located in the posterior end of the haemocoel followed by duct of ovotestis, albumen gland, fertilization chamber, male channel, female channel and penis (Fig. 2, a & b).

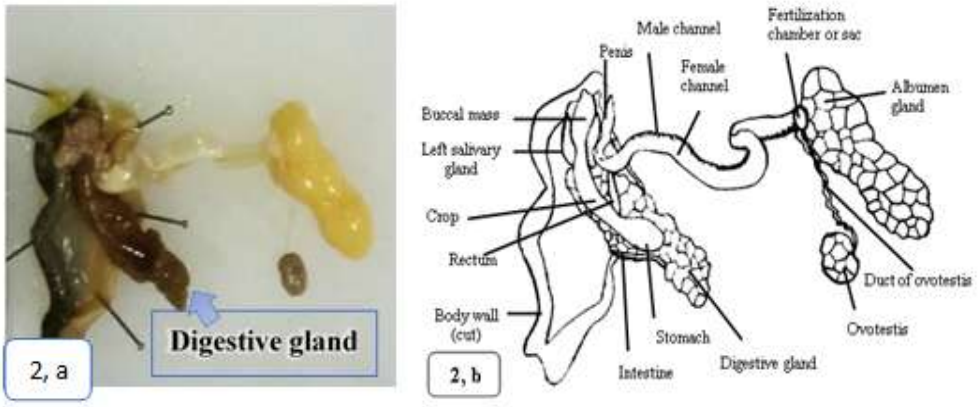


Fig. 2: A photograph of a dissected untreated specimen of *L. maximus* (a) and a diagrammatic drawing of the internal organs (b).

Bioassay:

The resulted estimated values of LC_{50} and LC_{90} obtained from the susceptibility test of the thymol against *L. maximus* were 269.77 ppm and 362.79 ppm respectively, as described in Table (1) and Figure (3).

Table 1: Molluscicidal activity of thymol against the slug *Limax maximus*, after 48 hours of treatment.

Concentration	Dead/total	Observed mortality %	Expected mortality %
Control	0/20	0	-
200	4/20	20	14.5152
250	6/20	30	43.5881
300	16/20	80	71.6006
350	17/20	85	88.3056
400	20 (19.5)/20	100	95.7776
Slope		9.2490+/-1.6107	
X^2		3.0562	
LC_{50}		269.77	
LC_{90}		362.79	

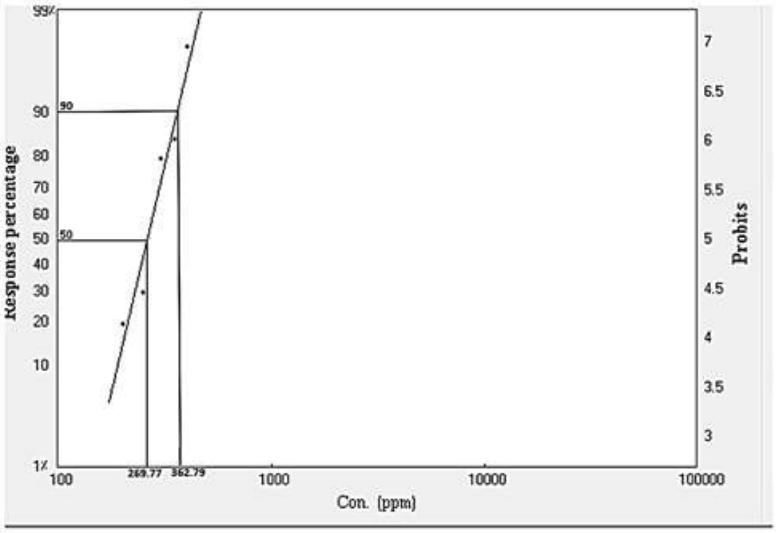


Fig. 3: Regression mortality line of the treated slug *Limax maximus* with different concentrations of thymol.

Histological and ultrastructural studies of digestive gland:

Histological examination:

The digestive gland of *L. maximus* composed of columnar epithelial cells; named digestive and secretory cells arranged around a central lumen. These cells rested on a basement membrane (Fig. 4). Digestive cells are simple columnar cells with round apices and narrow bases. They are characterized by several vesicles of different sizes and elliptical or irregular basal nucleus (Fig. 5). On the other hand, secretory cell is pyramidal or conical-shaped with broad bases and pointed apices. Their nucleus is spherical in shape, basophilic and basal (Fig. 6).

Examination of the digestive gland of treated slugs with LC_{50} of thymol showed slight histological changes in the two cell- types whereas LC_{90} revealed severe histological changes, including: vacuolated cytoplasm (Figs. 7-11), some degenerated cells of digestive and secretory cells (Figs. 7&8), abnormal star-shaped and

degenerated nuclei (Figs. 8, 9 & 11) as well as some pale toxic agents inside the cells (Fig. 7) and in the lumen.

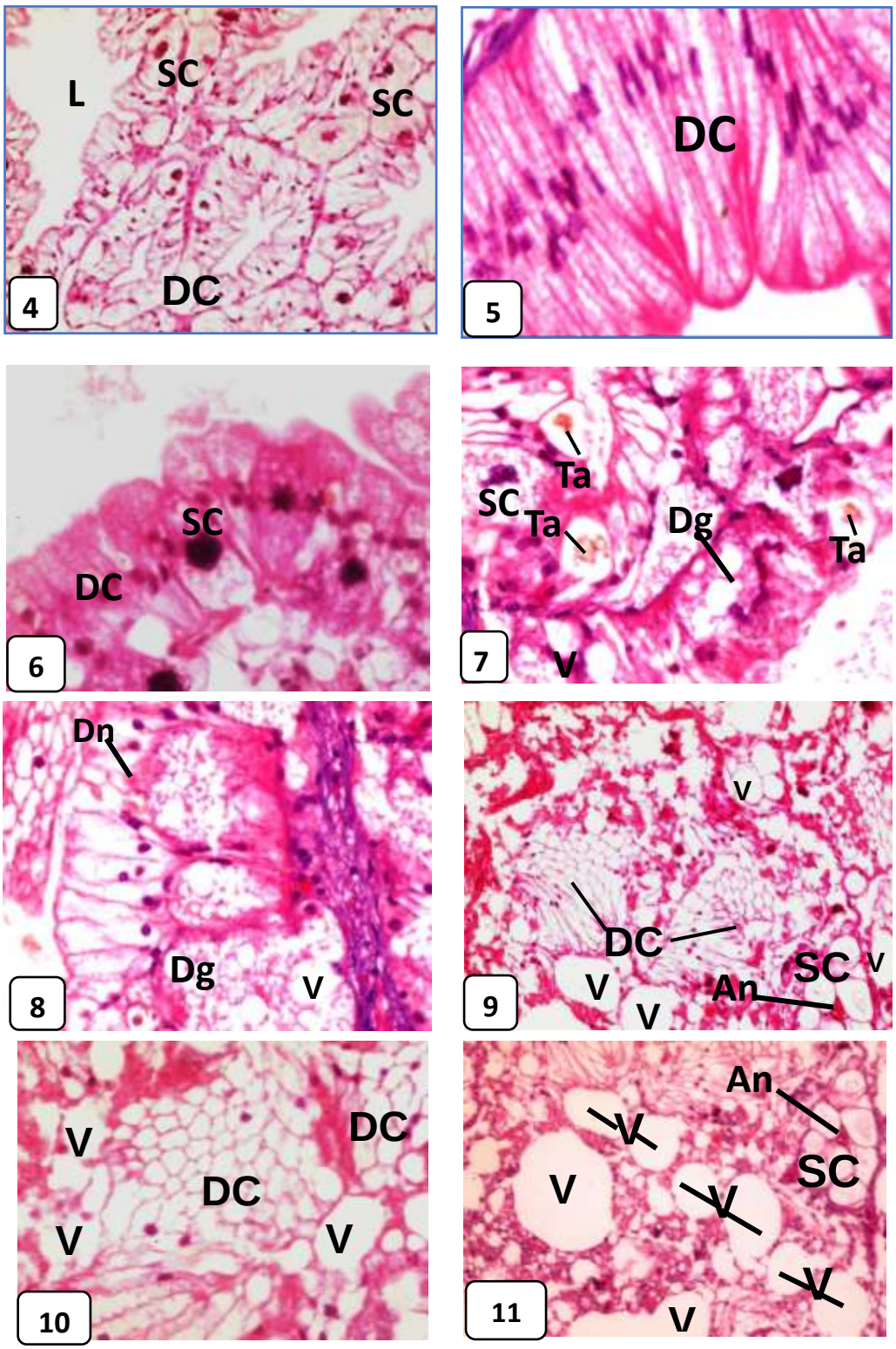
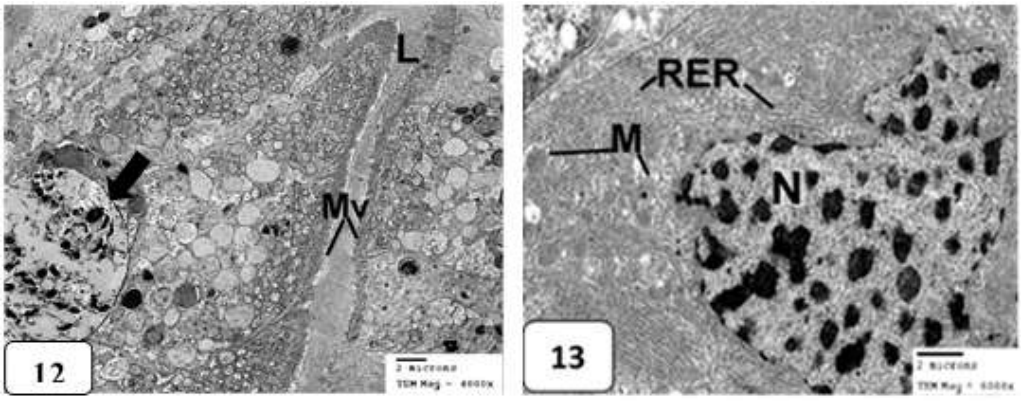


Plate 1: Photomicrographs of digestive gland of *L. maximus* stained with H&E. **Figs. 4-6:** untreated group showing digestive cells (DC), secretory cell (SC) and lumen (L). **Figs. 7&8:** treated group with LC_{50} of thymol for 48 hrs. showing vacuoles (V) inside digestive cells (DC) and secretory cells (SC), degenerated cells (Dg) beside some toxic agent (Ta) and degenerated nucleus (Dn). **Figs. 9-11:** treated group with LC_{90} of thymol for 48 hrs. showing vacuoles (V), digestive cells (DC), secretory cells (SC) and abnormal star-shaped nucleus (An). (Figs. 4,7&9) (X=400) and (Figs. 5,6, 8, 10 &11) (X=1000).

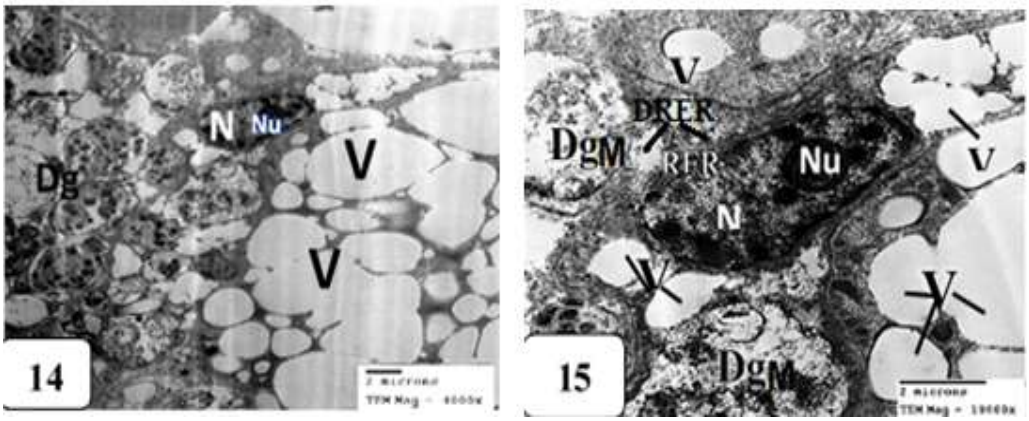
Ultrastructural examination:

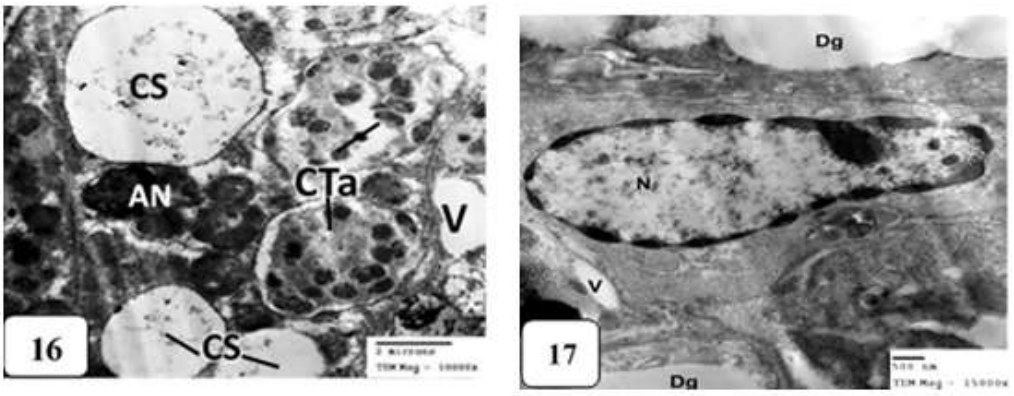
Examination of digestive gland of *L. maximus* by TEM revealed that digestive gland consists of the two cells, named secretory and digestive. These cells are simple columnar epithelial cells and their finger-like projected microvilli present apically, that may be simple, facing the lumen. Pinocytotic vesicles of various dimensions revealed in the apical cytoplasm of the digestive cell whereas calcium spherules in the secretory cell detected (Fig. 12). The nucleus is basal and spherical or elliptical or irregular in shape surrounded by rough endoplasmic reticulum and mitochondria (Fig. 13).



Figs. 12&13: TEM of sections in the digestive cells of untreated *L. maximus.*
Fig. 12: Illustrating digestive lobule open in the lumen (L), microvilli (Mv) are observed at the apical part of digestive and secretory cells, calcium spherules in the secretory cell (black arrows) (X=4000).
Fig. 13: Revealing the digestive cell-nucleus (N), rough endoplasmic reticulum (RER) near the nucleus and mitochondria (M) (X=6000).

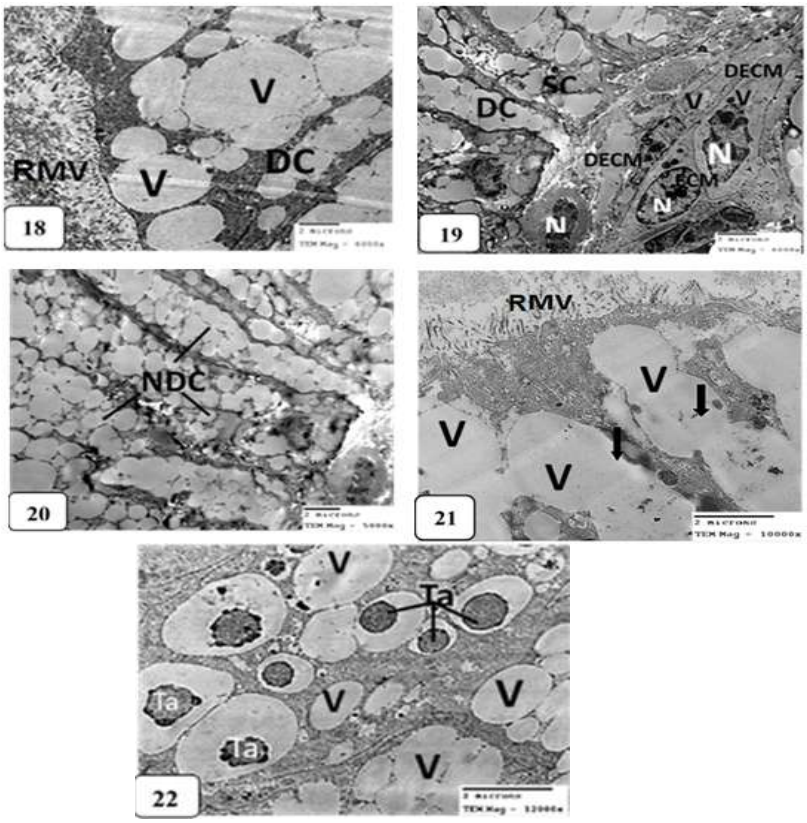
Fine structural micrograph of digestive gland of the treated slugs with LC_{50} and LC_{90} thymol for 48 hrs. showed many alternations in digestive gland, increased with LC_{90}, as compared with untreated samples. This changes include: various cellular vacuoles in varied sizes inside the cells (Figs. 14-19, 21 & 22), degeneration of some cells (Figs. 14 & 17), degenerated mitochondria and RER (Fig. 15), ruptured microvilli (Fig. 18 & 21), abnormal nucleus (Fig. 16) scattered toxic agent (Fig.16 & 22), degenerated-extracellular matrix (Fig.19), accumulation of calcium spherules in secretory cells (Fig. 16) and nearly necrosis of the two types of cells (Figs. 18 & 20-22).





Figures 14-17: TEM of sections in the digestive gland of treated *L. maximus* with LC_{50} of thymol for 48 hrs.

Fig. 14: Showing nucleus (N), nucleolus (Nu), vacuoles (V) and degenerated cell (Dg) (X=4000). **Fig. 15:** revealing nucleus (N), nucleolus (Nu), vacuoles (V), degenerated-rough endoplasmic reticulum (DRER) and degenerated mitochondria (DgM) (X=10000). **Fig. 16:** Illustrating vacuoles (V) abnormal nucleus (AN), numerous calcium spherules (CS) and cysts of numerous toxic agents (CTa). (X=10000). **Fig. 17:** Showing intratubular connective tissue, nucleus (N), small vacuoles (V) and degenerated cell (Dg) (X=15000).



Figures 18-22: TEM of sections in the digestive gland of treated *L.* maximus with LC_{90} of thymol compound.

Fig. 18: Showing digestive cell (DC), rupture of microvilli (RMv) and numerous vacuoles of various sizes (X=6000). **Fig. 19:** Showing normal small vacuoles (V), beside degenerated extracellular matrix (DECM) (X=6000). **Fig. 20:** Showing nearly necrosis of digestive cells (NDC), after disappearing of nucleus and majority of cellular organelles (X=5000). **Fig. 21:** Showing fused-wide vacuoles and (V) rupture of microvilli (RMv) and nearly necrosis of two types of cells (X=10000). **Fig. 22:** Showing numerous vacuoles of various sizes (V), toxic agents (Ta) inside the cells and nearly necrosis of two types of cells (X=12000).

DISCUSSION

The giant garden slug *Limax maximus*, tiger slug or great grey slug, is belonging to family Limacidae (Gaitán-Espitia, *et al.*, 2012). The present results of morphological features and anatomy of the internal organs of *L. maximus* are agree with results of Beltagi, *et al.* (2016).

Histological inspection of the normal digestive gland of *L. maximus* confirms the existence of two main cell types namely: digestive and secretory cells. Digestive gland was reported to be composed of two cell types: digestive and excretory or secretory cell by both Abdel-Haleem (2013) in *L. maximus* and Ibrahim (2006) in *Biomphalaria alexandrina.* In contrary, Zaldibar, *et al.* (2007) described two cell types; digestive and basophilic cells in the digestive gland of the snails *Eobania vermiculata* and *Littoria littorea*. However, Morten (1979) described three types of cells; digestive, calcium and thin cells in the slug *Deroceras caruanae*. In addition, the digestive gland had been revealed that it consists of three types of cells; digestive, calcium and excretory cells by Lopes, *et al.*, (2001) in the land snail *Oxychilus atlanticus*, Chabicovsky, *et al.* (2004) in land snail *Helix pomatia*, Abo Bakr (2011) in land snail *E. vermiculata*, Sharaf, *et al.* (2015) in land snail *Helicella vestalis* and Mustafa and Awad, (2018) in the slug *Lehmannia marginata*. Moreover, four cell types: digestive, calcium, excretory and thin cells were found in *E. vermiculata* by Hamed, *et al.*, (2007).

The present histological results of the digestive gland of thymol-treated *L. maximus* with LC_{50} and LC_{90} for 48 hrs. are similar and agree with the reported results of Abo Bakr (2011) and Yousef (2011),as well as with results of Hamed, *et al.*, (2007) in the same gland of treated land snail *E. vermiculata* with methomyl pesticide in spate of they found four cells: digestive, calcium, excretory and thin cells.

The present cytoplasmic vacuolation and degeneration of secretory cells in the digestive acini of treated *L. maximus* are in accordance with results of Abdel-Haleem (2013) in the same species, who used plant extracts of three plants *Euphorbia splendens, Ziziphus spina-Christi* and *Ambrosia maritimaon* against two freshwater snails *B. alexandrina* and *B. truncates*, and agree with Saad, *et al.* (2012) in the treated snail *B. alexandrina* with extracts of two plants *Cestrum diurnum* and *Casimiroa edulis*. In addition, Sharaf, *et al.* (2015) observed additional histological changes in the digestive gland of treated land snail *H. vestalis* with methiocarb and chlorpyrifos-pesticides included; severe tubular disruption, nuclear pyknosis and necrosis of tubules. Moreover, Mustafa (2018) reinforced the present results in another gland, salivary gland, where he found vacuolated cytoplasm and degenerated nuclei after treated with LC_{90} of thymol against *L. maximus*.

Ultrastructural inspection of the normal digestive gland of *L. maximus* confirms the existence of two main cell types namely: the digestive cells and secretory cells. These results fit well with Aly (2007) in the same species. On the contrary, Nath, *et al.* (2015) found that the digestive gland of the slug, *Laevicaulis alte* is composed of digestive cell, excretory cell and calcium cell.

The present ultrastructural results on treated target slugs agree with finding of Hamed, *et al.* (2007), who found severe cytoplasmic vacuolation, accumulation of residual bodies or yellow granules and reduction in microvilli of the digestive gland of the land snail *E. vermiculata* treated with methomyl, topically or by poison baiting technique. In addition, the present findings agree with Abdel-Haleem (2013) in the freshwater snails *B. alexandrina* and *B. truncates* treated with the Egyptian plant

extracts *E. splendens*, *Z. spina-Christi* and *A. maritimaon*, who found cytoplasmic vacuolation, swelling of secretory cell and accumulation of residual bodies.

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ARABIC SUMMARY

التغيرات النسيجية والتركيبية الدقيقة في الغدة الهضمية للبزاقة المصرية " *Limax maximus* " (Linnaeus, 1758)التي تم معالجتها بمركب الثيمول thymol النباتي المضاد للرخويات، مع التنويه بدراسات بيولوجية

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تضمن العمل البحثي الحالي دراسات بيولوجية (دراسة شكل خارجي وتشريح) وهذا بجانب التغيرات النسيجية والتركيبية الدقيقة والناشئ عن مركب الثيمول النباتي كطعم سام على الغدة الهضمية لبزاقة الحدائق العملاقة المصرية "*ليماكس ماكسيمس*" (عائلة: لايميسيدي) بعد معالجة البزاقة بنصف التركيز المميت (LC_{50}) والتركيز القبل مميت (LC_{90}) لمدة ٤٨ ساعة. ومركب الثيمول يسمى كيميائياً "تربينويد أحادي" ويتواجد في نباتات عديدة. وتم تعيين القيم التقديرية للتركيزات (LC_{50} وLC_{90}) للثيمول وكانت ٢٦٩.٧٧ و٣٦٢.٧٩ جزء في المليون على التوالي. وتأثير LC_{90} على الغدة الهضمية كانت تغيرات نسيجية حادة وتشوهات تركيبية دقيقة مثل: فجوات سيتوبلازمية ووجود عوامل سمية مبعثرة وانحلال بعض الانوية والخلايا وتمزق الخملات الدقيقة وزيادة في كريات الكالسيوم بالخلايا الافرازية، وفجوات واسعة مندمجة.
لذلك مركب الثيمول ربما قد يكون ذو قيمة كبيرة في مكافحة البزاقة موضع هذا البحث؛ وذلك كمبيد آمن واقتصادي للرخويات دون التسبب بضرر للنظام البيئي المحيط، وذلك بدلا من استخدام مبيدات آفات كيميائية التي باستطاعتها أن تحدث تلوث للبيئة.