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Histopathological alterations in the foot and digestive gland of the land snail, *Monacha* sp. (Gastropoda: Helicidae) treated with some plant oils and neomyl

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Abstract

The aim of this study was to test if the essential plant oils: Clove oil, *Syzygium aromaticum* (*S. aromaticum*); black cumin oil or black seed oil, *Nigella sativa* L (*N. sativa*) and mustard oil, *Brassica alba* (*B. alba*) have a molluscicidal potential that induced histological alternations against the land snail *Monacha* sp. (Muller) (Gastropoda: Helicidae) comparing with neomyl as a slandered chemical molluscicide. The tested oils were applied separately as a spray with sublethal concentration (60% of LC50 /24 hrs.) to the snails and their food. The estimated value of LC50 was 0. 125%, 4.17%, 9.29 % and 15.29 % of neomyl, clove oil, black cumin oil and mustard oil respectively, and the values of (60% of LC50) were 0.075, 2.502, 5.574 and 9.174% respectively. The effect was observed on the histological structure of the foot and hepatopancreas after four days of treatment. Snails in both control group and the vehicle (1% tween 80) treated group exhibited nearly the same normal histological structures of the examined tissues, whereas those treated with sublethal concentrations of the tested compounds exhibited substantial damages almost proportional to the potency of the applied compounds. Neomyl was the most effective one followed by clove oil, black cumin oil and mustard oil which is the least effective one. Thus, it can be concluded that these tested oils obtained from plants are toxic to the snail *Monacha* sp. and can introduce economic control of its population without causing environmental pollution.

Introduction

Terrestrial gastropods from the most important threats of sustainable agriculture in many parts of the world (Barker, 2002). Moreover, they play an important role in transmitting and spreading diseases to cultivated plants (Kandil *et al.*, 2020). Land snails are considered one of an economic importance among pest attacks different types of plants (Ismail and Abdel Kader, 2011). *Monacha cartusiana* (Muller) (Gastropoda: Helicidae) is a well-known species in this category for its great damage to many vegetable crops in the Egyptian coastal areas (El-Okda, 1983). Growers and farmers have often had trouble in controlling land gastropods with conventional molluscicides and need to use non-conventional methods (Schuder *et al.*, 2003). Synthetic chemical molluscicides had a toxic effect 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). In contrast, 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 and EI-Kassas, 2013). Essential oils from plants serve as excellent products of botanical origin due to their high bioactive potential, easy availability and economic viability (Lahlou, 2004). Essential clove oil is one such example, which has exhibited biological activity on a wide range of organisms ranging from microorganisms to humans (Kumar *et al.*, 2011). It is constituted by eugenol, which is regarded to be the source of its antifungal, anaesthetic and antiseptic properties (Rani *et al.*, 2012) and also its cytotoxicity (Watcharee *et al.* , 2012). The essential oil of black cumin seed, which has valuable phytomedicinal features contains a variety of active compounds, especially thymoquinoneis one which is the most attractive component in phytomedicinal studies. Genotypes, cultural practices and environmental conditions affect the essential oil composition (Burits and Bucar , 2000). The essential mustard oil has very high application value and can be used to suppress the growth of microorganism in seafood, such as *Helicobacter pyheri* and *Vibrio parahaemolyticus*. It also shows inhibitory effects on the growth of bacteria that cause food poisoning and fungi (Olivier *et al.* , 1999; Nielsen and Rios, 2000 and Shin and Kang, 2001). The oil exhibits significant inhibitory activities against *Aspergillus niger, A. Flavus, Trichoderma viride, Candida albicans, C. utilis, C. tropi-calis, Cryptococcus neoformans, Trichosporon mucoides,Trichophyton tonsuransand Geotrichum capitatum* (Musk and Johnson , 1993; Jiao *et al.*, 1994; Hashim *et al.*,1998 and Hou *et al.*, 2000). The main component of mustard oil is allyl isothiocyanate (Kirk *et al.*, 1964 and Kharchenko, 1964).

The present studies aim to examine the histological effects of these essential oils, clove oil, *Syzygium aromaticum* (*S. aromaticum*); black cumin oil or black seed oil, *Nigella sativa* L(*N. sativa*) and mustard oil, *Brassica alba* (*B. alba*) in the digestive gland and foot of *Monaca* snails before and after treatment with 60% of LC50 / 24 hrs. that applied as a spray under laboratory conditions.

Materials and methods

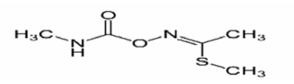
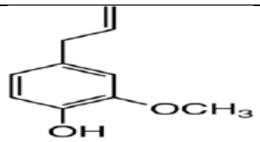
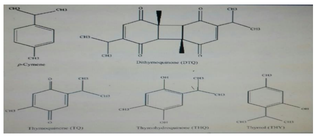
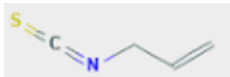
1. The tested land snail:

Monacha sp. (Mollusca: Gastropoda: Pulmonata: Stylommatophora: Helicidae). Adults of nearly equal shell diameter (16-18 mm) were collected during March (2020) from infested clover field at Soofea village, Awlad-Sakr district, Sharkia Governorate, Egypt. The collected snails were transferred to the laboratory in ventilated plastic container. In the laboratory, the healthy individuals were selected and kept for acclimatization in a glass aquarium containing moist clay soil to a depth of about 10 cm and were provided daily with fresh cabbage leaves for two weeks before any tests (Hemmaid *et al.*, 2017). The aquarium was covered with muslin cloth on the top. The water were added to provide suitable humidity for snail activity and the continuous cleaning of the aquarium achieved (Ustina *et al.*, 2018) by removing left-over food when the new food was introduced and the soil was adjusted at 75 ± 5 % relative humidity (Hemmaid *et al.*, 2017).

2. The tested compounds:

Table (1) included the compounds were tested in the present study.

Table (1): Showed neomyl and the three botanic essential oils.

	The compound	Group or Family	Specialization	Name	Formula	Structure
1	**Neomyl (Lannate ® 90% SP)**	Chemical group: Carbamates	Non-specific molluscicide	**Trade name:** Neomyl 90% WP **Common name:** methomyl **Chemical name:** S-methyl N (methylcarbamoyloxy) thioacetimidate l		)C5H10N2O2S(
2	**Clove oil**	Family: Myrtaceae.	Specific molluscicides: Botanical essential oils (ESO)	**Scientific name**: Clove **Biological name**: *Syzygium aromaticum / Caryophyllus aromaticus / Eugenia caryophyllata* **Other names**: Clove, clovos, caryophyllus		)C10H12O2(
3	**Black seed oil**	Ranunculaceae	Specific molluscicides: Botanical essential oils (ESO)	**Scientific name:** Black cumin **Biological name:** *Nigella sativa L* **Other names:** black cumin, black seed, black caraway		
4	**Mustard oil**	*Cruciferae*	Specific molluscicides: Botanical essential oils (ESO)	**Scientific name**: mustard **Biological name**: *Brassica alba /Brassica nigra* Koch. */Brassica juncea* (L.) Coss. */Brassica carinata* **Other names**: white mustard /black mustard /brown or Indian mustard/ Ethiopian mustard		C4H5NS Or CH2=CHCH2N=C=S

3. Experimental design:

Only healthy adult snails from the aquarium were selected for the experiment and distributed at the plastic boxes (Ten snails in each box). The snails were divided into three groups viz., control, vehicle treated group and clove oil, black cumin oil and mustard oil treated group with three replicates for each. The snails in control group were maintained at optimum conditions without any treatment, those in the vehicle treated group sprayed with 1% tween 80 solution, and those in the oils treated group were sprayed with 60% value of (LC 50 / 24 hrs.) of the tested oils prepared in 1% tween 80 solution (v/v) as shown in Table (2).), but neomyl concentration was prepared in water (W/V). Values of LC 50/24 hrs. were determined using the spraying technique based on the % percentage. The snails were starved for 24 hours then introducing considerable amount of lettuce leaves. The spraying technique for the tested compounds as well as the vehicle was applied on the snails during their foraging their food, to contact their food and foots. During the four days post- spraying, no removing left-over food and no new food was introduced.

Table (2) : Concentrations of the tested compounds.

The compound	LC 50 / 24 hrs. %	60% of (LC 50 / 24 hrs.) %
Neomly	**0.125**	**0.075**
Clove oil	**4.17**	**2.502**
Black cumin oil	**9.29**	**5.574**
Mustard oil	**15.29**	**9.174**

4. Histological studies:

Four days post- spraying application , snails from each group (control group, vehicle control treated group and essential oils (clove oil, black cumin oil and mustard oil) treated group, were processed for analysis of histological changes by hematoxylin and eosin staining, The whole mount of soft tissues of *Monacha* sp. were carefully taken out of their shells and dropped immediately into the Bouin's solution as a fixative for 24 hours, dehydrated in ascending grades (70% to 100%) of ethyl alcohol in subsequent steps and cleared in xylene. The fixed tissues were embedded in paraffin wax and sectioned to obtain 4μm sections that mounted on glass slides then stained with hematoxylin and eosin method according to (Cowie, 1984). They were examined on light microscope and photographed by using a microscopic camera.

Results and discussion

1. The foot :

1.1. The normal foot:

The snails in control group exhibited normal histology of foot (Figure 1) with very muscular foot usually forms a typical creeping sole, occupying the whole of the ventral surface and generally not delimited from the body sides (Abdel-Rahman *et al.,* 2012).The foot tissues contain mucocytes, protein cells, columnar muscle fibers and pigment cells. Pigment cells are the most abundant in the foot (Birgül *et al.*, 2004). A single outermost cuticular layer of epithelial cells (Epithelial covering) mostly ciliated covers the foot for protective . Inner to this lining there is a layer of tall columnar epithelium with unicellular glands which secrete mucous. Embedded in between are transversely inter woven muscle fibers, called as longitudinal muscle fibers. Major part of the foot muscles is represented by thickly arranged oblique muscle fibers (Nagare, 2013).

1.2. The treated foot:

The snails in vehicle treated group (Figure 2) exhibited normal histology as control group barring slight shrinkage and rupture of muscular tissue, migration and accumulation of the pigment cells and slight distortion of epithelial covering **.** *Monacha* snails exposed to neomyl (Figure 3) exhibited destruction of the epithelial covering and the muscular tissue. In snails treated with clove oil (Figure 4), toxic precipitation nearby the margin, destruction of the epithelial covering, complete destruction and lyses of the muscular tissue. Snails exposed to black cumin oil (Figure 5) observed toxic precipitation) towards the epithelial covering, distortion and destruction of the muscular tissue and the epithelial covering. The foot of *Monacha* sp. treated with mustard oil (Figure 6) revealed shrinkage of the muscular tissues, slight toxic precipitation in-between. An excessive mucous secretion and complete withdrawal of the whole body inside the shell was observed due to direct effect of the tested oils's sprays on the epithellial cells of *Monacha* snail's foot.

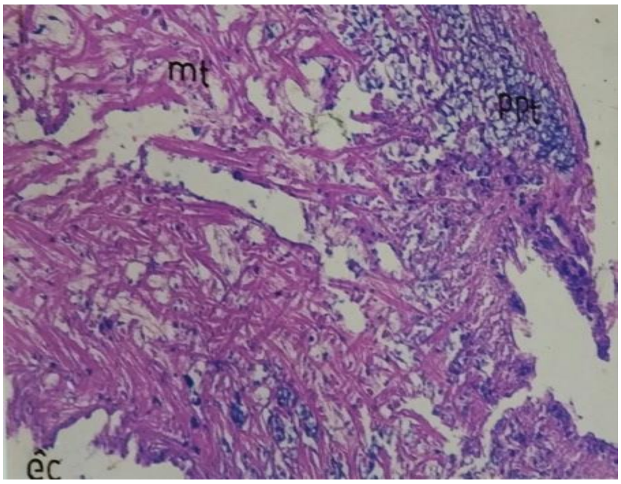


Figure (1): Photomicrograph of a section through normal foot of *Monacha* sp. control. H. & E. stain. (200X) showing muscular tissue (mt), pigment cells (c) and epithelial covering (ec).

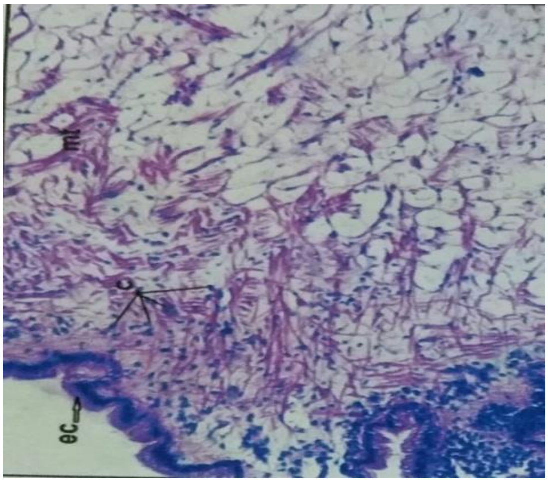


Figure (2): Photomicrograph of a section through foot of *Monacha* sp. treated with vehicle control H. & E. stain. (200X) showing slight shrinkage and rupture of muscular tissue (mt), migration and accumulation of the pigment cells (c) and slight distortion of epithelial covering (ec).

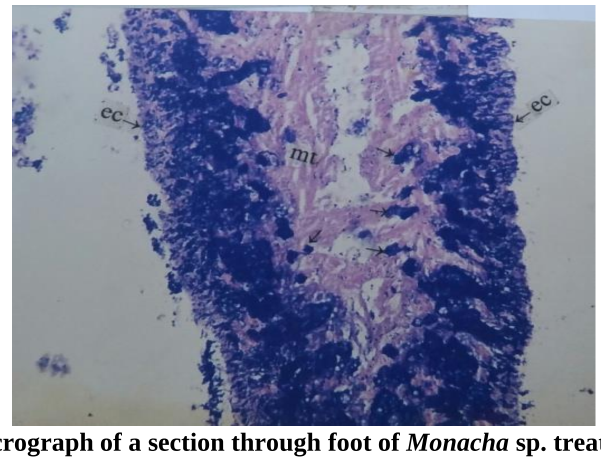


Figure (3):Photomicrograph of a section through foot of *Monacha* sp. treated with neomyl H. & E. stain. (200X) showing neomyl precipitation (arrows), destruction of the epithelial covering (ec) and the muscular tissue (mt).

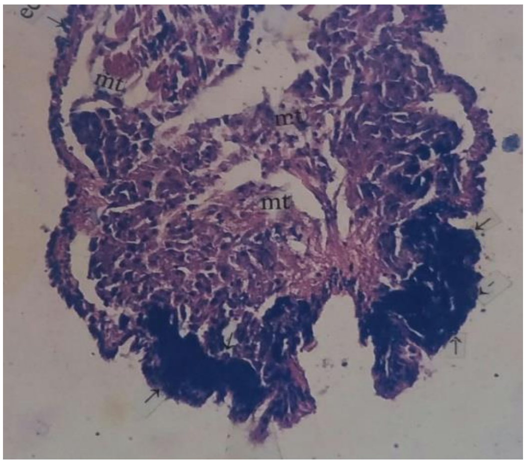


Figure (4): Photomicrograph of a section through foot of *Monacha* sp. treated with clove oil H. & E. stain. (200X) showing toxic precipitation nearby the margin (arrows), destruction of the epithelial covering (ec) , complete destruction and lysis of the muscular tissue (mt).

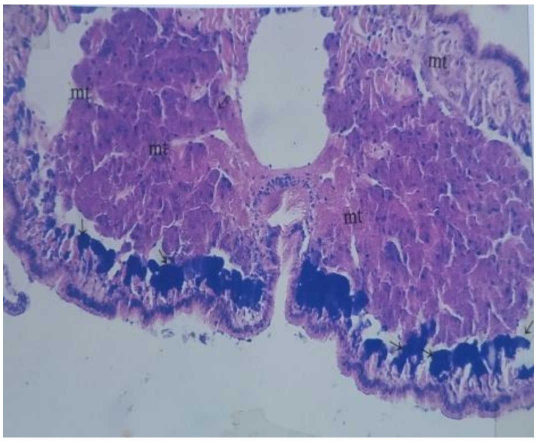


Figure (5): Photomicrograph of a section through foot of *Monacha* sp. treated with black cumin oil H. & E. stain. (200X) showing toxic precipitation (arrows) towards the epithelial covering (ec) , distortion and destruction of the muscular tissue (mt) and the epithelial covering (ec).

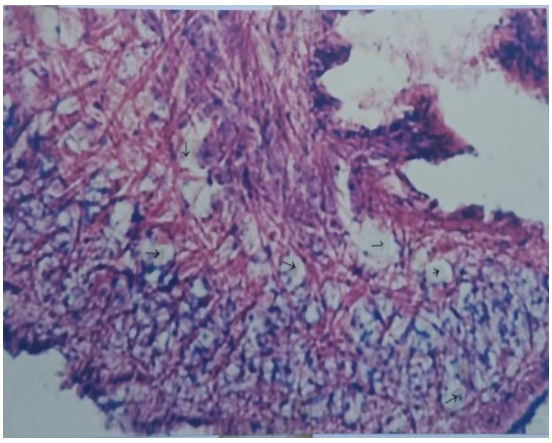


Figure (6): Photomicrograph of a section through foot of *Monacha* sp. treated with mustard oil H. & E. stain. (200X) showing shrinkage of the muscular tissues, slight toxic precipitation in-between (arrows) and distortion of the epithelial covering (ec).

2. The hepatopancreas:

2.1.Normal hepatopancreas (Control group):

Hepatopancreas of molluscs involved in extracellular and intracellular digestion of food material; absorption of nutrients; storage of lipids, glycogen and minerals and it plays also a major role in detoxification (Beeby and Richmond, 1988 and Henry *et al.*, 1991). The morphology and histology of the hepatopancreas of the land snail *Monacha* sp. has been described in several studies (Aioub *et al.,* 2000; Abdel-Rahman *et al.*, 2012; Parvate and Thayil, 2017; Hemmaid *et al.*, 2017 and Ustina *et al.*, 2018). The hepatopancreas of *Monacha* snail Figure 7 (a,b) is a bilobed tubuloacinar gland located in the dorsal portion of the animal. The gland is covered by the general covering integument of the visceral mass which consists of a single layer of epithelial cells rest on a delicate basement membrane. The tissue of the hepatopancreas comprises numerous, compressed small blind-ending tubules. Between the tubules, loose connective tissues and hemolymphatic spaces were present. The tubules lined by columnar epithelial cells of unequal heights and containing basal nuclei (Yonge, 1983). These cells rest on a thin basement membrane known as basal lamina. The epithelium lining the digestive tubules consists of four different cellular populations, digestive cells, excretory cells, calcium cells and thin cells (Sherifa *et al.*,2007). The two main cell types are the digestive and excretory cells (Figure 7b). Digestive cell is by far the most numerous elements in the wall of the digestive gland tubules. They are long columnar with domed distal apices and flat bases by which they rest on very thin basement membrane. They vary greatly in length within the same tubule. The nucleus is basal, usually oval but may be spheroidal or even irregular. They show important morphological alterations according to the digestive activity of the animal. Excretory cells are present in much smaller number than the digestive cells. They are shorter pyramidal, globular or cone-shaped, but sometimes may be columnar. They are markedly shorter than the digestive cells and therefore appear wedged in between groups of the digestive cells. They are characterized by the presence of a single large vacuole often in the form of a large brown body. The free surface of the cell possesses a well-developed brush border. The nucleus is small and pressed flat against the cell base.

2.2.Treated Hepatopancreas:

The hepatopancreas in Vehicle treated snails exhibited histology close to the normal one in the control group (Figure 7c). The tissue of the hepatopancreas of *Monacha sp.* exhibited marked histological alterations in response to treatment by neomyl, clove oil, black cumin oil and mustard oil as follows: Neomyl (Figure 8) showed cell's necrosis, degeneration of the digestive tubules and neomyl precipitation. In snails treated with clove oil (Figure 9), detachment of the integument (itg), complete distortion of digestive tubules and toxic precipitation full the digestive tubules and in between. Black cumin oil (Figure 10) showed shrinkage and collapse of the digestive tubules and slight toxic precipitation (arrows). The hepatopancreas of *Monacha* sp. treated with mustard oil revealed negligible toxic precipitation, collapse and shrinkage of the digestive tubules and detachment of the covering membrane (itg) (Figure 11).

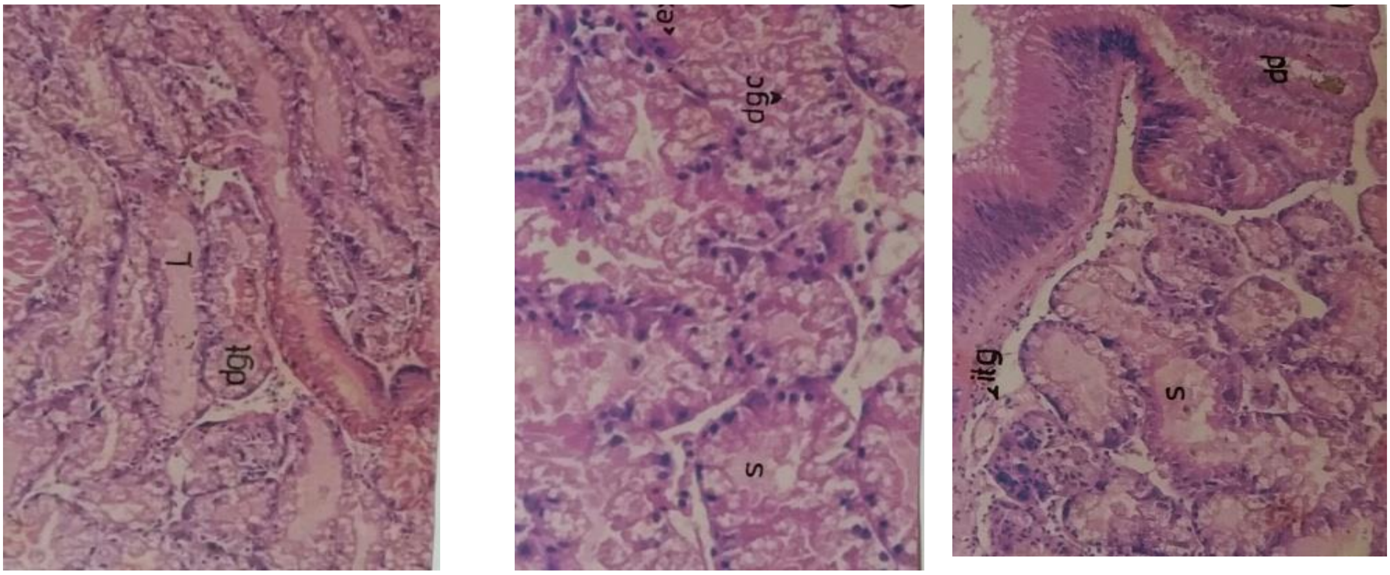


Figure (7a) Figure (7 b) Figure (7c)

Figure (7): Photomicrograph of a section through normal hepatopancreas of *Monacha* sp. control (a,b) ; Vehicle control (c) : H. & E. stain. (200X) showing (a): digestive gland tubule (dgt), lumen of digestive gland tubule (L),, (b): digestive cell (dgc), excretory cell (exc), secretion (S), (c): digestive ducts (dd), secretion (S) and the epithelial covering (integument) (itg).

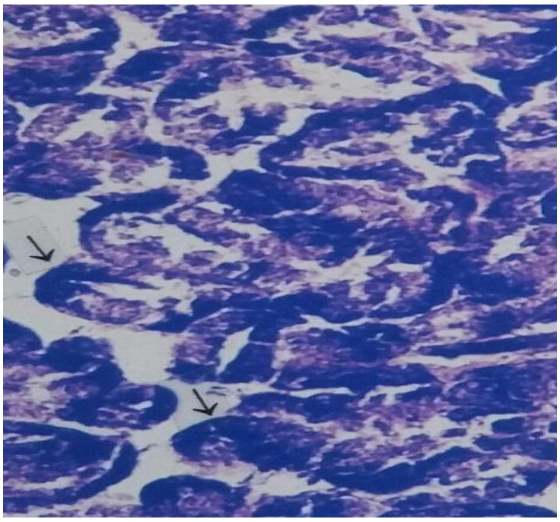


Figure (8): Photomicrograph of a section through hepatopancreas of *Monacha* sp. treated with neomyl H. & E. stain. (200X) showing severe toxic precipitation cell's necrosis, degeneration of the digestive tubules and neomyl precipitation (arrows).

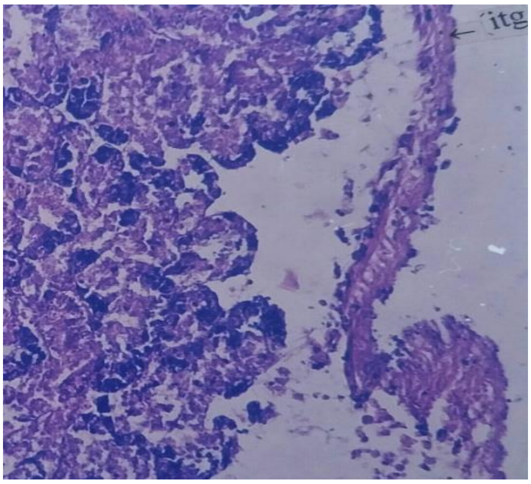


Figure (9): Photomicrograph of a section through hepatopancreas of *Monacha* sp. treated with clove oil H. & E. stain. (200X) showing detachment of the integument (itg), complete distortion of digestive tubules and toxic precipitation full the digestive tubules and in between.

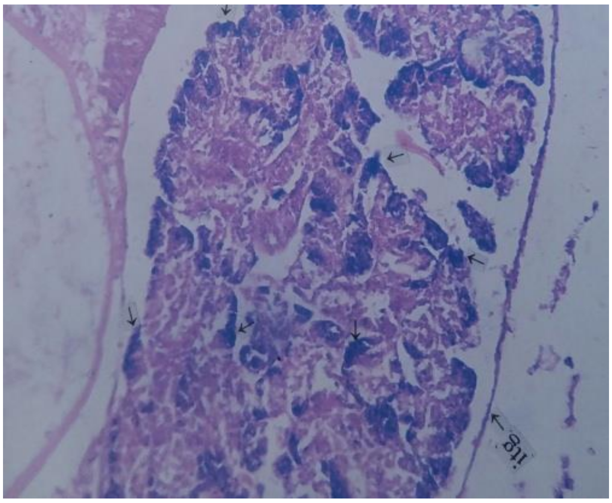


Figure (10): Photomicrograph of a section through hepatopancreas of *Monacha* sp. treated with black cumin oil H. & E. stain. (200X) showing shrinkage and collapse of the digestive tubules and slight toxic precipitation (arrows).

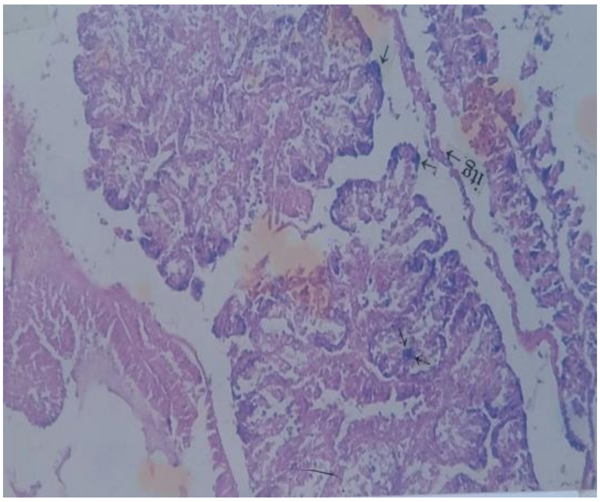


Figure (11): Photomicrograph of a section through hepatopancreas of *Monacha* sp. treated with mustard oil H. & E. stain. (200X) showing negligible toxic precipitation (arrow), collapse and shrinkage of the digestive tubules and detachment of the covering membrane (itg).

Plant essential oils are potential candidates for snail control, due to their selective action, and little or no harmful effects on the non-target organisms and the environment (Isman, 1999). The toxic effect of any compound on an organism can be evaluated by observing the histopathological changes caused by the compound (Parvate and Thayil, 2017). The digestive glands of molluscs are known as target organs for toxicants and the changes in the cytoarchitechture of digestive gland of snails is used as a biomarker of induced toxicity (Triebskorn and Kohler, 1996).Thus any structural damage to the digestive gland affects the animals in multiple ways. The present results are in accordance with the data obtained in the studies of Zhou *et al.* (1993) on the snail *Biomphalaria glabrata* treated with niclosamide and extracts of *Eucalyptus camaldulensis*, that showed irregularity and shrinkage in the digestive cells (Hepatocytes) and calcium cells of hepatopancreas along with breakage of some digestive cells and leakage of its secretory material into the lumen. Similar observations such as shrinking of lumen of digestive tubule, degeneration and necrosis of cells along with atrophy of the connective tissue of digestive gland were noted on exposure of *Lymnaea luteola* to Paraquat (Kanapala and Arasada, 2013). Irreversible necrotic changes were noticed in the digestive gland of *Lymnaea stagnalis* following exposure to thiodan (Unlü *et al.*, 2005).

The present results also agree with the studies on the snail *Archachatina marginata* exposed to sublethal concentrations of Cu salt wherein there was clogging of hepatocytes observed along with peripheral thickening of hepatic tubules (Otitoloju *et al.*, 2009). The data obtained by Parvate and Thayil (2017) on the snail *A. fulica* illustrated that the land snail *Achatina fulica* in the vehicle treated group had histology close to normal but the digestive cells of the hepatopancreas were degenerated in snails exposed to both lower as well as higher concentration of clove oil roughly in proportion to the dose administered, along with peripheral thickening of hepatic tubules, and atrophy of connective tissue in snails exposed to lower sub-acute dose.

Clustering of secretory material exuded from the damaged hepatocytes was also observed which was massive in snails treated with higher sub-acute dose of clove oil. Lahlou (2004) revealed that clove oil possesses strong bioactivity. It has also exhibited ovicidal properties against eggs of several varieties of snails including *A. fulica* (Hollingsworth *et al.*, 2012). Kamel *et al.* (2017) illustrated that the application of 1/2 LC50 of newmyl resulted in cytoplasmic degeneration in digestive cells of the land snail *Eobania vermiculata* and LC50 of newmyl showed that the digestive cells were widely degenerated and harboring pleomerphic mitochondria. 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 LC90 of thymol against L. maximus. Ustina *et al.*, (2018) revealed that the effect of LC90/48 hrs. on the digestive gland of the Egyptian giant garden slug, *Limax maximus* 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. Heiba *et al.* (2002) revealed that the pathological alterations in the digestive gland of the land snails *E. vermiculata* and *M. contiana* exposed to lannate are probably due to accumulation of the insecticide in the cells of the digestive gland. This damage could be correlated with the disturbed enzyme activities in the different species.

Comparable results were obtained in the current investigation wherein the land snail *Monacha* sp. in the vehicle treated group had histology close to the normal. Neomyl caused necrosis, degeneration of the digestive tubules and toxic precipitation. Clove oil exhibited detachment of the integument, complete distortion of digestive tubules and toxic precipitation full the digestive tubules and in between. Black cumin oil showed shrinkage and collapse of the digestive tubules and slight toxic precipitation. The hepatopancreas of *Monacha* sp. treated with mustard oil revealed negligible toxic precipitation, collapse and shrinkage of the digestive tubules and detachment of the covering membrane. The foot of snails treated with vehicle showed histological similarity to the control group barring slight shrinkage and rupture of muscular tissue, migration and precipitation of pigment cells and slight distortion of epithelial covering. Snails exposed to neomyl exhibited destruction of the epithelial covering and the muscular tissue. Clove oil resulted in toxic precipitation nearby the margin, destruction of the epithelial covering, complete destruction and lyses of the muscular tissue of the foot. Back cumin oil observed toxic precipitation towards the epithelial covering, distortion and destruction of the muscular tissue and the epithelial covering.

The foot of *Monacha* sp. treated with mustard oil revealed shrinkage of the muscular tissues and slight toxic precipitation in-between. These observations are in congruence with the results of the earlier and recent study using crude extracts of camellia seed and mangosteen pericarp, which affected cells in the foot by relaxing muscle fibers and creating gaps between epithelial cells and connective tissue, resulting in the dearangement of the cilia in the snail *Bithynia siamensis* (Aukkanimart *et al.*, 2013). Degenerative changes in the muscle fiber, protein and pigment cells of the foot were noticed in the snail *Lymnaea stagnalis* exposed to thiodan in pesticide-free freshwater (Unlü *et al.*, 2005). The results here also agree with the recent study of Parvate and Thayil (2017) who found that the foot of snail *A. fulica* treated with both lower as well as higher subacute dose of clove oil showed increase in the size of empty spaces within the mesoepithelial cells and muscle fibers, which was more pronounced in higher subacute dose. Further at higher subacute dose the empty spaces within the muscle fibers were also infiltrated with inflamed cells. An excessive mucous secretion and complete withdrawal of the whole body inside the shell was observed in the present study,due to direct effect of the tested oils's sprays on the epithellial cells of *Monacha* snail's foot. This observation agrees with Triebskorn *et al.* (1998) who said that the topical application of the molluscicides on snails primarily target the epithelial cells of skin including the mucus cells.

In fact, one of the first responses of molluscs to stress is increased mucus production and secretion (Triebskorn and Ebert, 1989). Profuse mucus production was observed in snails exposed to both lower (20% of LD 50/24 hrs.) as well as higher dose (60% of LD 50/24 hrs.) of clove oil. This is in accordance to the observations of Desai *et al.* (2015) who had exposed the snail *A. fulica* to various synthetic molluscicides. The mucus forms a protective barrier between the toxin and the surface of epithelial cells and helps in diluting the toxin (Triebskorn and Ebert, 1989).

In conclusion, these results indicated that the tested essential oils, clove oil, black cumin oil and mustard oil are highly effective against *Monacha* snail and the histopathological alterations caused were proportionate to the potential of the tested oil. Thus, these oils may be of great value in the field to control the population of the target herbivorous land snail *Monacha* sp., being eco-friendly as safe and economic molluscicide, which no harm upon ecosystems instead of using chemical pesticides that could pollute the environment.

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