

Toxicity of Thymol on the Ultra-Scanning Structure of Skin and Digestive Gland Proteins of the Two Slugs '*Limax maximus* and *Lehmannia marginata*'

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ABSTRACT

Background: thymol is a natural derivative of herb thyme and extracted from *Thymus vulgaris*.

Aim of the work: the present investigation was carried out to study the side effects of thymol on the skin and protein content in the digestive gland of both slugs: *Limax maximus* and *Lehmannia marginata*.

Materials and Methods: the slugs are classified into three groups: the first one served as a control, the second and third groups fed on LC₅₀ and LC₉₀ of thymol for 48 hours.

Results: the histological and scanning electron microscopic (SEM) observations of skin of LC₉₀-treated slugs exhibited erosion of the epithelial cells, hypoplasia of the connective tissues with increased mucus secretion. Moreover, the different protein bands of the control and treated slugs with LC₅₀ and LC₉₀ of thymol were demonstrated by SDS-PAGE technique. A total number of 37 different protein bands were ranged from 5.181 to 84.375 kDa.

Conclusion: the present study supported the use of thymol as a molluscicide agent on the skin and digestive gland proteins of both slugs (*Limax maximus* and *Lehmannia marginata*) that offers a safe alternative to other more persistent chemical pesticides that can be dispersed in runoff and produce subsequent contamination.

Keywords: Digestive Gland, Proteins, Skin, SEM, Slugs, Thymol

INTRODUCTION

The development of botanical molluscicides as a possible substitute for chemical molluscicides are gaining wide attention. Molluscicides of plant origin are environmentally friendly, culturally more acceptable than synthetic ones, less expensive, rapidly biodegradable and have low toxicity to non-target organisms^(1&2). Thymol (also known as 2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, C₁₀H₁₄O, found in oil of culinary herb thyme and extracted from *Thymus vulgaris*⁽³⁾. The Native Americans recognized this plant as strong antiseptic plant, and used poultices of the plants for skin infections and minor wounds⁽⁴⁾. Moreover, Nieto⁽⁵⁾ reported that thymol was also used as a rapidly degrading, non-persistent pesticide. Some monoterpenoids (such as camphor, menthol and limonene) consist of two isoprene units and have the molecular formula C₁₀H₁₆.

The skin of gastropods especially in the foot regions is composed of three main regions: epithelial layer, compact fibrous and muscular layer. Such observations were carried out by the most extensive studies on the skin of gastropods such as Prior and Gleperin⁽⁶⁾ and Mustafa⁽⁷⁾ on *Limax maximus*; Godan⁽⁸⁾ on the terrestrial pulmonates. Wilbur⁽⁹⁾ studied the Mollusca skin and found that the epidermis was a single layer of cells resting on a basement membrane and supported by a sheet of connective tissue. The epithelium itself consisted of three main types, namely epidermal, ciliated and glandular cells. Mohamed and Sheir⁽¹⁰⁾ studied the

comparative analysis of soluble protein extracts of the digestive gland and female gonads by using SDS-PAGE on the freshwater Bivalves *Caelatura teretiscula* and *Caelatura nilotica*. Bakry⁽¹¹⁾ studied the effect of LC₂₅ of the menthol extract from the plants: *Guayacum officinalis*, *Atrplex stylosa* and *Euphorbia splendens* for two weeks on the snail *Biomphalaria alexandrina*. Analysis of snail's soft tissues indicated that the tested plant extracts effected on the protein patterns. The electrophoretic pattern of total protein of snail's soft tissues showed differences in number and molecular weights of protein bands. Moreover, Yousef⁽¹⁾ studied the effect of LC₂₅ and LC₅₀ of the monoterpenoids, thymol and nicotine on the electrophoretic pattern of total protein of the digestive gland of *Eobania vermiculata*. Saidi et al.⁽¹²⁾ used antifungal, molluscicidal and larvicidal assessment of anemonin and *Clematis flammula* extracts against mollusks *Galba truncatula* and intermediate host of *Fasciola hepatica* in Tunisia. Faria et al.⁽¹³⁾ reported the molluscicide (*Manikara subseicea*) crude extract from leaves showed an efficient method to control the schistosomiasis disease and being able to reduce intermediate host snail *Biomphalaria glabrata* number.

MATERIALS and METHODS

Experimental Animals

The two terrestrial pulmonate slugs *Limax maximus* and *Lehmannia marginata* had been collected from the garden of Faculty of Education, Ain Shams University, Cairo, whereas the used plant molluscicide (thymol) was obtained from Sigma-

Aldrich. The slugs were classified into three groups: the first one served as a control, the second and third groups were fed on the lower and higher doses (LC₅₀&LC₉₀) of thymol for 48 hours.

Bioassay

Six concentrations of thymol (50, 100, 200, 300, 400&500 ppm) for 30 slugs/ concentration were used. Controls were similarly without adding thymol. Mortalities were recorded in the control and treated samples after 48 hrs. Regressing lines between tested concentrations and corresponding mortalities were drawn with Excel 2010, LC₅₀ and LC₉₀ were calculated by **Finny** ⁽¹⁴⁾.

Histological Preparation and SEM of Skin

The normal structure of the slug's skins was studied with the routine haematoxylin-eosin stain preparation (H&E). At the end of the post treatment period (48 hours), slugs were dissected, the parts of upper skin were excised and fixed immediately in aqueous Bouin's fixative for 24 hrs. Then they were dehydrated through ascending series of ethanol followed by clearing in terpineol for 48 hrs. Finally, the tissues were embedded in paraffin wax. Sections, 6 µm thick, were cut and stained with haematoxylin and eosin. Finally, the slides were examined by light microscope and photographed using light microscope Olympus CX31 connected with digital camera model No. E- 330 at central Lab., Education Faculty, Ain Shams University.

For SEM examination, the skins of two slugs from each species (control and treated with LC₉₀ of thymol) were placed into 3% glutaraldehyde with phosphate buffer (pH 7.3). Then target tissues were dehydrated in graded series of ethanol (70-100), and subsequently dried with critical-point-dryer **Russell and Daghlian** ⁽¹⁵⁾. The dried material was coated by gold sputter coater (JEOL model) and samples examined by JEOL-JSM-5300 LV reflection scanning electron microscopy at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Electrophoresis Separation of Digestive Gland Proteins

Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used to resolve and compare the protein patterns of the digestive gland of control specimens with those of the specimens treated with LC₅₀ and LC₉₀ of thymol according to the method of **Laemmli** ⁽¹⁶⁾.

RESULTS

Bioassay

The present results, thymol was effected on the slugs *L. marginata* more than *L. maximus*. LC₅₀ and LC₉₀ were calculated, the lower dosages were obtained of LC₅₀ of thymol (225.18 & 122.23 ppm/each slug) on *L. maximus* and *L. marginata*, respectively. In addition, the higher dosages were obtained of LC₉₀ of thymol (442.21 & 386.96 / each slug) on the both types for 48 hours respectively in **table 1**.

Histological and SEM Studies

The skin of *L. maximus* is formed of three layers namely epidermis, dermis and hypodermis. The epidermis is formed of an epithelial layer; it consists of a single layer of cuboidal or simple columnar cells resting on a basement membrane. The epithelial layer is containing scattered secreting unicellular mucous gland which secretes mucus. This layer is thrown up into numerous invaginations scattered in different sites forming furrows. The dermis is formed of a loose network of collagen fibres and layer of pigment cells. Hypodermis is situated between the muscle bundles. It consists of a fine network of loose connective tissue

(**Figs.1 &2**).

Examination of sections of the skin of *L. maximus* treated by LC₉₀ of thymol showed erosion of epithelial cells in the epidermal layer (**Fig.3**) with hypoplasia in the connective tissue and fragmentation of muscle fibres in the muscles tissues (**Fig.4**). The composition of the skin of *L. marginata* is similar to the *L. maximus* and the upper surface of it is flat without any infusions and adhesion of the skin (**Figs. 5 &6**). Sections of the skin of *L. marginata* treated by LC₉₀ of thymol showed wide inter space in the dermal layer (**Fig.7**). Also, hypoplasia of connective tissue and fragmentation of muscles fibres were detected (**Fig.8**). As revealed by scanning electron microscopy, the superficial layer of the epidermis of the two slugs comprised polygonal cells; the mucus was recognized on the skin surface (**Figs. 9, 10, 13 &14**). Increased mucus secretion on the upper surface of the skin of *L. maximus* was realized in a vesicle shape (**Fig. 11**). Also, the deep groves appeared in skin of group treated with LC₉₀ of thymol (**Figure 12**). As shown in **Figures 15 and 16** abundant mucus was secreted on the upper surface of the skin of *L. marginata* with presence of slightly insertions between the cells treated with LC₉₀ of thymol.

Electrophoresis Examination of Protein Native Protein

The native protein polyacrylamide gel electrophoresis (PAGE) in the present study was shown in **Figures 17&18**. Their data were represented in **table 2**. The separated bands were read according to their relative fragmentation (Rf) values and their amount percent (Am) or density as represented in **table 2**. The number of native protein bands produced for control digestive gland sample of *L. maximus* (lane 1) was three bands, their Rf value ranged between 0.15-0.32. Band number 3, with Rf value 0.15, showed the highest percent density in the digestive gland and its amount percent was 9.89%, while band number 5, with Rf value 0.32 showed the lowest density with amount 7.63%.

The number of native protein bands produced for LC₅₀ of thymol digestive gland sample of *L. maximus* (lane 2) was four bands, their Rf values ranged between 0.023-0.33. Band number 3, with Rf value 0.14, showed the highest percent density in the digestive gland its amount percent was 9.07%, while band number 5, with Rf value 0.33 showed the lowest density with amount 3.35%. The number of native protein bands produced for LC₉₀ digestive gland sample of *L. maximus* (lane 3) was five bands, their Rf value ranged between 0.029-0.44.

Band number 3, with Rf value 0.14, showed the highest percent density in the digestive gland; its amount percent was 9.47%, while band number 5, with Rf value 0.32 showed the lowest density with amount 2.47%. The number of native protein bands produced for the control digestive gland sample of *L. marginata* (lane 4) was four bands, their Rf value ranged between 0.046-0.43.

Band number 3, with Rf value 0.15, showed the highest percent density in the digestive gland; its amount percent was 4.65% while, band number 4, with Rf value 0.43 showed the lowest density with amount 3.31%. The number of native protein bands produced for LC₅₀ digestive gland sample of *L. marginata* (lane 5) was three bands, their Rf value ranged between 0.083-0.26. Band number 2, with Rf

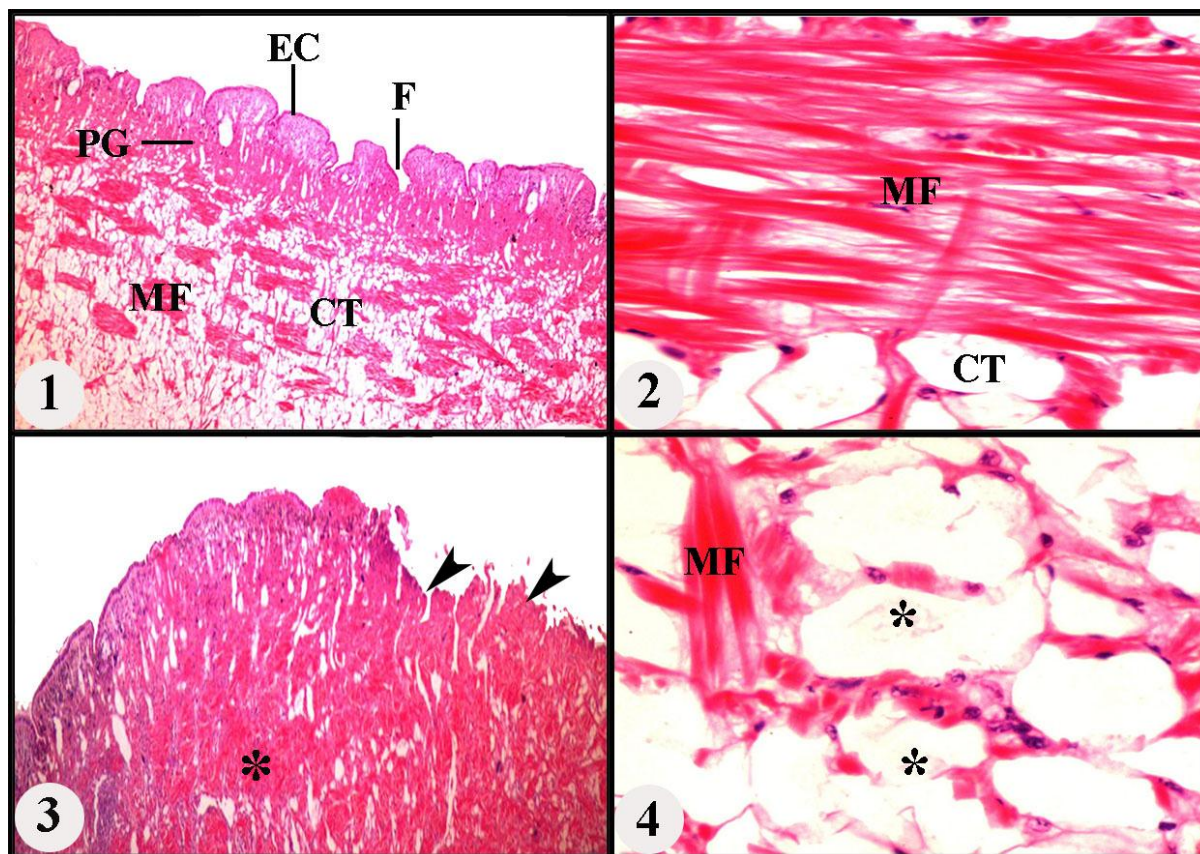
value 0.15, showed the highest percent density in the digestive gland; its percent was 4.34%, while band number 5, with Rf value 0.26 showed the lowest density with amount 4.04%. The number of native protein bands produced for LC₉₀ digestive gland sample of *L. marginata* (lane 6) was two bands, their Rf value ranged between 0.15-0.44. Band number 6, with Rf value 0.44, showed the highest density in the digestive gland; its percent was 3.81%, while band number 3, with Rf value 0.15 showed the lowest density with amount 3.26%.

SDS-PAGE Observation

Digestive gland protein of the control and treated slugs; *L. maximus* and *L. marginata* were electrophoretically separated by using SDS-PAGE. The separating gel was at 12% polyacrylamide at pH 8.8. **Figures 19&20** showed the electrophoretic patterns of the digestive gland proteins from the control slugs, *L. maximus* (lane 1), and thymol-treated slugs (lanes 2 and 3, treated with LC₅₀ and LC₉₀, respectively). Lane 4 of the digestive proteins of the control slugs, *L. marginata* (lanes 5 and 6, treated with LC₅₀ and LC₉₀ respectively), and thymol-treated slugs, *L. marginata*. Standard protein markers (lane M) were separated and used as a reference for the molecular weights (M.wt.) determination of the separated protein bands.

Table 3 showed comparison between the different protein bands of the control and treated slugs separated by SDS-PAGE. A total number of **37** different protein bands, with M. wt. ranged from 5.181 to 84.375 kDa. Only eight protein fractions were separated from digestive gland of the control slugs; *L. maximus*.

A number of seven and four protein fractions were separated from slugs; *L. maximus* treated with LC₅₀ and LC₉₀ of thymol, respectively. Only six protein fractions were separated from digestive gland of the control slugs, *L. marginata*. A number of 6 protein fractions were separated from slugs, *L. marginata* treated with LC₅₀ and LC₉₀ of thymol.



Figures 1&2: Photomicrographs of a section of the skin of a control slug, *L. maximus*.

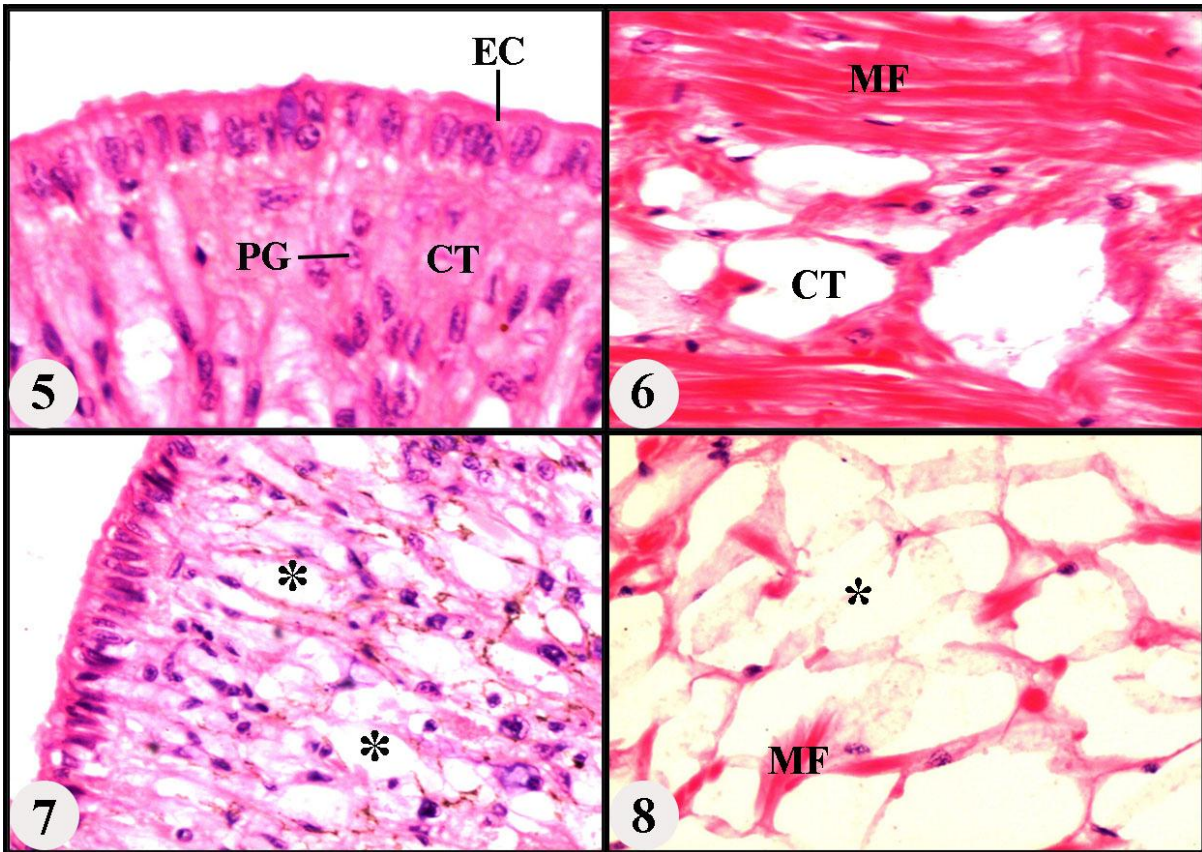
Fig. 1: Showing epithelial cells (EC) in the epidermis layer, furrow (F), pigment granule (PG), muscle fibres (MF) and connective tissue (CT). (H&E, X200)

Fig. 2: Revealing the dermal layer which is formed of network of muscle fibres (MF) and connective tissue (CT) situated in hypodermis layer. (H&E, X1000)

Figures 3&4: Photomicrographs of a section of the skin of the slug, *L. maximus* treated with LC₉₀ of thymol for 48 hrs.

Fig. 3: Showing erosion of epithelial cells (arrowheads) and compact muscle fibres (*). (H&E, X200)

Fig. 4: Illustrating that the hypoplasia of connective tissues (*) and fragmentation of muscle fibres (MF). (H&E, X1000)



Figures 5&6: Photomicrographs of a section of the skin of a control slug, *L. marginata*.

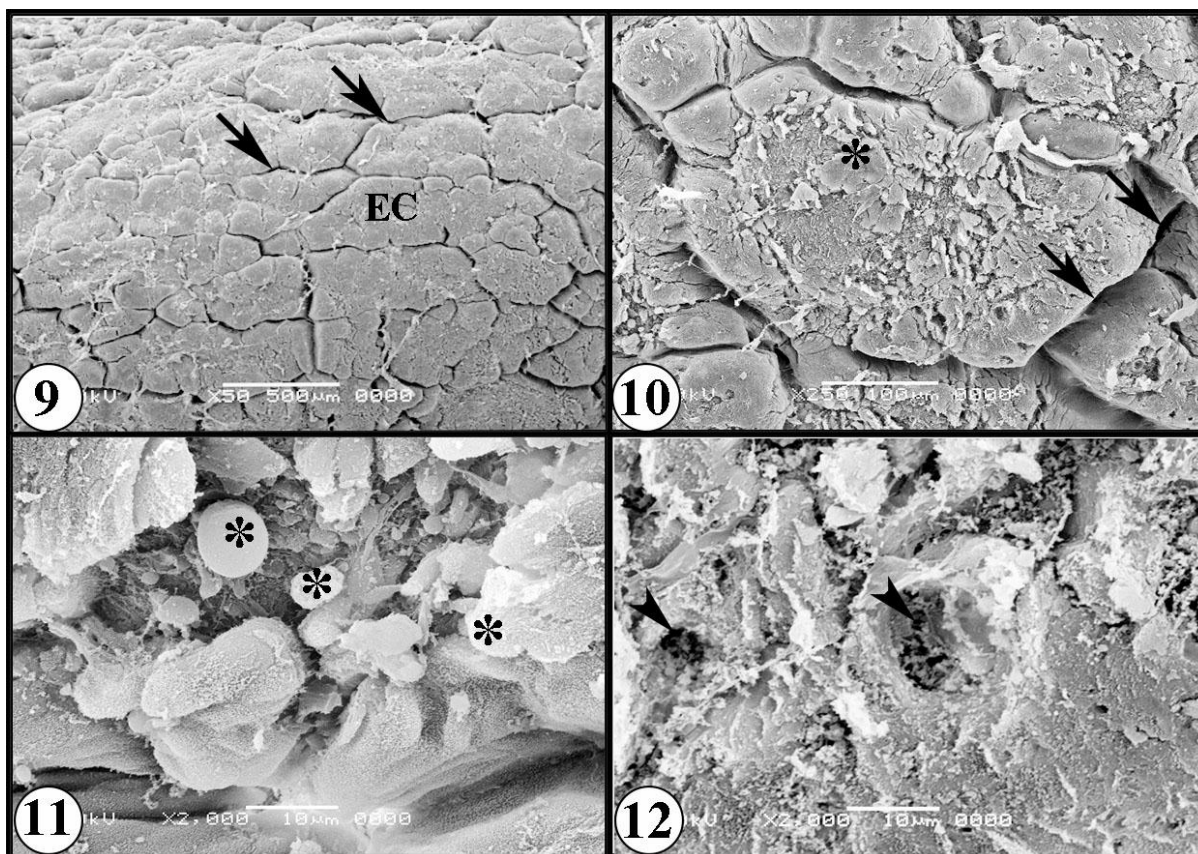
Fig. 5: Showing straight epithelial cells (EC) in the epithelial layer without any furrows, presence pigment granule (PG) and connective tissue (CT). (H&E, X400)

Fig. 6: Illustrating the muscle fibres (MF) in the dermis layer and abundant connective tissues (CT) in the hypodermis layer. (H&E, X1000)

Figures 7&8: Photomicrographs of a section of the skin of the slug, *L. marginata* treated with LC₉₀ of thymol for 48 hrs.

Fig. 7: Showing wide interspaces in the dermal layer and hypoplasia (*) of connective tissues. (H&E, X200)

Fig. 8: Revealing hypoplasia of connective tissue (*) and fragmentation of muscle fibres (MF). (H&E, X.1000)



Figures 9&10: Scanning electron micrographs of the skin of a control slug, *L. maximus*

Fig. 9: Showing the surface of the epithelial cells (EC), these cells are polygonal, differ in size and surrounded by boundary tissues (arrows). (Scale bar=100 μ m)

Fig. 10: High magnification part of the previous figure showing deep grooves present between the epithelial cells (arrows) and presence the mucus secretion (*).

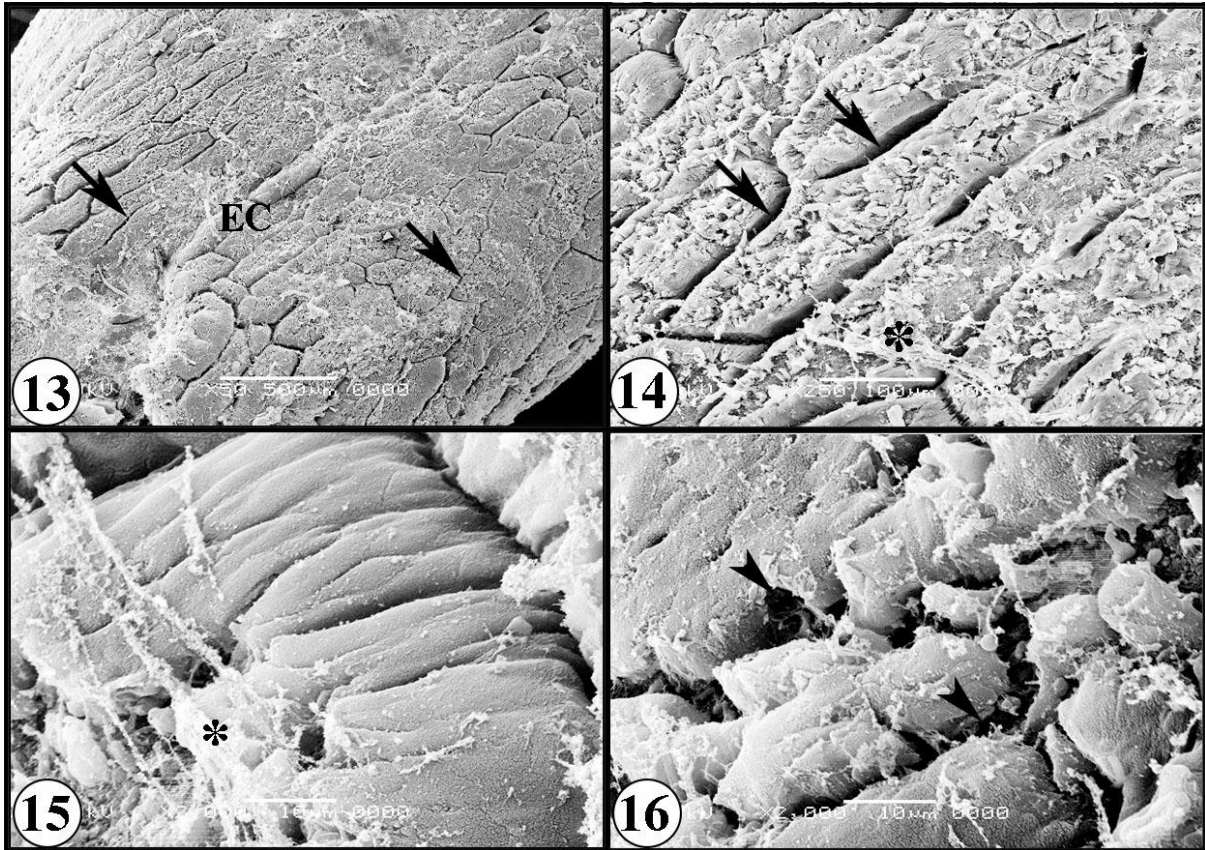
(Scale bar=100 μ m)

Figures 11&12: Scanning electron micrographs of the skin of the slug, *L. maximus* treated with LC₉₀ of thymol for 48 hrs.

Fig. 11: Revealing that the mucus secretion increase on the upper surface of the skin and it's appeared in the vesicles (*). (Scale bar=10 μ m)

Fig. 12: Illustrating the mucus secretion of the epithelial cells in deep grooves (arrowheads).

(Scale bar=10 μ m)



Figures 13&14: Scanning electron micrographs of the skin of a control slug, *L. marginata*.

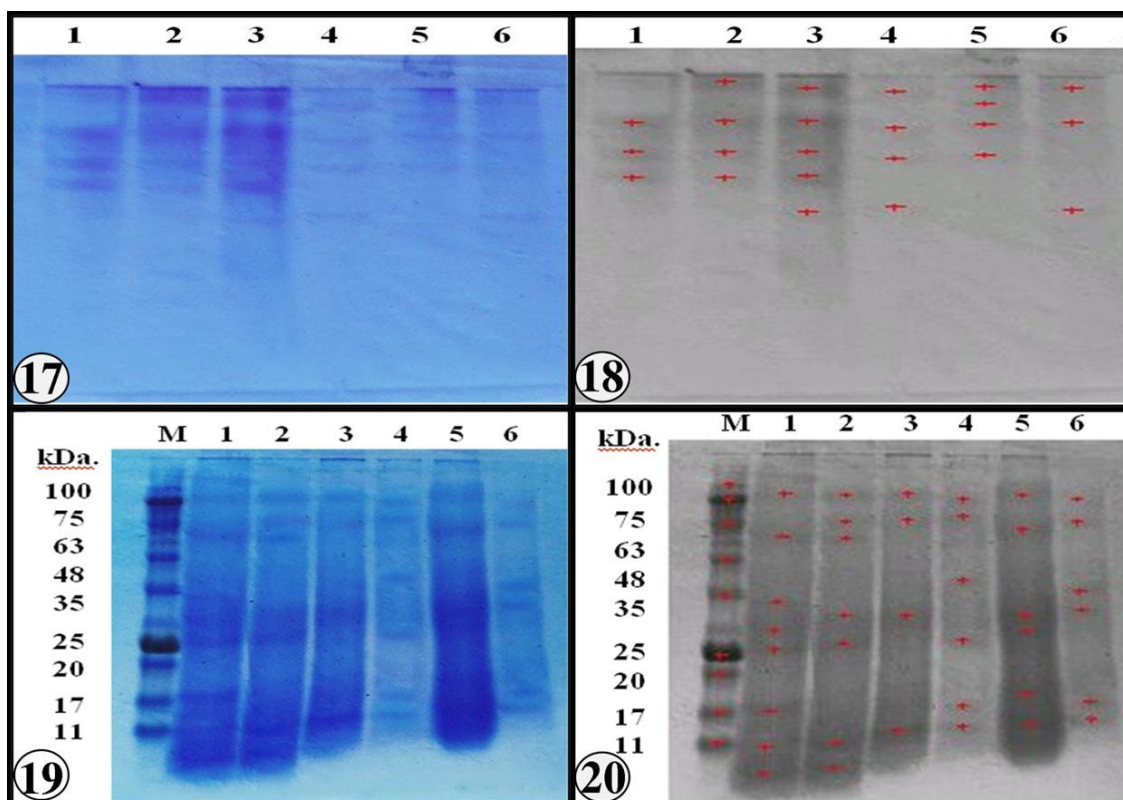
Fig. (13): Showing the surface of the epithelial cells (EC). These cells are polygonal in shape, differ in size and surrounded by boundary tissues (arrows). (Scale bar=500 μ m)

Fig. 14: High magnification of part of the previous figure showing the surface of the epithelial cells is polygonal in shape (arrows). The mucus secretion (*) is appeared on the surface. (Scale bar=100 μ m)

Figures 15&16: Scanning electron micrographs of the skin of the slug, *L. marginata* treated with LC₉₀ of thymol for 48 hrs.

Fig. 15: Illustrating the distribution of mucus secretion (*) on the upper surface (Scale bar=10 μ m)

Fig. 16: Showing presence of slightly insertions between the cells (arrowheads). (Scale bar=10 μ m)



Figures 17& 18: digestive gland proteins patterns of the control (lanes 1&4) and thymol-treated slugs (lanes 2, 3, 5&6) as separated by PAGE.

Figures 19& 20: digestive gland proteins patterns of the control and thymol- treated slugs as separated by SDS-PAGE.

M. Marker, M. wt. standard proteins

Lane1: Control slug, *L. maximus*.

Lane2: LC₅₀ of thymol-treated *L. maximus*.

Lane3: LC₉₀ of thymol-treated *L. maximus*.

Lane4: Control slug, *L. marginata*

Lane5: LC₅₀ of thymol- treated *L.marginata*.

Lane6: LC₉₀ of thymol-treated *L. marginata*.

Table 1: bioassay of thymol against slugs *L. maximus* and *L. marginata*

Tested animal	<i>L. maximus</i>	<i>L. marginata</i>
Slope	0.1843	0.1511
C ₅₀ (with confidence limits*)	225.18 (234.56 ,216.17)	122.23 (130, 114.90)
C ₉₀ (with confidence limits*)	442.21 (460.63,424.52)	386.96 (411.66 ,363.7)

Three replicates/ conc. (30 slugs/ conc.) *Confidence limits at 95% probability.

Table 2: relative fragmentation (Rf) and amount percent (Am %) of native protein of the digestive gland of the two slugs *L. maximus* and *L. marginata* of the control and those treated groups with LC₅₀ & LC₉₀ of thymol for 48 hrs.

Lanes:	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6	
Rows	Rf	Am%	Rf	Am%	Rf	Am%	Rf	Am%	Rf	Am%	Rf	Am%
R1			0.023	4.32	0.029	7.54	0.046	4.31				
R2									0.083	4.13		
R3	0.15	9.89	0.14	9.07	0.14	9.47	0.17	3.52	0.15	4.34	0.15	3.26
R4	0.24	8.07	0.25	6.64	0.24	2.47	0.27	4.65	0.26	4.04		
R5	0.32	7.63	0.33	3.35	0.33	7.5						
R6					0.44	2.81	0.43	3.31			0.44	3.81

Table 3: relative fragmentation (Rf), molecular weight (M.wt) and amount percent (Am %) of fractionated protein of the digestive gland of the two slugs *L. maximus* and *L. marginata* of the control and treated groups with LC50&LC90 of thymol for 48 hrs.

lanes:	Marker		Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6	
Rows	wt. (kD)	Am%	wt. (kD)	Am%	wt. (kD)	Am%	wt. (kD)	Am%	wt. (kD)	Am%	wt. (kD)	Am%	wt. (kD)	Am%
r1	100	2.55												
r2	75	7.85	84.375	4.02	82.813	4.05	84.375	3.53	75	4.33	82.813	5.08	76.563	2.2
r3	63	7.56			63.96	2.72	64.44	2.39	66.36	5.51			63.48	4.1
r4			57.615	6.61	56.846	2.9					60.308	4.03		
r5	48	7.96												
r6									40.474	4.57				
r7	35	7.41											36.368	4.08
r8			33.906	4.03										
r9					31.719	2.88	31.563	4.37			31.563	5.48	32.656	5.98
r10			29.063	3.46							29.063	3.12		
r11					27.031	2.88			27.5	5.03				
r12	25	11.9	25.938	4.03										
r13	20	8												
r14											18.537	3.81	17.878	3.76
r15	17	8.3	17.146	3.73					17.512	4.45				
r16											14.818	5.94	15.727	6.66
r17							13.545	8.2	14.273	5.84				
r18	11	7.87	10.273	4.13	11.182	6.12								
r19			5.1818	2.73	6.4545	6.98								

DISCUSSION

The mortality of slugs during calculation of LC₅₀ and LC₉₀ of thymol was interpreted by limited works. Such as detected *in vivo* exposure of *Lymnaea acuminata* to thymol indicated a significantly alter acetyl cholinesterase, lactic dehydrogenase, succinic dehydrogenase and cyto-oxidase activity in the nervous tissue of snails⁽¹⁷⁾. Such neurotoxicity of snails was concerning neurotransmission mechanisms through a complex interaction between the different neurotransmitters, as interpreted by the previous authors. Otherwise, N-glycosylation potential of the gastropods *Limax maximus* and others was analyzed by investigation of the N-glycan structures of the skin and viscera glycoproteins to uncover new means for pest control of some species and to identify carbohydrate-epitopes, which might be relevant for immune response⁽¹⁸⁾. On the same manner, the thymol was the potent one at least LC₅₀ and LC₉₀, whereas it showed considerable molluscicidal effect against *Biomphalaria alexandrina*, *Bulinus truncatus* and *Lymnaea natalensis*, that caused a highly significant reduction of total cercarial production per snail occurred in the experimental snails as compared to the control⁽¹⁹⁾. The Mollusca epidermis is involved in functions as diverse as those of respiration, assimilation, osmoregulation, locomotion and reproduction⁽⁹⁾. In this study, skin of the slugs; *L.*

maximus and *L. marginata* showed that the epidermis is a single layer of cells resting on a basement membrane and supported by a sheet of connective tissue. These results agree with results of a previous author⁽⁹⁾. Presence of pigment granules in the skin of the slugs may be attributed to form melanocytes which had aged and died deposits and their melanin is found on the side of the epidermal cells away from the body surface. These results are in accordance with those obtained by Fox⁽²⁰⁾ on mollusks. The epithelial layer of the skin is characterized by containing scattered secreting unicellular mucous glands that secrete mucus that may facilitate the slug motion and protect the body surface. This is confirmed by El-Harty⁽²¹⁾ who found that the mucous glands of *Physa acuta* are distributed over the surface of the body. The histological changes in the skin treated with LC₉₀ of thymol exhibited vacuolation of both epithelial cells and connective tissues. These results are similar to those recorded by El-Harty⁽²¹⁾ on freshwater snails affected by cadmium and copper. The degeneration of muscles fibres are similar to those obtained by Mustafa⁽⁷⁾ on the *L. maximus* affected by chemical molluscicides osbac and cyanox. Results of the current study indicated that skin of *L. maximus* treated by LC₉₀ of thymol showed degrees of degeneration in some epithelial cells with fragmentation of muscle fibres in

the muscular tissues. Such effects might be due to that thymol is lipophilic, enabling it to interact with the cell membrane of fungus cells, altering cell membrane permeability permitting the loss of macromolecules⁽⁵⁾.

At scanning EM level, the present study revealed increased accumulated mucus that covered the upper surface of skin of the slugs administered thymol extract at LC₉₀ dosage. These results agree with results of **Godan**⁽⁸⁾ on the terrestrial snails and slugs which contained special gland for secrete a cover of mucus, which served as a protection against desiccation. Mucus is a complex aqueous fluid that owes its viscoelastic, lubricating and hydration properties to the glycoprotein mucin combined with electrolytes, lipids and other smaller proteins⁽²⁹⁾. The skin of terrestrial mollusks is of importance as the structure which was exposed to contact with molluscicides **Godan**⁽⁸⁾.

Zone electrophoresis offers a simple, rapid technique for comparing and characterizing proteins. Acrylamide gel is more satisfactory in both degrees of resolution and number of protein components detected by **Hubby**⁽²³⁾. Generally, **Dutta et al.**⁽²⁴⁾ declared that the changes in the electrophoretic protein profile were considered as a good monitor of environmental pollution. After using polyacrylamide gel electrophoresis (PAGE) technique, the pattern of digestive gland native proteins in the present investigation recorded distinct **21** main zones in both the control and thymol- treated slugs (*L. maximus* and *L. marginata*). These zones were included on: albumin, fast α -globulin, transferrin (β - globulin), slow α -globulin, post transferrin and immunoglobulin. Moreover, the most anodal zone was the albumin zone, whilst the most cathode one was the immunoglobulin zone. The appearance of new protein band may was illustrated by **Cholod**⁽²⁵⁾ who explained that the pre-albumin region was the lowest molecular weight and under the influence it was defined as the post- albumin region due to its migration distance. Also **Dutta et al.**⁽²⁴⁾ reported that the new bands formed in the protein electrophoresis were due to the breakdown of high molecular weight bands. The proteins of the digestive glands of all the control and thymol-treated slugs were separated electrophoretically they were measured by using image analysis techniques. The disappearance in certain protein bands of the treated slugs may be attributed to the effects of treatment of thymol which inhibited the synthesis and expression process of these deleted proteins (qualitative effect). **Mohamed and Sheir**⁽¹⁰⁾ reported that protein analysis revealed several qualitative differences whereas the most obvious results in the total intensity was remarkable decrease

in the digestive gland of the Bivalves *C. teretiuscula* and *C. nilotica*, whilst it increased in the female gonads, one and three days post- exposure to abamectin. In addition, even the band remained after treatment, it usually differs in the amount of protein and this may be explained by that treatment of thymol could not inhibit the synthesis of this protein type, but may be affected only on the quantitative level. These results agree with those of **Yousef**⁽¹⁾ on the snails *Eobania vermiculata*. **In conclusion**, the present study supported the use of thymol as a molluscicide agent on the skin and digestive gland proteins of both slugs; *Limax maximus* and *Lehmannia marginata* that offers a safe alternative to other more persistent chemical pesticides that can be dispersed in runoff and produce subsequent contamination.

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