وزارة الزراعة واستصلاح الاراضى
Ministry of Agriculture and Land Reclamation
مركز البحوث الزراعية
Agricultural Research Center

**Egyptian Journal of Plant Protection Research Institute**

www.ejppri.eg.net

# Response of land snails to diclofenac-potassium under laboratory and field conditions

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**ARTICLE INFO**

*Article History*
Received: 6/ 10/2022
Accepted:21/12 /2022

***Keywords***

Diclofenac-potassium, *Eobania vermiculata*, mantle cavity, lipid, peroxidase and molluscicides.

## Abstract

Mantle cavity is the lung roof of land snails. The lung has a single opening for breathing in the right side. The present study was conducted to evaluate the impact of diclofenac-potassium compound (drug) on the mantle of land snail *Eobania vermiculata* (Müller) (Gastropoda: Helicidae), and its efficiency as a molluscicide under field conditions. Snails were treated with a series of concentrations of the tested compound, using the contact method, for one week. The medium lethal concentration ($LC_{50}$) was calculated after seven days of treatment. Lipid peroxidase activity was determined in the snails treated with ½ $LC_{50}$ of the tested compound. The mantle tissues were scanned after one week of treatment. Moreover, the tested compound was evaluated as a spray against land snail, *E. vermiculata*, under field conditions for 21days in infested citrus nurseries, compared with methomyl, the recommended molluscicide by the Egyptian Ministry of Agriculture and land Reclamation (MALR). Results revealed that 2.5% of diclofenac- potassium caused 90% mortality for tested animals and the $LC_{50}$ was 1%. Further, the compound caused full dehydration of the body water contents of the tested animals. Also, it induced a remarkable increase in lipid peroxidase activity in the snails. The compound also induced damage to respiratory epithelia and infiltration of immune cells. Under field conditions, the compound achieved 91.5% reduction in snail populations, compared with 80.5% for methomyl. Therefore, diclofenac-potassium could be recommended as a promising molluscicide under Egyptian agriculture field conditions.

## Introduction

Terrestrial snails are widespread in Egypt. They are important crop pests because most of them are omnivorous feeders. They feed on stems, bulbs, leaves and tubers vegetable, ornamental and fruit crops (Barker, 2002). The chocolate band snail *Eobania vermiculata* (Müller) (Gastropoda: Helicidae), is one of the most prevalent species in Egypt attacking fruit and ornamental plants (Ali and Ramadane, 2020). Land snails have a mantle cavity in the pallial lung. It has a single opening on the right side, the breathing pore (Sturm *et al*., 2006). Mantle is a specialized organ. It is not only enduring the glands which excrete the shell, but also it acts as a barrier between the hard limestone crust and the snail itself (Kumar and Priyadarsini, 2014).

Land snail control is difficult due to its hard shells and mucus.

Traditional chemical pesticides are commonly used, however, they cause environmental pollution and harm to non-target species. For these reasons, researchers should be investigating new and safe substances to control these pests.

Diclofenac-potassium is an anti-inflammatory drug, the high doses cause the toxic effects that induces oxidative stress, mitochondrial damage and interaction between reactive metabolic with cellular macromolecules, leading to changes in proteins structure (Owumi and Dim 2019; Galati *et al.,* 2002 and Masubuchi *et al.,* 2002).

The aim of the present work is to study the effect of diclofenac-potassium on the respiratory of land snails, as a non-traditional molluscicide, under laboratory and field conditions.

**Materials and methods**

**1.Tested compounds:**

**1.1. Diclofenac – potassium (50 mg granules)**

**Trade name:** Catafast, The $LD_{50}$ (Medium lethal dose) for rats is 345 mg / kg b.w. (Arzneimittel Forschung 1993 ). It was produced by Novartis Pharma (S.A.E.) -Cairo, Egypt.

**1.2. Methomyl:**

Trade name: Lannet (90 % powder) is a carbamate insecticide recommended by Ministry of Agriculture and Reclamation against land snails infesting agricultural crops. It is used as bait at the rate of 8-10 kg /faddan. The $LD_{50}$ for rats is 17 -24 mg /kg. It was obtained from Kafr El-Zayat Co., Egypt.

**2.Tested animals:**

The adult individuals of chocolate band snail, *E. vermiculata*, were collected from citrus nursery trees at Abu Rawash area, Giza Governorate, Egypt, coordinate N “30°.8” E31. 5“26”. Animals were transported to the laboratory of Plant Protection Research Institute, Agricultural Research Center, Giza Governorate, Egypt (N 30°.2”44” E 31° 12 “26”). Snails were placed in small glass boxes, containing 8-10 cm moist soil, provided with fresh green lettuce leaves, covered by white cloth and secured with a rubber bands to prevent animals from escaping. They were kept under 20±2° c in the laboratory for 15 days for acclimatization.

**3. Laboratory tests:**

**3.1. Contact method (Thin layer film technique):**

Serial concentrations of diclofenac–potassium (0.3, 0.6, 1.3 and 2.5 %) were tested against *E. vermiculata* using the thin layer film technique (Ascher and Mirian, 1981). On the surface of each petri-dish two ml of each concentration was put with water. Water was evaporated under room temperature, leaving a residual layer/film of the tested compound. The snails were offered individually to each concentration of the tested compound for one week. A parallel control test was conducted using plain water. Mortality percentages were calculated with a recording of the toxicity observations. The $LC_{50}$ (Medium lethal concentration) value was calculated according to Finney (1971) after one week of treatment.

**3.2. Biochemical studies:**

**3.2.1. Lipid peroxidase (LPO) determination:**

Lipid peroxidase was estimated in land snails *E. vermiculata,* after one week of treatment with ½ LC of diclofenac-potassium.

**3.2.2. Sample preparation:**

The shell of treated and untreated snails was removed, and one gram of tissue was homogenized under cooling by homogenizer for three min., with sodium chloride (10 ml) 0.9 *N*, and then centrifuged (4000 r.p.m. (Round per minute), for 15 min.). The enzyme activity was determined at 534 nm., according to Ohkawa *et al.* (1979). Data were statistically analyzed by ANOVA (Analysis of Variance) and LSD (Least Significant Difference) (P < 0.05) using CoStat Program (Glenn, 2005).

**3.3. Histopathological Studies:**

After one week of treatment with $LC_{25}$ of diclofenac-potassium, the mantle of treated and untreated animals was separated. Samples were fixed in 10 % formalin for 24 hrs. Then washing was done with tap water, and after that the samples were put in serial dilutions of alcohol (Methanol, ethyl and absolute ethyl) for dehydration. The samples were cleared in xylene and embedded in paraffin at 56 C degrees in a hot air oven for 24 hrs. Tissues were put in paraffin wax blocks for sectioning at four microns thickness by slide microtone. The tissue sections were collected on glass slides, and stained by hematoxylin and eosin stain for routine examination by light electric microscope (Banchroft *et al.*, 1996).

**4. Field experiments:**

The efficiency of diclofenac-potassium was evaluated as a spray compared with methomyl against land snails, *E. vermiculata*, on citrus nursery trees at Abu Rawash area, Giza Governorate, Egypt, coordinate (N 30º.̓8” E 31 .5 “26”). Six plots were chosen (Each of 60 $m^2$), with at least 10 meters distance in-between, 2 replicates for each compound and 2 for the control. Live snails were counted on random trees in each plot, pre- and post-treatments, at 1, 3, 7, 15 and 21 days of treatment. Population reduction percentages of snails were calculated after 21 days of treatment according to Henderson and Tilton (1955**).**

**5. Statistical analysis:**

The results were analyzed as one-way ANOVA, using ANOVA in SAS software, version 9.1, SAS Institute carry, NC USA (SAS Institute, 2008) and means were compared by Turkey's HSD (P<0.05 level) using the same program.

**Results and discussion**

**1. Laboratory studies:**

**1.1. Toxic effect of diclofenac-potassium:**

Data in Table (1) shows the effect of diclofenac-potassium against land snail, *E. vermiculata*, after one week of treatment using the contact technique. The results obtained that the tested concentrations *i.e.,* 0.3, 0.6, 1.3 and 2.5 % caused 10, 30, 50 and 90 % mortality, respectively. The $LC_{50}$ value was 1 % after one week of treatment. These results pointed out that mortality percentages increased with increasing concentration of the compound.

**Table (1): Toxic effect of diclofenac-potassium against land snail *Eobania vermiculata* after one week of treatment using thin film layer application.**

| Concentration % | Mortality % | $LC_{50}$ % |
|---|---|---|
| 0.3 | 10.0 | |
| 0.6 | 30.0 | 1.0 |
| 1.3 | 50.0 | |
| 2.5 | 90.0 | |

The increase in compound concentrations reduced the ability to expel the mucus from snail bodies. These results agreed with Mobarak and Kandil (2021). They indicated that, the mortality percentage of *Massylae vermiculata* (Müller) (Gastropoda: *Helicidae)* increased with increasing the Cuprol compound concentrations. Also, Mobarak *et al.* (2021) reported that, the mortality percentages increased with increasing acetylcysteine compound concentrations against *Monacha cartusiana* (Müller) (Gastropoda: Hygromiidae*).* Eshra

(2014) mentioned that copper hydroxide was more toxic than methomyl against *E. vermiculata*, after three days of treatment, whereas $LC_{50}$ was 3.31 and 3.75 %, respectively. Moreover, Mobarak *et al.* (2017) indicated that, the $LC_{50}$ of acetylsalicylic acid was 210.6 ppm, after one week of treatment, using the thin film layer technique. Hegab *et al.* (2013) showed that, 7% concentration of copper sulfate achieved 85% mortality against *Helicella vestalis* (Pfeiffer) (Gastropoda: Geomitridae), after one week post treatment. Parvate and Thayil (2017) indicated that, the $LC_{50}$ of clove oil was 7.9 % against land snail, *Achatina fulica* Bowdich (Gastropoda : Achatinidae) after 24 hrs. of exposure.

### 1.2. Clinical symptoms:

The observations of the clinical symptoms after treatment with diclofenac-potassium on land snail *E. vermiculata*, using the contact technique, were described in Table (2). The results revealed that the body weight of treated land snails decreased from 4.5 g to 2.5 g compared with untreated snails (4.5 g body weight). The treated snails choked and left the whole body from the shell. In addition, the body water content was dehydrated after the snails were treated with the tested compound. These symptoms may be attributed to the loss of a lot of body water contents.

**Table (2): Clinical symptoms of land snail *Eobania vermiculata* treated with diclofenac-potassium using thin film layer technique.**

| Parameter | Untreated snails | Clinical symptoms observations on treated snails |
|---|---|---|
| **Body weight (g)** | 4- 4.5 | 2.5- 2.8 |
| **Body status** | Normal | Choking and leaving the whole body from the shell and full dehydration |

The same results occurred with Mobarak *et al.* (2021), who found that the body weight of *Helix aspersa* Muller (Gastropoda: *Helicidae)* was decreased after being treated with clodinafop-propargyl after seven days of exposure. Also, clove oil caused a strong toxic effect on the epithelial cells of the skin including the mucus cells (Triebskorn *et al.*, 1998).

### 1.3. Impact of $LC_{25}$ of diclofenac-potassium on lipid peroxidase activity:

Data in Table (3) showed the effect of ½ $LC_{50}$ of diclofenac-potassium on the land snail, *E. vermiculata*, after one week of exposure. The results revealed that the tested compound caused remarkably significant increasing in the level of enzyme activity to 40.3 n mol /g in treated snails compared with 25.7 n mol /g in untreated snails. These results mean that the compound induced trouble in the enzyme activity. This imbalance leads to an imbalance between antioxidants and free radical oxygen, resulting in tissue damage and oxidative stress.

**Table (3): Effect of ½ $LC_{50}$ of diclofenac-potassium on lipid peroxidase (LPO) (n mol/ g tissue) in land snail, *Eobania vermiculata*, after one week of treatment.**

| Lipid peroxidase n mol/ g tissue | | |
|---|---|---|
| **Control (mean ± SE)** | **Treatment (mean ± SE)** | **LSD** |
| **25.7± 1.1$^{b}$** | 40.3±2.1$^{a}$ | 6.7 |

**$P < 0.05$.**
*** Data are expressed as mean ± SE.**
*** Means, which share the same superscript symbol(s), are not significantly different.**

This result was compatible with Al-Daihan *et al.* (2010) and Mobarak and Kandil (2021). They reported that cuprol compound caused increase in the enzyme activity of treated snails compared with untreated land snail *M.*

*vermiculata* . Moreover, Srikanth *et al.* (2021) proved that, the effect of silica nanoparticles caused increase in the LPO activity in water snail, *Pila virens* (Lamarck) Gastropoda: Ampullariidae) , after treatment with 165 Mg/L for 24 hrs. Also, Caixeta *et al.* (2020) showed that CuoNPs, CdNPs and $Sio_2$ NPs caused oxidative stress on lipid pyroxidase and catalase in land snails leading to death.

### 1.4. Histopathological studies.

The histopathological effect of ½ $LC_{50}$ of diclofenac-potassium on mantle cavity tissue (The roof of lung) of snail was observed in Figure (2) compared with untreated snails in Figure (1). In the normal mantle of the untreated snail with a thin inner mantle layer and outer ciliated epithelial layer, the respiratory tissue spaces (lacunae) showed infiltration by granulocytes within intraepithelial spaces (Figure 1). While Figure (2) shows the mantle cavity of a treated snail that shows damage to respiratory epithelia (Lacunal wall) and heavy infiltration of immune cells.

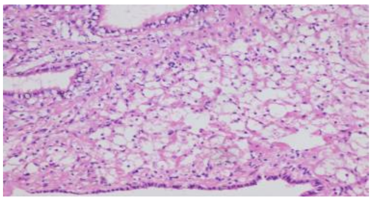

**Figure (1):** Normal mantle cavity with thin inner mantle layer and outer ciliated epithelial layer. The respiratory tissue spaces (Lacunae) showed infiltration by granulocytes within intraepithelial spaces. H&E X 400.

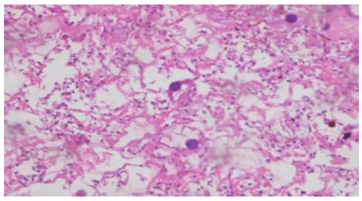

**Figure (2):** Mantle cavity treated with diclofenac-potassium showing damage of respiratory epithelia (Lacunal wall) and heavy infiltration of immune cells. H&E X 400.

These results indicate the inability of the snails to breathe due to the treatment led to laceration of mantle tissue (Lung tissue). This damage may be due to the compound caused a severe increase in the LPO enzyme activity led to this damage in the mantle tissue, the snails cannot breathe resulting in suffocation, leaving the whole body out of the shell. Higher doses of diclofenac-potassium over long time lead to liver damage and acute kidney injury (Owumi and Dim, 2019). Mobarak and Kandil (2021) investigated that cuprol compound induced remarkable thickening and hyalinization of lacunal wall in the mantle of the *M. vermiculata*. Otludil and Ayaz (2020) observed that copper sulfate caused degeneration in the connective tissues of the digestive cells of snails after exposure.

### 2. Field studies:

Table (4) tabulated the field efficacy of diclofenac-potassium compared with methomyl compound against land snail, *E. vermiculata,* using the spray technique. The results revealed that the compound achieved 91.5 % reduction in snail' population compared with 80.5% for methomyl after three weeks of application. Diclofenac–potassium achieved significant reduction ($P < 0.05$) in snail population.

**Table (4): Field performance of diclofenac-potassium against land snail, *Eobania vermiculata*, compared with methomyl compound after three weeks of treatment using spray technique.**

| Treatment | Rate of application g / L | No. of survival pre-treatment | | No. of survival post-treatment | | Population reduction % |
|---|---|---|---|---|---|---|
| | | No. | Mean ± SE | No. | Mean ± SE | |
| **Diclofenac** | 25 | 396 | 78.8± 5.1[a] | 41 | 8.2± 1.5[b] | 91.5 |
| **Methomyl** | 20 | 157 | 31.8± 1.9[b] | 36 | 7.2± 0.9[b] | 80.5 |
| **Control** | - | 515 | 103.0± 4.3[a] | 599 | 119.0± 8.2[a] | |
| **LSD** | | 37.16 | | 14.88 | | |

**P < 0.05.**

*** Data are expressed as mean ± SE.**

*** Means, which share the same superscript symbol(s), are not significantly different.**

These results may be attributed to severe oxidative stress that occurred by diclofenac-potassium in snails that led to death. Mobarak and Kandil (2021) pointed out that cuprol compound caused 87.4 % population reduction compared with methomyl, which gave 72 % after treatment, against *M. vermiculata*. Chen *et al.* (2019) indicated that the novel molluscicide mixture of kaoline compound with black carbon caused 95% reduction in *Oncomelania hupensis* Gredler (Gastropoda :Pomatiopsidae) snail populations using 2.0 g / $m^2$ as a spray method.

It was concluded that the diclofenac-potassium compound affected the roof of the mantle (Lung) of the snails leading to death and caused a reduction in the snail populations in the field, therefore, it was recommended as a promising molluscicide under Egyptian agriculture conditions.

**Acknowledgement**

We would like to send our deepest gratitude to Dr. Aly H. El-Sherbiny, Professor of Vertebrate Ecology, at the Harmful Animals Research Department, Plant Protection Research Institute, Agricultural Research Center for correcting the manuscript's language, and for scientific reviewing. Great and special thanks to Dr. Waheed Gabr, Professor at the Harmful Animals Research Department, Plant Protection Research Institute, Agricultural Research Center, for correcting the manuscript scientifically.

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