Antagonistic Effect of Some Antifungal Substances against Fungi Causing Rots of the Glassy Clover Snail Baits

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Abstract: Mouldiness is one of the most common microbiological defects found in molluscicide baits. Total count of fungi which associated with metaldehyde, methomyl and salicylic acid baits was detected under laboratory and field conditions. The antagonistic effect of four common antibiotics (locasten, mycostatin, terbin and trosyd) was estimated against these fungi. Moreover, effect of antibiotics on the molluscicidal activity of baits against *Monacha cartusiana* snails was assessed under the laboratory and field conditions. Result showed that in the laboratory, *Aspergillus flavus* and *Rhizopus stolonifer* were the most predominant fungi on the tested and control baits. But *Aspergillus niger* and *Fusarium subglutinans* were appeared only on salicylic acid and metaldehyde baits, respectively. In the field, *A. flavus* and *R. stolonifer* were the two only fungi which associated with salicylic acid and metaldehyde baits, respectively. Regarding to antibiotics, the highest effect of locasten was showed against *A. niger* and *R. stolonifer* by MIC value 0.2%. While, the highest activity of mycostatin and terbin was recorded against *R. stolonifer* by MIC values 0.04 and 0.02%, respectively. The highest antifungal effect of trosyd was exhibited against *R. stolonifer* by MIC value 0.08%. About the effect of baits alone and at mixing with antibiotics against *M. cartusiana* snails, in the laboratory methomyl with terbin, methomyl with trosyd and methomyl with mycostatin were the most potent baits against snails. Under field conditions, mycostatin increased the molluscicidal activity of salicylic acid and methomyl baits against snails and it showed also a high ability for attract snails to baits. For this reason, this antibiotic considered a promising antifungal and attractive material can add to the different molluscicide baits for control land snails in the future studies.

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1. Introduction

Land snails are a serious pest cause immense damage to the vegetation including vegetables, horticultural parts and field crops in most area in Egypt **(Mahrous *et al.*, 2002 and Gabr *et al.*, 2006)**. *Monacha cartusiana* is the most abundant snail in all localities at Sharkia Governorate **(Shetaia, 2010)**. The use of bait is the most common method in the control of gastropods **(El–Massry, 1997)**. Poisonous baits containing wheat bran and sugar cane syrup can attract *M. cartusiana* snails far from 100 cm. For this baits considered the best technique to reduce numbers of *M. cartusiana* snails in different Egyptian fields **(Ismail *et al.*, 2014)**. Mold contamination loss the baits quality as a result of possible formation of mycotoxins and other metabolites which have unpleasant odor alienating land snails. *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* spp. were the commonly toxigenic fungi which contaminate the rice bran **(Jayaraman and Indira, 2009)**. Temperature is one of the main virulence factors affecting on fungi **(Rhodes, 1988)**. 25°C was the optimum temperature for the growth of *A. niger*, *A. flavus* and *Aspergillus fumigatus* **(Ved and Dharam, 1996)**. Antibiotics are antimicrobial factors and broad spectrum drugs, have their selective effects on various groups of microbes as fungi or bacteria **(Mohamed *et al.*, 2005)**. The inhibition effects of antibiotics on microbes have been reported in the reviews by **Halling – Sorensen *et al.* (1998)** and **Thiele – Bruhn (2003)**. Mycostatin possessed a fungistatic and fungicidal activities against *Aspergillus* spp., *Candida* spp. and *Cryptococcus neoformans* **(Elizabeth *et al.*, 1998)**. Terbin also proved to be highly active against dermatophytes (MIC range, 0.001 to 0.01 microgram / ml), aspergilli (MIC range, 0.05 to 1.56 micrograms / ml) **(Petranyi *et al.*, 1987)**. It has also a highly antifungal activity against Zygomycetes, *Aspergillus* spp., *Fusarium* spp. and *Paecilomyces* spp. **(Michael *et al.*, 2013)**. At the same trend, Tioconazole (trosyd) is an antimicrobial agent has a broad spectrum of activity *in vitro* against dermatophytes **(Clissold and Heel, 2012)**. With regard to molluscicides, metaldehyde was a successful molluscicide has high toxic effect against the *M. cartusiana* snail **(El Akhrasy, 2010)**. Moreover, methomyl and salicylic acid represent also a superior compounds in controlling the same snail species under laboratory and field conditions **(Shokry, 2013)**. Combination of antibiotics with other antifungal agents increased its

toxicity and antifungal activity. Antibiotics causing mortality of land snails by affecting on it's immunocompetence. Furthermore, it has a bad effect on the gut of *Helix aspersa* terrestrial snail **(Ansart *et al*., 2002)**.

This study aimed to prevent the growth of fungi that causing rot of certain molluscicide baits by using some common antibiotics. The study also extends to investigate the effect of these antibiotics on the toxic activity of these baits against *M. cartusiana* individuals.

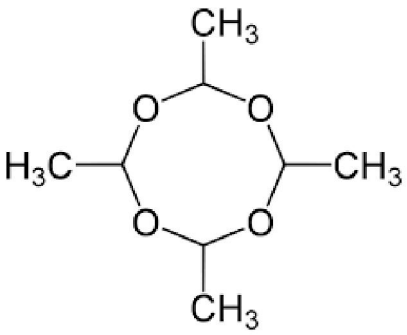
2. Materials and Methods

2.1. Tested molluscicides

I - Metaldehyde

* **Trade name:** Gastrotex (5%) white or colorless crystalline solid

* **Structure formula:**



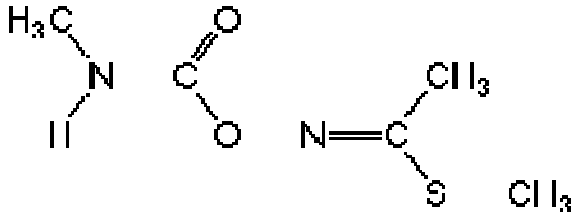
* **Chemical name:** 2,4,6,8-tetramethyl-1,3,5,7-tetraoxocanemetaceta- ldehyde

* **Molecular formula:** $C_8H_{16}O_4$

II – Methomyl

* **Trade name:** Lannate (90%) white powder

* **Structure formula:**



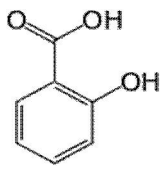
* **Chemical name:** S-methyl-N-{(methyl carbamoyl) oxy}thio acetimidate

* **Molecular formula:** $C_5H_{10}N_2O_2S$

III – Salicylic acid

* **Trade name:** Mediplast (95%) white crystalline powder

* **Structure formula:**



* **Chemical name:** 2-Hydroxy-benzoic acid

* **Molecular formula:** $C_7H_6O_3$

2.2. Survey of fungi associated with the molluscicide baits

Poisonous baits of three common molluscicides (metaldehyde 5%, methomyl 90% and salicylic acid 95%) were prepared with concentrations 1, 1.5 and 2% from the active ingredient of metaldehyde, methomyl and salicylic acid, respectively. Baits were prepared by adding the amount of each tested molluscicide required to obtain the appropriate concentration to 5 parts of sugar cane syrup and mixed with 94, 93.5 and 93 parts of wheat bran for the tested baits, respectively **(El-Okda, 1981)**. In the laboratory, fifty grams from each bait were introduced into plastic box (10 cm diameter). Four replicates were prepared for each concentration and other eight boxes were prepared by the same manner as a control, four boxes from them not containing any molluscicide and sugar cane syrup and the other four replicates prepared with sugar cane syrup for detect the ability of fungi for growing in the presence and absence of the sugar cane syrup as a main nutrient material of most fungi. The boxes were covered with muslin cloth and secured with rubber band. All boxes were examined daily for observed the appearance of fungi on baits. On the other hand, at April 2015 the same molluscicide baits were prepared another one time (100 g) on a plastic pieces put in a field of lettuce not infested with land snails at El- Ashraf village, Zagazig district, Sharkia Governorate, Egypt. This design for tested the effect of field conditions on the appearance of fungi on baits. Examination of baits was occurred daily for 21 days to record the fungi which associated with it. The mean of air temperature during the experiment period was about 18°C in the laboratory and 20.43°C in the field.

2.3. Enumeration and isolation of baits fungi

Fungi of baits were counted, the tips of fungal spores were removed and placed on mycological medium, that is potato dextrose agar (PDA: 300 g / l diced potatoes, 20 g / l dextrose and 20 g / l agar) supplemented with antibiotic benzyl penicillin (3mg / 100 ml) in a sterile petri dishes. The plates were incubated at 28°C for 7 days then examined **(Yin *et al*., 2012)**.

2.4. Identification of fungi

Fungal colonies which isolated from baits were identified at the Mycology Research & Plant Diseases Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Egypt. There were identified according to **Domsch and Anderson (2007)**.

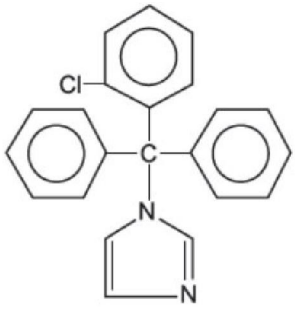
2.5. Antifungal agents

Four common antibiotics were selected for using as antifungal agents against the fungi which associated with baits in the laboratory and field. These antibiotics were obtained from El Ezaby Pharmacy, Cairo, Egypt.

A- Locasten

*** Trade name:** Locasten (each 20 ml solution contains 200 mg of clotrimazole).

*** Structure formula:**



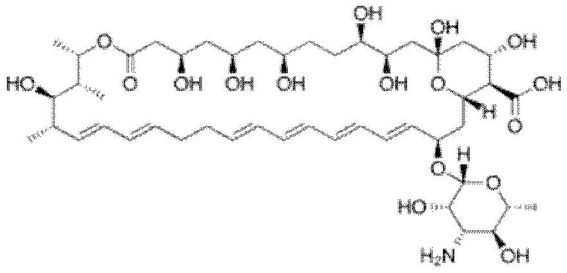
*** Chemical name:** Clotrimazole

*** Molecular formula:** $C_{22}H_{17}ClN_2$

B- Mycostatin

*** Trade name:** Mycostatin (each ml of suspension contains 100,000 units nystatin).

*** Structure formula:**



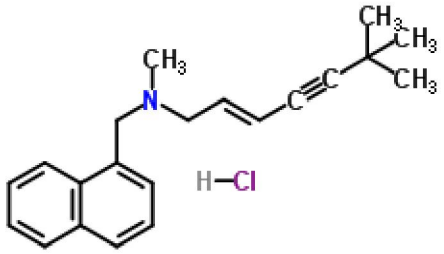
*** Chemical name:** Nystatin

*** Molecular formula:** $C_{47}H_{75}NO_{17}$

C- Terbin

*** Trade name:** Terbin (each ml of solution contains 1.0% w/w of terbinafine HCl)

*** Structure formula:**



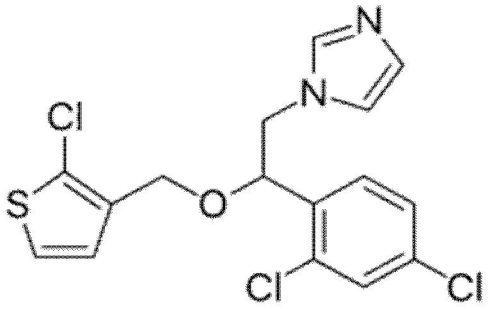
*** Chemical name:** Terbinafine

*** Molecular formula:** $C_{21}H_{25}N$

D- Trosyd

*** Trade name:** Trosyd (white crystalline powder 1%).

*** Structure formula:**



*** Chemical name:** Tioconazole

*** Molecular formula:** $C_{16}H_{13}N_2OSCl_3$

2.6. Minimum inhibitory concentrations test

This susceptibility test was performed against the fungi which associated with the laboratory and field baits. MIC values of the tested antibiotics were determined against these fungi. The concentrations (0.1, 0.2, 0.3 and 0.4%) of locasten, (0.01, 0.02, 0.04 and 0.1%) of mycostatin, (0.01, 0.015, 0.02 and 0.025%) of terbin and (0.04, 0.06, 0.08 and 0.1%) of trosyd were prepared in glass bottles (100 ml), each bottle contained 50 ml sterilized potato dextrose broth as diluent (Photo 1). The tested fungi inoculated in each bottle separately. Bottles without any antibiotic concentrations were prepared act as a control to ensure the ability of the organism to grow in the medium. All bottles were incubated at 27°C for 10 days and examined for observed the fungal growth **(Xu *et al.*, 2009)**.

* Minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that showed complete growth inhibition in liquid media.

Photo (1): Bottles contained 50 ml sterilized potato dextrose broth. 2.7. Survey of fungi associated with the molluscicide baits mixed with antibiotics

This experiment occurred for explore the actual ability of antibiotics in inhibition the growth of fungi on baits. The tested concentration of molluscicide baits; 1% of metaldehyde, 1.5% of methomyl and 2% of salicylic acid were prepared by adding the amount of each tested molluscicide required to obtain the wanted concentration to 5 parts of sugar cane syrup and mixed with 94, 93.5 and 93 parts of wheat bran. Control baits without molluscicide and sugar cane syrup (control A) and with sugar cane syrup (control B) was also prepared. Four plastic boxes (10 cm diameter) were prepared for each bait. The antibiotic values 0.4, 0.1, 0.025 and 0.1% of locasten, mycostatin, terbin and trosyd, respectively were prepared by dissolving in 50 ml sterilized distilled water then mixed individually with each bait in boxes. All boxes covered with muslin cloth and secured with rubber band. Baits examined daily for investigate the ability of antibiotics in preventing the fungal growth. At March 2016, the same baits were prepared another one time by the same manner on a plastic pieces placed in a lettuce field at El-Ashraf village, Zagazig district, Sharkia Governorate, Egypt. 0.4, 0.1, 0.025 and 0.1% of locasten, mycostatin, terbin and trosyd, respectively were mixed individually with each bait on the plastic pieces. Baits observed daily for 21 days for detect the effect of antibiotics on the inhibition of fungal growth on baits actually under the field conditions. Mean of temperature during the experiment period was about 17.22°C in the laboratory and 20.19°C in field.

*** Collection and rearing of snails**

Adults of *Monacha cartusiana* with shell diameter (12–13 mm) were collected from infested field cultivated with lettuce at El-Ashraf village, Zagazig district, Sharkia Governorate, Egypt. Snails were transferred directly to the laboratory and kept in a glass container (30 × 30 × 50 cm^3) contained moist clay soil and covered with muslin cloth for prevent snails escaping. The snails were supplied daily with fresh cabbage leaves for two weeks before any test for acclimatization **(Abd El-Aal, 2001)**.

2.8. Effect of the molluscicide baits prepared with and without antibiotics on the *M. cartusiana* snails under laboratory conditions

This test was carried out for investigate the effect of antibiotics on the toxicity of tested molluscicide baits against *M. cartusiana* snails in the laboratory. Metaldehyde, methomyl and salicylic acid baits at 1, 1.5 and 2% concentrations, respectively and control baits (without molluscicide and sugar cane syrup and with sugar cane syrup) were prepared exactly as mentioned in the previous experiments. On the other hand, the same baits were prepared again by the same manner with adding antibiotics which only prevented the fungal growth when mixed with each bait previously. Four replicates were prepared for each bait of the two groups (contains and not contains

antibiotics). Ten adults of *M. cartusiana* were introduced in each replicate. All boxes were closed with muslin cloth for prevent snails escaping. Mortality percentages of *M. cartusiana* snails were recorded after 1, 3, 7, 14 and 21 days in all boxes for make a comparison between the mortality which achieved by the molluscicides only and with antibiotics. Mortality percentages were corrected according to **Abbott's formula (1925)**.

2.9. Field application of the molluscicide baits prepared with and without mycostatin against *M. cartusiana* snails

This experiment was conducted for investigate the effect of mycostatin on the efficiency of tested molluscicides against the land snail *M. cartusiana* under field conditions. Mycostatin was selected in this trial specifically unlike the other antibiotics due to it's ability in preventing the fungal growth in the all tested baits previously. At April 2016, two lettuce fields heavy infested with *M. cartusiana* snails were chosen in this application. These fields located at El-Ashraf village, Zagazig district, Sharkia Governorate, Egypt. The first field includes the molluscicide baits metaldehyde, methomyl and salicylic acid at 1, 1.5 and 2%, respectively. These baits prepared by adding the amount of each tested molluscicide required to give the appropriate concentration to 5 parts of sugar cane syrup and mix with 94, 93.5 and 93 parts of wheat bran for the mentioned concentrations, respectively. In the second field, metaldehyde, methomyl and salicylic acid baits at the concentrations 1, 1.5 and 2%, respectively were prepared another one time by the same manner but with mixing with mycostatin. Mycostatin added to each bait as 10 ml / 50 ml water. Control baits were designed without any molluscicide or antibiotic. In the two fields, each bait prepared as 100 g introduced on plastic pieces put at different distance in the field. Each tested and control bait was replicated for three times and before placing each bait a live snails were counted and then recorded after 1, 3, 7, 14 and 21 days. Reduction percentages were calculated according to the formula of **Henderson and Tilton (1955)**. Mean of temperature during this application was about 25.80°C.

*In the all previous field experiments air temperature was obtained from Metereological station of Abu-Kabier, Sharkia Governorate, Egypt.

3. Results and Discussion

3.1. Total count of fungi isolated from the molluscicide baits under laboratory and field conditions

As cleared in Table (1) *Rhizopus stolonifer* was the most predominant fungus on metaldehyde, methomyl and salicylic acid baits in the laboratory. It was recorded the highest mean 46 colonies on the metaldehyde bait (Photo 2A) comparing with 9 and 7.7 colonies on methomyl and salicylic acid baits, respectively with a high frequency of occurrence (3H) on the three tested baits. This fungus appeared as mean 26 and 23.7 colonies on the bran bait without sugar cane syrup (control A) and with sugar cane syrup (control B), respectively. Under field conditions, the same fungus appeared on the metaldehyde bait only with mean 11 colonies with low frequency of occurrence (1 L) on the three tested baits compared with 6 and 37.7 colonies on the control baits (A) and (B), respectively. *Aspergillus flavus* was the second common fungus on the laboratory baits (Photo 2B) recorded it's highest mean of appearance 7.5 colonies on methomyl bait and gave high frequency of occurrence (3 H) on the three tested baits. This fungus recorded means 10.2 and 13.7 colonies on control (A) and (B) baits, respectively. In the field, it was appeared only on salicylic acid bait with mean 9.25 colonies and it doesn't appear at all in both control baits. *Aspergillus niger* grew only on salicylic acid bait at the laboratory (Photo 2C) with mean 5.2 colonies and low frequency of occurrence (1L) on the three tested baits and never appeared on the control baits. This fungus not appeared on any tested or control baits in the field. Under the laboratory conditions, *Fusarium subglutinans* gave mean of appearance 5.2 colonies on the metaldehyde bait. This fungus not recorded any appearance on the other tested and control baits and also not find on the all baits at the field. Little is known about the fungal population associated with baits as cleared during collection of information about this subject. Our results indicated that is a little fungi associated with salicylic acid bait under laboratory and field conditions. These findings were supported by **Abdel-Monaim (2013)** stated that salicylic acid considered as a very important chemical inducer prevent the appearance and growth of fungi specially, *Fusarium oxysporum*. At the same trend, **Ibrahim (2015)** reported that *Aspergillus* spp., *F. oxysporum* and *Fusarium moniliforme* highly reduced in presence of salicylic acid which greatly inhibiting the growth of these fungi. This acid may prove to be a superior antifungal agent can prevent the fungal growth under field conditions **(Prithiviraj *et al*., 1997)**. Pesticides causes inhibition of spore germination and vegetative development (mycelial growth), and they also reduce the viability of conidia **(Parvatha, 2016)**.

Table 1. Total mean count of fungal species associated with baits under laboratory and field conditions.

Isolates	Baits under laboratory conditions						Baits under field conditions					
	Metaldehyde	Methomyl	Salicylic acid	FO	Control (A)	Control (B)	Metaldehyde	Methomyl	Salicylic acid	FO	Control (A)	Control (B)
Aspergillus flavus	4.0 ± 1.4	7.5 ± 3.0	7.0 ± 1.0	3 H	10.2 ± 2.7	13.7 ± 2.0	-	-	9.25 ± 1.3	1 L	-	-
Aspergillus niger	-	-	5.2 ± 1.8	1 L	-	-	-	-	-	-	-	-
Fusarium subglutinans	5.2 ± 3.5	-	-	1 L	-	-	-	-	-	-	-	-
Rhizopus stolonifer	46.0 ± 3.4	9.0 ± 2.6	7.7 ± 0.4	3 H	26.0 ± 3.0	32.7 ± 2.5	11.0 ± 0.8	-	-	1 L	6.0 ± 2.4	37.7 ± 3.1

Control (A) = bran without sugar cane syrup Control (B) = bran with sugar cane syrup
H = high frequency (3 occurrence) L = low frequency (1 occurrence) FO = frequency of occurrence

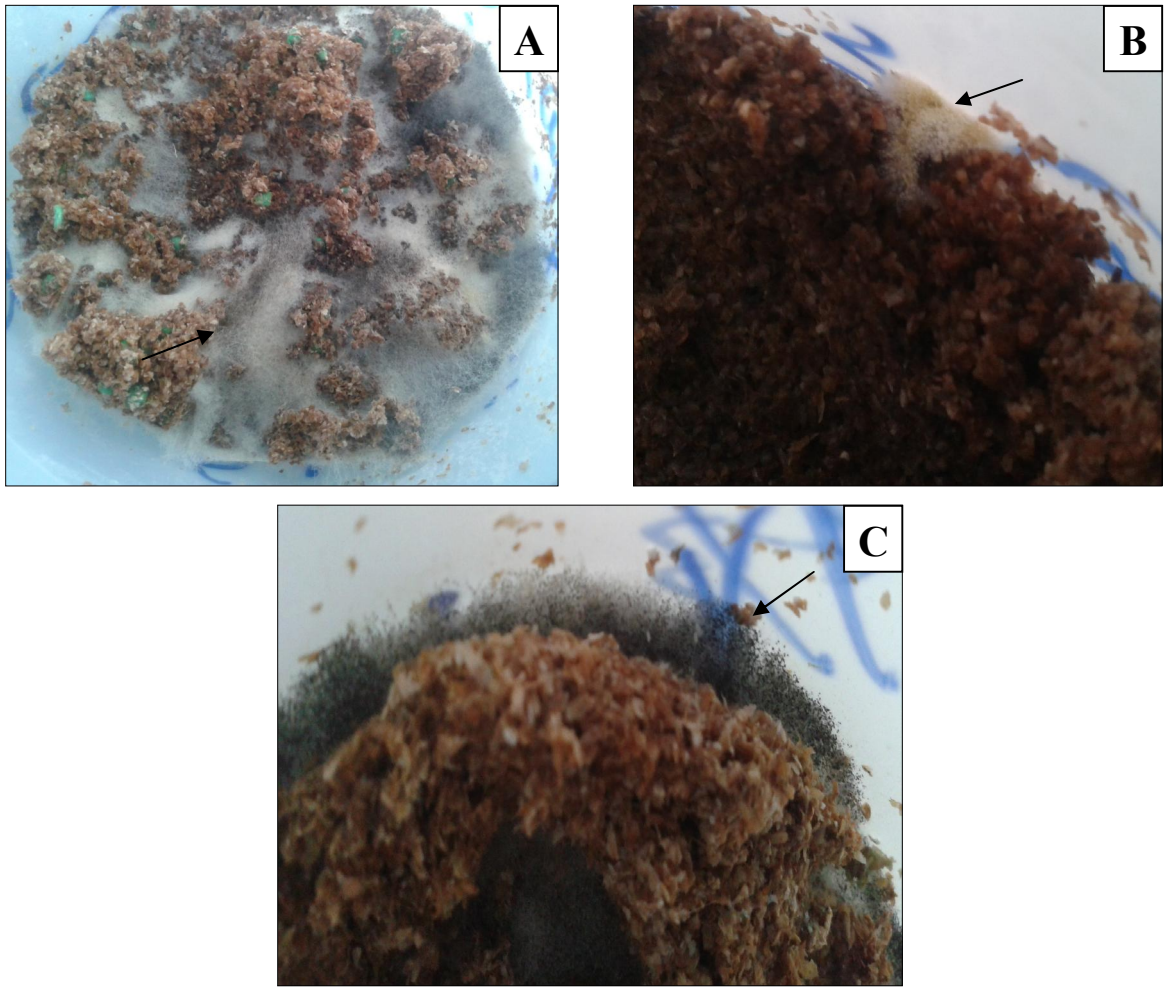


Photo (2 A – C): Fungal growth on baits. A, Metaldehyde bait infested with *R. stolonifer*. B, Methomyl bait infested with *A. flavus*. C, Salicylic acid bait infested with *A. niger*.

Moreover, **Jayaraman and Indira (2009)** showed that *A. flavus*, *A. niger* and *Penicillium* spp. were the toxigenic fungi which recorded in the rice bran samples. These fungi found in these samples less in number (1 one to 14 cfu / g) with an average of 2 cfu / g. 66.7 % of *A. flavus* strains isolated from the bran samples. Our results also agree with **Ioannis *et al.* (2011)** cleared that *Aspergillus* and *Mucor* were the common genera which causing the bran contamination. *A. niger*, *A. flavus* and *A. ochraceous* usually appeared on the bran samples. *A. niger* was the dominant fungus associated with the all samples

(Rai *et al*., 1990). Number of fungi was different from laboratory to field this may be due to the variation in temperature. This observation confirmed by **Morrondopelayo *et al*. (1992)** indicated that different climatic factors specially temperature had a greater influence on the fungal growth. Temperature is one of the main virulence factors affecting on fungi **(Rhodes, 1988)**. 25ºC was the optimum temperature for the growth of *A. niger*, *A. flavus* and *A. fumigatus* fungi **(Ved and Dharam, 1996)**.

3.2. Determination of minimum inhibitory concentrations (MIC) of antibiotics against tested fungi

Minimum inhibitory concentration (MIC) is the lowest concentration which prevent the growth of microorganism. The MIC values of tested antibiotics against *Aspergillus flavus*, *Aspergillus niger*, *Fusarium subglutinans* and *Rhizopus stolonifer* fungi which causing baits rot were recorded. As shown in Table (2) and Figure (1) locasten recorded the same MIC value 0.2% against *A. niger* and *R. stolonifer* fungi while it was gave 0.3 and 0.4% values against *A. flavus* and *F. subglutinans*, respectively. On the other hand, mycostatin recorded 0.1% against *A. flavus* and *A. niger* fungi, 0.02 and 0.04% against *F. subglutinans* and *R. stolonifer*, respectively. 0.01% was the MIC value which achieved by terbin against *A. flavus*, *A. niger* and *F. subglutinans* fungi but it gave 0.02% value against *R. stolonifer*. Trosyd recorded the same MIC value 0.1% against *A. niger* and *F. subglutinans* while it was achieved 0.04 and 0.08% values against *A. flavus* and *R. stolonifer*, respectively. These results were in harmony with those obtained by **Rania *et al*. (2012)** demonstrated that locasten had a highest inhibitory effect on the fungal species, *A. flavus* and *Alternaria alternate*. While, the other antibiotics as locatret and betnovate had a limited effect against these fungi. On the other hand, **Gonzalez *et al*. (1996)** recorded that mycostatin is a broad spectrum antifungal agent which is active *in vitro* and *in vivo* against *Aspergillus* spp. Thus, **Elzabeth *et al*. (1998)** showed that mycostatin possessed a fungistatic and fungicidal activities against 200 isolates of *Aspergillus* spp. MIC ranges at which 50 and 90% of the *Aspergillus* isolates were inhibited or killed. The MIC_{50}s of mycostatin for *A. flavus* and *Aspergillus fumigatus* were 1 and 8 Mg / ml.

Table 2. Minimum inhibitory concentration (MIC) of antibiotics against tested fungi

Tested fungi	MIC values of antibiotics (%)			
	Locasten	Mycostatin	Terbin	Trosyd
Aspergillus flavus	0.3	0.1	0.01	0.04
Aspergillus niger	0.2	0.1	0.01	0.1
Fusarium subglutinans	0.4	0.02	0.01	0.1
Rhizopus stolonifer	0.2	0.04	0.02	0.08

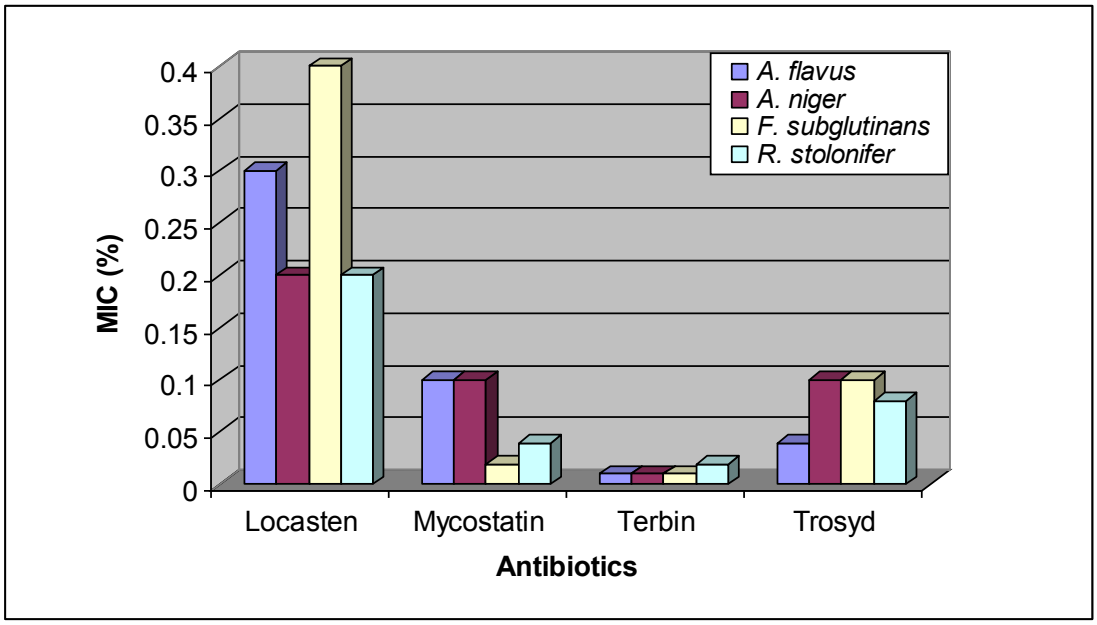


Fig. 1. Determination of minimum inhibitory concentration (MIC) of the antibiotics against tested fungi.

On the contrary, **Abd EL Aziz (2011)** reported that mycostatin has a low antifungal activity against *A. flavus* but it was showed a high inhibition activity against *A. niger*. In general, mycostatin is effective against the genus *Aspergillus* **(Wallace *et al.*, 1997)**. Terbinafine is extremely potent against dermatophytes with MIC values (0.04 + / - 0.23) as (mean + / - SEM) **(Gupta and Kohli, 2003)**. It has a high antagonistic activity against *A. flavus* and *Penicillium chrysogenum* fungi **(Singh and Singh, 2003)**. At the same direction, **Caroline *et al.* (2001)** illustrated that the *in vitro* activity of terbinafine was inferior against *A. fumigatus* with MIC value 19.03 Mg / ml and superior against *A. flavus* with 0.10 Mg / ml, *Aspergillus tereus* with 0.16 Mg / ml and *A. niger* with 0.19 Mg / ml. **Bueno *et al.* (2010)** evaluated the antifungal activity of terbinafine and voriconazole against 103 species of *Candida*, 10 species of *Fusarium*. Voriconazole was the most active agent against *Candida* species, whereas terbinafine and voriconazole were most potent against dermatophytes. The lowest MIC_S for dermatophytes obtained by terbinafine, followed by voriconazole. Our results also strongly confirmed by **Abdel-Mallek (1995)** reported that the antifungal drug trosyd (tioconazole) considered as a standard inhibitor of *A. flavus*, *A. niger*, *P. chrysogenum*, *Penicillium funiculesum* and *Rhizopus stolonifer*.

3.3. Total count of fungi associated with the molluscicide baits mixed with antibiotics

Table 3. Total mean count of fungal species associated with baits mixed with antibiotics under laboratory conditions

Isolates	Metaldehyde with antibiotics				Methomyl withantibiotics				Salicylic acid withantibiotics				FO	Control (A)				Control (B)			
	Lo	M	T	Tr	Lo	M	T	Tr	Lo	M	T	Tr		Lo	M	T	Tr	Lo	M	T	Tr
Aspergillus flavus	--	--	--	--	--	--	--	--	--	--	--	3.7 ± 1.4	1 L	3.0 ± 1.7	--	--	--	--	--	--	--
Aspergillus niger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium subglutinans	-	-	31.7 ± 2.0	37.0 ± 3.3	-	-	-	-	-	-	-	-	-1 L	-	-	34.2 ± 3.5	44.0 ± 2.7	-	-	47.7± 2.2	86.0 ± 1. 0
Rhizopus stolonifer							-				-	-		2.0 ± 0.8							

Lo = locasten M = mycostatin T = terbin Tr = trosyd L = low frequency (1 occurrence)

Photo (3): Methomyl bait without fungi due to addition of locasten.

Data in Table (3) showed that *A. flavus* and *R. stolonifer* were the two fungi which appeared on the baits mixed with antibiotics under the laboratory conditions. *A. flavus* appeared only on the salicylic acid bait mixed with trosyd by mean 3.7 colonies with low frequency of occurrence (1 L) on the three molluscicide baits. It was find on control (A) bait mixed with locasten as 3 colonies and never exist on the same bait when mixed with the other antibiotics and also not find on the control (B) bait with antibiotics. *R. stolonifer* appeared on metaldehyde bait especially when mixed with each of terbin and trosyd with means 31.7 and 37 colonies, respectively and low frequency of occurrence on the three tested baits. This fungus appeared also on the control (A) bait when mixed with locasten, terbin and trosyd by means 2, 34.2 and 44 colonies, respectively. It was appeared on the control (B) bait mixed with terbin and trosyd only by means 47.7 and 86 colonies, respectively. *A. niger* and *F. subglutinans* fungi do not appeared at any tested or control baits mixed with antibiotics. It worth to note that is no fungi associated with methomyl bait at mixing with the all antibiotics (Photo 3). Under field condition, there is no any fungi grow on the all baits. It seems clearly from our results that mycostatin was the only antibiotic which prevent the appearance of all fungal species on all baits under the laboratory and field conditions. These results were supported by **Richardson and Warnock (1997)** showed that mycostatin has been a useful antifungal agent since the 1950s. It was effective against a variety of fungal species **(Sevtap *et al.*, 2002)**. Our findings also elucidate that the number of fungi on the control baits mixed with antibiotics was more than its count on the molluscicide baits mixed with the same antibiotics. This observation means that antibiotics not only the main reason in preventing the fungal appearance but the molluscicides has also an antifungal behavior. These results were in agreement with those achieved by **Da Rocha *et al.* (2015)** reported that salicylic acid considered as a very important antifungal agent. Its antimicrobial effect is due mainly to its chemical structure rather than its capacity to acidify the solution **(Benigne *et al.*, 2002)**.

At the same trend, **Amborabe *et al.* (2002)** explained that antimicrobial effect of salicylic acid was shown to be related to its molecular structure. **Shokry (2013)** added that methomyl, salicylic acid and chlorpyrifos were the most potent inhibitor which

completely prevent the growth of *Paecilomyces lilacinus*, *Paecilomyces variotii* and *Trichoderma album* fungi.

3.4. Efficiency of the molluscicide baits with and without antibiotics against *M. cartusiana* snail under laboratory conditions

The results in Table (4) explained the effect of the antibiotics addition to the tested baits and the baits only without antibiotics on the mortality of *M. cartusiana* snails in the laboratory. The obtained data indicated that mortality percentages of snails by all treatments were increased gradually until reached to its maximum value at the end of experiment after 21 days. Metaldehyde recorded its highest effect when mixed with mycostatin with general mortality mean 19.3%. It was followed by 14.66 and 6.62% mortality achieved by metaldehyde without antibiotics and metaldehyde with locasten, respectively. On the other hand, methomyl recorded its highest toxic effect when mixed with terbin gave 64.66% mortality. While, methomyl with trosyd, methomyl with locasten, methomyl with mycostatin and methomyl without antibiotics caused 57.96, 54.62, 53.96 and 50.62% mortalities, respectively. Salicylic acid exhibited its highest mortality 51.28% when mixed with terbin followed by 12.64, 3.96 and 3.3% mortalities recorded by salicylic acid with mycostatin, salicylic acid with locasten and salicylic acid only, respectively. While the bran baits mixed with each of mycostatin, trosyd and locasten exhibited 14.6, 11.96 and 10.64% mortalities, respectively. That is no any mortality achieved in the control baits which prepared without antibiotics. Generally, the mixing of methomyl with terbin was the most effective bait against the *M. cartusiana* individuals. These findings were agree with those obtained by **Gust *et al.* (2013)** recorded that antibiotic group represent a risk for gastropods, which affected on the immunocompetence by increasing hemocyte count, ROS levels and phagocytosis and decreased intracellular thiol levels. It showed also a high bad effect on the gut of the land snail, *Helix aspersa* **(Ansart *et al.*, 2002)**. On the other hand, it cannot be denied that the molluscicides used have participated strongly in the mortality of snail individuals. This fact has been confirmed by **Hendawy *et al.* (2015)** indicated that methomyl has a high toxic effect against the terrestrial snail, *Monacha cantiana*. Moreover, methomyl baits was found to be more toxic than methiocarb against *E. vermiculata* snail. It was highly affected on the snail nervous system by reducing the acetylcholines terase activity **(Essawy *et al.*, 2009)**.

Table 4. Effect of the molluscicide baits with and without antibiotics on *M. cartusiana* snail under laboratory conditions

Baits	Mortality percentages at different periods (days)					General mean
	1	3	7	14	21	
Metaldehyde	3.30	10.00	20.00	20.00	20.00	14.66
Metaldehyde + locasten	0.00	3.30	6.60	6.60	16.60	6.62
Metaldehyde + mycostatin	13.30	13.30	23.30	23.30	23.30	19.30
Methomyl	13.30	33.30	56.60	73.30	76.60	50.62
Methomyl + locasten	23.30	30.00	66.60	76.60	76.60	54.62
Methomyl + mycostatin	20.00	30.00	66.60	76.60	76.60	53.96
Methomyl + terbin	40.00	50.00	70.00	80.00	83.30	64.66
Methomyl + trosyd	23.30	40.00	66.60	76.60	83.30	57.96
Salicylic acid	0.00	3.30	3.30	3.30	6.60	3.30
Salicylic acid + locasten	0.00	0.00	6.60	6.60	6.60	3.96
Salicylic acid + mycostatin	3.30	6.60	10.00	20.00	23.33	12.64
Salicylic acid + terbin	36.60	36.60	56.60	63.33	63.33	51.29
Bran	0.00	0.00	0.00	0.00	0.00	0.00
Bran + locasten	3.30	3.30	13.30	13.30	20.00	10.64
Bran + mycostatin	3.30	10.00	20.00	20.00	20.00	14.66
Bran + trosyd	3.30	10.00	13.30	16.60	16.60	11.96

Shilpa *et al.* (2014) evaluated the toxic effect of the poison baits of metaldehyde 2.5%; copper sulphate and bleaching powder against the land snail, *Cryptozona semirugata*. Metaldehyde achieved the highest effect by recording 94.83% mortality followed by copper sulphate and bleaching powder causing 72.22 and 70.08% mortality of snails, respectively. Acetyl salicylic acid was effective against adults of *E. vermiculata* and *M. obstructa*. It's LC_{50} and LC_{90} values were 0.67 & 4.0 and 0.3 & 1.6 mg / cm^2 against the two snail species, respectively **(Ahmed, 2008)**. At the same trend, **Shokry (2013)** stated that salicylic acid at concentration 1% caused 100% mortality of *M. cartusiana* adult snails after one day of treatment. Whereas, methomyl exhibited the same effect at the concentration 1% also against the same

snail species after 3 days of treatment under the laboratory conditions.

3.5. Effect of the molluscicide baits with and without mycostatin on *M. cartusiana* snail under field conditions

The molluscicide baits prepared alone and at mixing with mycostatin were evaluated against *M. cartusiana* snails under field conditions. As cleared in Table (5) metaldehyde recorded the highest initial effect 51.88% reduction followed by methomyl and salicylic acid which recorded 38.97 and 33.38% reduction, respectively. While, when the same molluscicide baits mixed with mycostatin salicylic acid with mycostatin achieved the highest initial effect 56.76% followed by metaldehyde with mycostatin and methomyl with mycostatin with reduction 39.1 and 37.35%, respectively. These findings means that mycostatin has a high ability in increasing the toxic effect of salicylic acid bait against the snail individuals in comparing with the other two tested baits. At the same trend, metaldehyde exhibited also the highest residual effect with reduction equal 50.39% followed by salicylic acid and methomyl which recorded 43.82 and 37.07% reduction, respectively. But with regard to the mixing of mycostatin to these baits, metaldehyde with mycostatin was recorded the highest residual effect 49.15% reduction followed by 43.95 and 43.34% which showed by methomyl with mycostatin and salicylic acid with mycostatin, respectively. These results clearly indicated that mycostatin was increased the residual effect of methomyl against snails. It worth to mention that during the baits examination it was strongly observed that is a large number of *M. cartusiana* snails were attracted to the baits which contain of mycostatin. So this antibiotic can be considered as a promising molluscicide and attractive material which can add to the different poisonous baits for control land snails in the future studies. Our results were in agreement with those obtained by **El-Akhrasy (2010)** indicated that metaldehyde exhibited the highest reduction 55% of *M. cartusiana* snails at the concentration 5%. Followed by 51.4, 47.6, 45.4 and 42.8% reduction at 4, 3, 2 and 1% concentrations, respectively under the field conditions. At the same trend, **Hegab *et al.* (2006)** reported that metaldehyde gave 100% reduction of *M. cartusiana* snails in the field after 7 days of application.

Table 5. Reduction of *M. cartusiana* snails by the molluscicide baits with and without mycostatin under field conditions.

Baits	Reduction percentages at different period (days)							General mean
	1	3	Initial effect	7	14	21	Residual effect	
Metaldehyde	52.23	51.53	51.88	46.26	65.96	38.96	50.39	50.98
Metaldehyde + mycostatin	48.44	29.76	39.1	55.92	37.18	54.36	49.15	45.13
Methomyl	37.90	40.05	38.97	35.42	28.02	47.79	37.07	37.83
Methomyl + mycostatin	42.85	31.86	37.35	42.85	60.43	28.57	43.95	41.31
Salicylic acid	27.97	38.80	33.38	37.64	44.97	48.86	43.82	39.64
Salicylic acid + mycostatin	64.81	48.71	56.76	42.37	40.99	46.66	43.34	48.70

On the other hand, methomyl recorded 88.98 and 94.58% reduction of *M. cartusiana* snails under the field conditions after 3 and 21 days of treatment, respectively **(Ismail *et al.*, 2010)**. This molluscicide achieved 32.5% reduction as initial effect and 34.5% reduction as residual effect against the same snail species in the field **(Ismail *et al.*, 2015)**. In our results, the mixing of mycostatin with salicylic acid and methomyl gave a high toxic effect against *M. cartusiana* snails. This may due to the chemical structure of these materials. At this direction, **Radwan *et al.* (1992)** showed that the toxic effect of molluscicide against snails was strongly related to the basis of their chemical structure. Methomyl containing N. methyl group with an additional carbamyl moiety had a highest toxic activity against snails.

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