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Microcin as an Insecticidal Antibiotic Against *Trogoderma Granarium* Everts (Coleoptera: Dermestidae)

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Abstract
Microcin was extracted and formulated from *Citrobacter* spp. isolated from a healthy human. The results showed that there is a deleterious effect on the survival of khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), which is a major pest of stored grains. Microcin has been used as Bio-insecticides to overcome the disadvantages of chemical pesticides. Biological control is one of the main components of IPM in recent years. This laboratory study was to evaluate the insecticidal activity of antibiotic microcin. The oral delivery of microcin caused a dose-dependent effect, with 100% mortality of adult at concentrations of 62.5mg/ml after 10 days. Moreover, the toxicity of microcin was tested against the larvae, *T. granarium* which caused 30% mortality at 500 mg/ml after 10 days. Larvae were more tolerant than adults to the microcin. This study demonstrates the potential power of using microcin as a new approach for the control of *T. granarium*. Also, the results of this study showed that the germination of wheat grains treated with different concentrations (62.5, 125, 250 and 500 mg/ml) of microcin did not affect by the microcin treatment.
Key word: microcin, *Citrobacter* spp., antibiotic, insecticide, *T. granarium*

INTRODUCTION

The khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), is an economically important pest which mainly grows in stored grains and other stored food products(Lindgren et al., 1955; Scheff et al., 2020).That mainly infests cereal grains and pulses, including several plant and animal materials, particularly seeds ofwheat (Kavallieratos et al., 2019). It is native to India, but is currently distributed in Asia, Africa, the Middle East, and a few European countries (Eliopoulos, 2013).There are some factors that help this insect to spread and distribute; first, it can infest a wide variety of products and second, larvae have the ability to undergo facultative diapause, but not all stages enter this status (Denlinger, 1991). Diapausing *T. granarium* mature larvae have an increased tolerance to an extreme condition such as low and high temperature, inadequate food and insecticides (Bell, 1994). Those larvae can remain in diapause for more than three years before pupating and emerging adults(Nair and Desai, 1972). Diapausing larvae of the Khapra beetle are controlled by multiple genes and not all strains have obligate diapause (Day and White, 2016; Nair and Desai, 1973). Several studies have revealed that pyrethroids have a role in controlling of adults, however, larvae are more tolerant than adults(Chadda et al., 2004). Fumigation with

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methyl bromide has been widely used in the past to control *T. granarium.* However, this fumigant is an ozone depleting material, therefore, it has been banned in most countries (Bernstein, 2001; Fields and White, 2002; Hasan et al., 2020).

Currently, only the organophosphate Malathion and the Pyrethroid Permethrin are reported by the USDA-Animal Plant Health and Inspection Service (APHIS) for the control of *T. granarium* (Stibick, 2007). Numerous studies have reported a Malathion resistance world-wide in stored product insects during the last few decades (Collins, 2006; Wilches et al., 2019). For these reasons, alternative methods of this insect control should be developed.

In this study, we report the development of a unique method to control *T. granarium*by using microcin.

Microcin is bacteriocin with low molecular – weight (10000 Da) used as antimicrobial peptides secreted by members of the Enterobacteriaceae family and are involved in microbial competition within the intestinal tract(Fields and White, 2002). They are generally synthesized by the ribosomal pathway. The broad variety of microcin structures results in a diversity of mechanisms of action, such as the inhibition of vital enzymatic functions and damage to the inner membrane(Duquesne et al., 2007). In addition, target intracellular enzymes responsible for DNA/RNA structure or synthesis (Morin et al., 2011).In this study, microcin was usedto investigate the insecticidal potential of the targeting *T. granarium*, the susceptibility of larval instars and adults to microcin; and the effect of microcin on germination wheat grain.

MATERIAL AND METHODS

Insects: A culture of *T. granarium* was provided by Entomology Laboratory in the College of Agriculture-Univ. of Kufa. The culture was maintained on wheat grains (*Triticum aestivum* L.), containing 5% brewer's yeast, at 30±2°C and 70% ±5 relative humidity.

Bacteria; *Citrobacter* spp. was isolated from stool samples from healthy then the diagnosis of bacterial isolates was performed using selective media (CHROM agar).

Isolation and Purification of Microcin: The method described by (Piskunova, 2017) with some modification was carried out for isolation of microcin from *Citrobacter* spp. The bacterial isolates were cultured on M63 minimum media (Elbing and Brent, 2002) and incubated for 18 hr at 37˚C. The cells were removed by centrifuging and the culture medium was loaded onto a Syringe filter. The Syringe filter was firstly washed with distilled water and then with 0.1% aqueous trifluoroacetic acid, then the bounded materials were eluted stepwise with 10% acetonitrile in 0.1% trifluoroacetic acid. The 10% acetonitrile fraction was concentrated by lyophilization. The high performance liquid chromatography (HPLC) in the laboratory of the Ministry of Science and Technology was achieved by preparation of C18-ODS colum (25 cm*4.6 mm) using mobile phase that consists of CH_3CN: D.W: Formic acid with a percentage of 60:30:10 the flow rates of the solution was 1m/min and Detector. Flo, Ex= 360nm, Em=450nm (Figure1).

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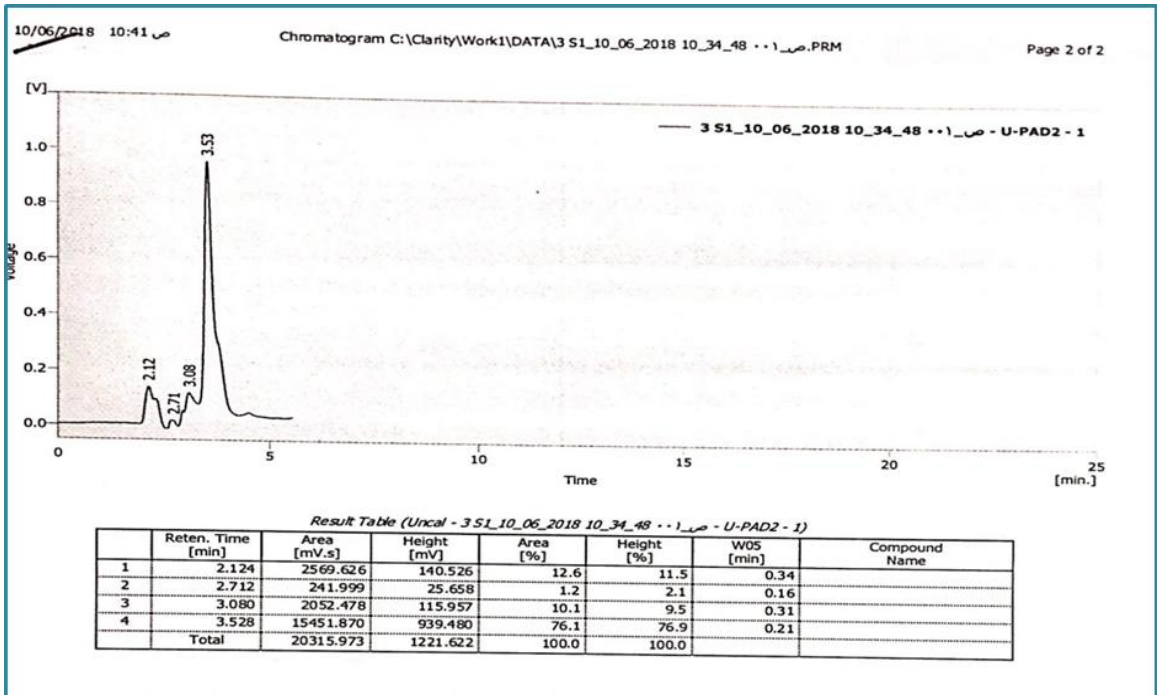

Result Table (Uncal - 3 S1_10_06_2018 10_34_48 ••_ص - U-PAD2 - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.124	2569.626	140.526	12.6	11.5	0.34	
2	2.712	241.999	25.658	1.2	2.1	0.16	
3	3.080	2052.478	115.957	10.1	9.5	0.31	
4	3.528	15451.870	939.480	76.1	76.9	0.21	
	Total	20315.973	1221.622	100.0	100.0		

Figure1.HPLC chart for purification of microcin from *Citrobacter* spp.

Efficiency of the Tested microcin on Adults and Larvae of *T. granarium* Beetle: Wheat grains were treated with the tested microcin for protection against larvae (~1–2 weeks old), and adults (~1–2days old) of *T. granarium* at concentration levels of 62.5, 125, 250 and 500 mg/ml. Each concentration was applied in four replicates each consisting of 10 insects. The treatment of wheat grains was carried out by dipping wheat grains in the aqueous solution of microcin at the tested concentration levels twice consecutively for 5 seconds and subsequently spread on top of plastic sheets to dry for 90 min. The control treatment was carried using water only and replicated four times. Then, 10 adults and larvae of *T. granarium* were transferred to treated wheat grains which were put in an 85×75 mm plastic jar and kept at 30±2°C and 70%±5 r.h, according to the method described by Kestenholz et al. (2007). The glass jars were covered with cotton cloths held on with rubber bands. The number of dead adults and larvae in each jar was counted every two days for 10 days and the percentage of insect mortality was recorded.

Germination seed: The wheat seeds were treated with different concentrations (62.5, 125, 250 and 500 mg/ml) of microcin. The treated seeds were then dried to prevent infestation. Ten selected seeds from each treatment were placed on filter papers inside a 9 cm in diameter Petri-dish for germination test with four replications for each treatment. The well germinated seeds in each Petri-dish were counted after 10 days of treatment and expressed in percentages described by Mamun and Shahjahan (2011).

Statistical analysis: Insect mortality was analyzed using Kaplan-Meier survival analysis and the Sigma Plot program, (version 12.5, Systat. Software Inc., San Jose, USA) and insect cumulative mortality was corrected according to Abbott's formula (Abbott, 1987). Consumption and germination results were analyzed treatment with one-way ANOVA

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followed by the Tukey's test to compare differences in the effect of various concentrations of microcin (Minitab, State College, PA, USA).

RESULTS AND DISCUSSION

The present study evaluated the efficacy of using microcin as an antibiotic extracted from *Citrobacter* spp. against*T.granarium* insects. The results indicated that there is insecticidal activity against insect's larval and adult instars. In addition, results showed that the mortality rates of adults were significantly higher than fifth instar larvae.

Demonstration of insecticidal activity of Microcin against adult of *T. granarium:* Experiments were carried out to test the efficacy of microcin against *T. granarium*. Adults were fed with drenching wheat grains at different concentrations (62.5, 125, 250 and 500 mg/ml) of microcin with Four biological replicates, 10 insects/rep. for each dose. Abbott's formula was used to correct survival data relative to control (untreated control).

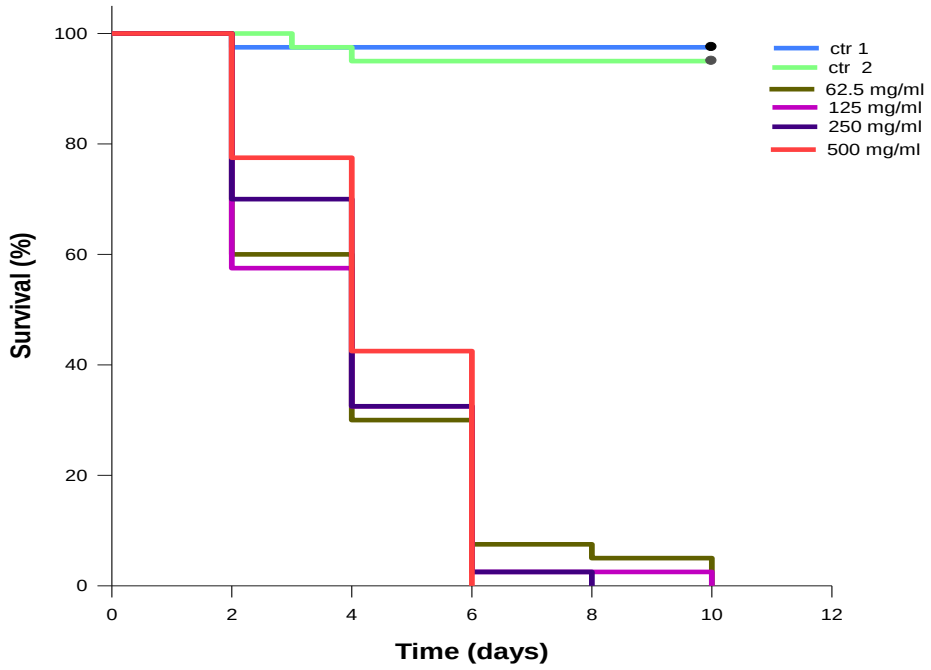

Figure 2. Survival of *T. granarium* adults fed with microcin ($p<0.001$; n= 40 adult/treatment, as shown by Kaplan-Meier survival analysis). Control 1= adults fed on wheat grains, control 2 = adults fed on water socked wheat grains.

It can be seen from the Fig.2 that there was a clear decrease in survival (high mortality) when using microcin (an antibiotic prepared from *enterobacterace*) which gave the most effective and economical control of *T. granarium* compared with control 1 and 2 in the 10 days after treatment. The mortality of control groups was 2%, 5% for controls 1 and 2 respectively, were observed over the assay period, with no significant differences between the control groups. In contrast the mortality with microcin (Figure 2) was100% at concentrations 62.5, 125, 250 and 500 mg/ml after 10 days which did not differ among each other ($p >0.001$). The

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LC50 of the adult fed on microcin was 2.38 mg/ml on day 6. The present study confirmed that microcin can be used in the control of *T.granarium.* A possible explanation for microcin actions may be attributed to firstly; its ability to act as blocking vital functions in the target organism, inhibiting aspartyl-tRNA synthetase which is necessary for protein synthesis and inhibiting the DNA gyrase, resulting in double DNA breaks, secondly; microcin may block the secondary RNA polymerase channel, impairing transcription and acting on cytochromes to inhibit cellular respiration, or the ATP (Baquero et al., 2019; Etayash et al., 2016; Simons et al., 2020).

The present findings are inconsistent with findings reported by Heisey et al. (1988), who found that *Streptomyces griseus* bacteria produce a valinomycin, as an insecticidal and acaricidal antibiotic, where the LC_{50} values of mosquito larvae and Mexican bean beetle larvae were 3 and 35 ppm respectively. Another study has shown that 25 ppm of liuyangmycin (an antibiotic prepared from *Streptomyces griseolus*) resulted in 71.1% and 83% mortality to female adults and nymphs of *Polyphagotarsonemus latus on* green piper after 5 days respectively (Xie et al., 1992). Liu et al. (2008) also reported that the insecticidal activity of antibiotic quinomycin Against *Spodoptera exigua*, *Plutella xylostella* and *Aphis glycines* with LC50 values of 83.9 mg/ml, 144.2 mg/ml, and 246.6 mg/ml, respectively.

Demonstration of insecticidal activity of Microcin against 5th instar larvae of *T. granarium*

The results of this study (Figure 3) show that fifth instar larvae fed on microcin soaked wheat grains at concentrations 62.5, 125, 250 and 500 mg/ml caused 17%, 22%, 25% and 30% mortality, 10 days post treatment, respectively. No larval mortality occurred in control1, and only 2 % for control 2 but there was no significant difference (P<0.001) in mortality between two controls. Khapra-beetle larvae were more resistant to wheat treated with microcin than adults. This may be attributed to the tendency of larvae to enter diapause (Honey et al., 2017). This has increased the larvae resistance to them making it especially difficult to control. Larvae deeper in diapause have a slower metabolism, higher production of stress proteins, and as a result, increased tolerance to extreme developed conditions (Fields et al., 2011; Lee et al., 1998). Burges (1963) found that larvae of *T. granarium* enter diapause at 25 °C in crowded cultures (25 larvae/g diet). *T. granarium* insect is one of the most serious pests of agriculture throughout the world (Ahmedani et al., 2007; Singh et al., 2017). This importance is due to its wide host range and ability to diapause in the larval stage, this makes khapra beetle to be a global pest. Some studies have revealed that adults of this insect that have passed through diapause are larger and have higher fecundity than their non-diapausing counterparts, which promotes a rapid population growth post-diapause (Gothi et al., 1984; Karnavar, 1972, 1984).

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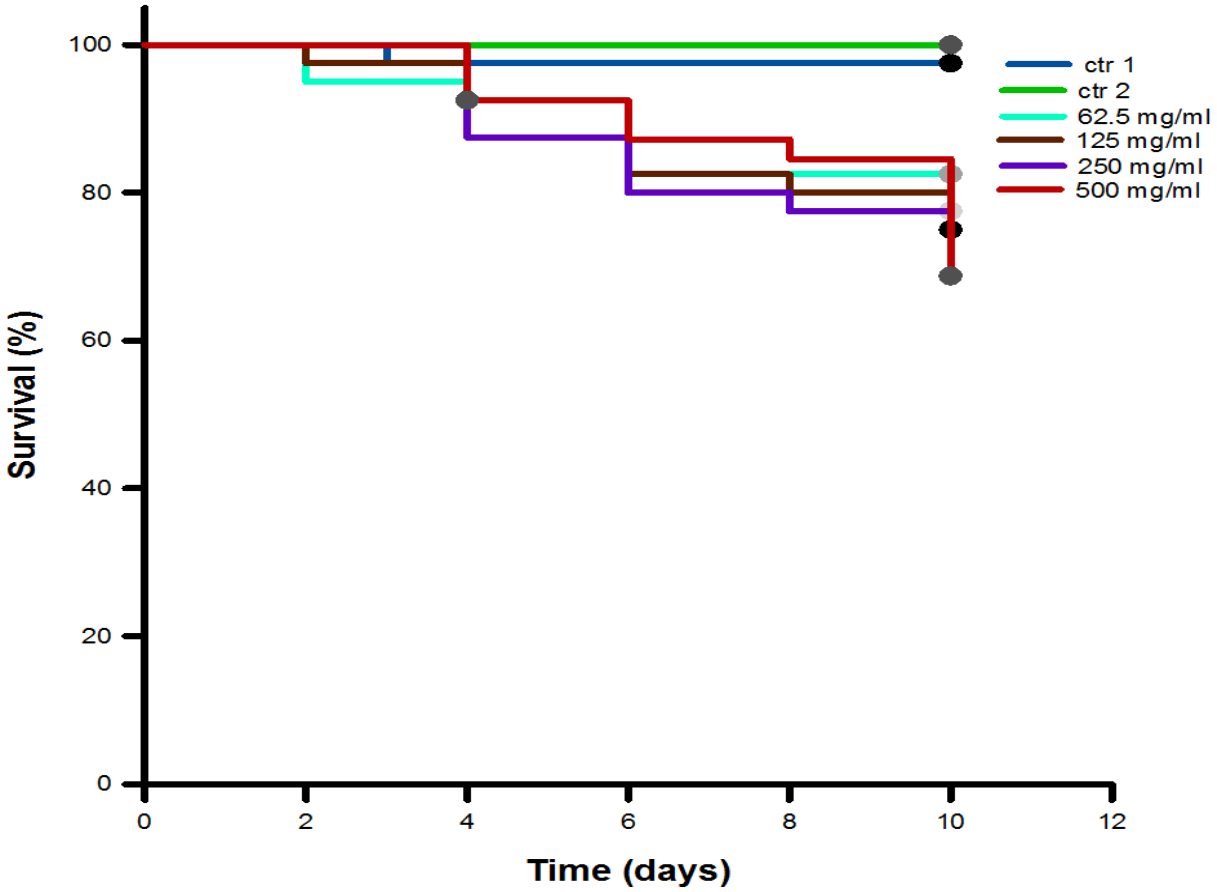

Figure 3. Survival of *T. granarium*larvae fed with microcin ($p<0.001$; n= 40 larvae/treatment, as shown by Kaplan-Meier survival analysis). Control 1= larvae fed on wheat grains, control 2 = larvae fed on drenching wheat grains with water.

Consumption

For the fifth instar larvae that survived all 21 days of the experiment, there were slightly consumed in diet across studied treatments (Figure 5). Statistical analysis revealed no significant difference between treatments and control 2.

Germination

The results of this study (Table1) show that wheat grain treated with microcin at concentrations 62.5, 125, 250 and 500 mg/ml resulted in germination rates of 97%, 93%, 90% and 97%, respectively after 10 days, with no significant differences ($P>0.001$) between treated seeds and control. Findings of his study demonstrated that microcin can be used to protect wheat grain without adverse effects on seeds germination.

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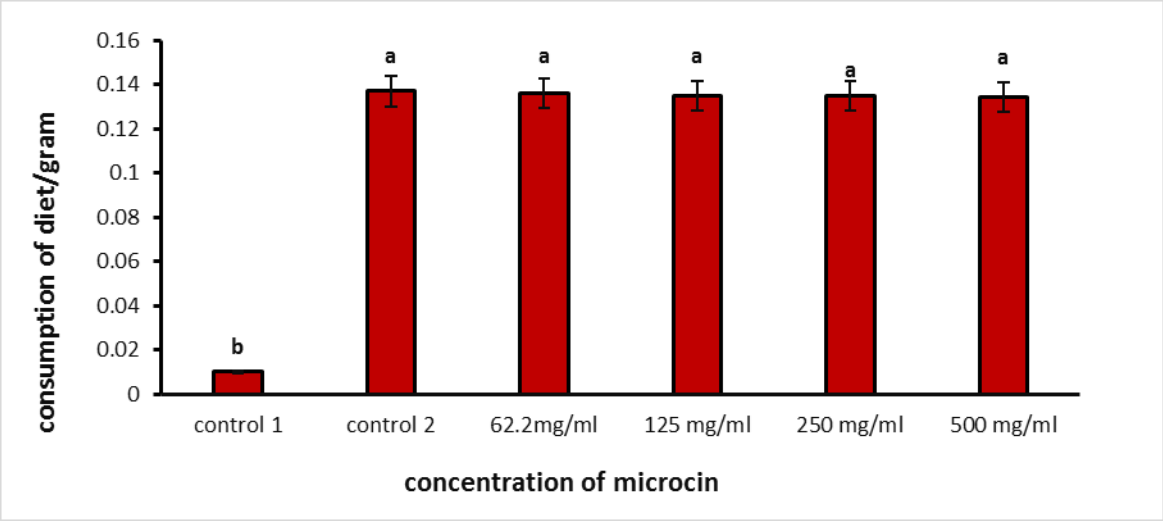

Figure 4: Daily consumption of *T. granarium*larvae fed with drenching wheat grains at different concentrations of microcin. Different letters indicate a significant difference between groups ($P < 0.05$, one-way ANOVA, post- hoc Tukey test). n= 50 larva/treatment. Control 1= untreated grains, control 2 = larvae fed with drenching wheat grains at water.

Table 1. Effect of microcin on seed germination

Treatment	Germination
Control	97%[a]
62.5 mg/ml	97%[a]
125 mg/ml	93%[a]
250 mg/ml	90%[a]
500 mg/ml	97%[a]

CONCLUSION

This study is considered the first step toward more investigation about using microcin as insecticide alternative for controlling the stored product pests. This will help to reduce the environmental pollution and the adverse effect on human health resulted from using insecticides.

REFERENCE

[1]. Abbott W S (1987) A method of computing the effectiveness of an insecticide. Journal of the American Mosquito Control Association *3*, 302-303.

[2]. Ahmedani M S, Khaliq A, Tariq M, Anwar M and Naz S (2007) Khapra beetle (Trogoderma granarium Everts): A serious threat to food security and safety. Pak J Agri Sci *44*, 481-493.

[3]. Baquero F, Bravo-Vazquez D A, Lanza V F, Baquero M-R and Del Campo R (2019) Microcins, peptide antimicrobials from Enterobacteriaceae in the eco-active intestinal chemosphere. Frontiers in Microbiology *10*, 2261.

[4]. Bell C (1994) A review of diapause in stored-product insects. Journal of Stored Products Research *30*, 99-120.
[5]. Bernstein S (2001) The compromise of liberal environmentalism (Columbia University Press).
[6]. Burges H (1963) Studies on the dermestid beetle Trogoderma granarium Everts. VI.—Factors inducing diapause. Bulletin of Entomological Research *54*, 571-587.
[7]. Chadda I, Jayraj K and Dhuri A (2004) New method of phosphine and carbon dioxide application and its optimization. Paper presented at: A paper presented at "International Conference on Controlled Atmosphere and Fumigation in Stored Products" held in Gold Coast, Australia on.
[8]. Collins P (2006) Resistance to chemical treatments in insect pests of stored grain and its management. Paper presented at: Proceedings of the 9th International Working Conference on Stored Product Protection.
[9]. Day C and White B (2016) Khapra beetle, Trogoderma granarium interceptions and eradications in Australia and around the world.
[10]. Denlinger D L (1991) Relationship between cold hardiness and diapause. In Insects at low temperature (Springer), pp. 174-198.
[11]. Duquesne S, Destoumieux-Garzón D, Peduzzi J and Rebuffat S (2007) Microcins, gene-encoded antibacterial peptides from enterobacteria. Natural product reports *24*, 708-734.
[12]. Elbing K and Brent R (2002) Media preparation and bacteriological tools. Current Protocols in Molecular Biology *59*, 1.1. 1-1.1. 7.
[13]. Eliopoulos P (2013) New approaches for tackling the khapra beetle. CAB reviews *8*, 1-13.
[14]. Etayash H, Azmi S, Dangeti R and Kaur K (2016) Peptide bacteriocins-structure activity relationships. Current Topics in Medicinal Chemistry *16*, 220-241.
[15]. Fields, P., Subramanyam, B., and Hulasare, R. (2011). Stored product protection.
[16]. Fields P G and White N D (2002) Alternatives to methyl bromide treatments for stored-product and quarantine insects. Annual review of entomology *47*, 331-359.
[17]. Gothi, K., Tamhankar, A., and Rahalkar, G. (1984). Influence of larval diapause on male response to female sex pheromone in Trogoderma granarium, Everts (Coleoptera: Dermestidae). Journal of stored products research *20*, 65-69.
[18]. Hasan M M, Aikins M J, Schilling M W and Phillips T W (2020) Comparison of Methyl Bromide and Phosphine for Fumigation of Necrobia rufipes (Coleoptera: Cleridae) and Tyrophagus putrescentiae (Sarcoptiformes: Acaridae), Pests of High-Value Stored Products. Journal of Economic Entomology *113*, 1008-1014.
[19]. Heisey R M, Huang J, Mishra S K, Keller J E, Miller J R, Putnam A R and D'Silva T D (1988) Production of valinomycin, an insecticidal antibiotic, by Streptomyces griseus var. flexipertum var. nov. Journal of Agricultural and Food Chemistry *36*, 1283-1286.
[20]. Honey S F, Bajwa B, Mazhar M S and Wakil W (2017) Trogoderma granarium (everts)(Coleoptera: Dermestidae), an alarming threat to rice supply chain in Pakistan. International Journal of Entomological Research *5*, 23-31.
[21]. James R, Lazdunski C and Pattus F (2013) Bacteriocins, microcins and lantibiotics, Vol 65 (Springer Science & Business Media).

[22]. Karnavar G (1972) Mating behaviour and fecundity in Trogoderma granarium (Coleoptera: Dermestidae). Journal of Stored products research *8*, 65-69.
[23]. Karnavar G (1984) Studies of the influence of larval treatment on the fecundity of the stored grain pest, Trogoderma granarium Everts. Journal of the Entomological Society of Southern Africa *47*, 67-73.
[24]. Kavallieratos N G, Athanassiou C G, Boukouvala M C and Tsekos G T (2019) Influence of different non-grain commodities on the population growth of Trogoderma granarium Everts (Coleoptera: Dermestidae). Journal of Stored Products Research *81*, 31-39.
[25]. Kestenholz C, Stevenson P C and Belmain S R (2007) Comparative study of field and laboratory evaluations of the ethnobotanical Cassia sophera L.(Leguminosae) for bioactivity against the storage pests Callosobruchus maculatus (F.) (Coleoptera: Bruchidae) and Sitophilus oryzae (L.)(Coleoptera: Curculionidae). Journal of Stored Products Research *43*, 79-86.
[26]. Lee K-Y, Hiremath S and Denlinger D L (1998) Expression of actin in the central nervous system is switched off during diapause in the gypsy moth, Lymantria dispar. Journal of Insect Physiology *44*, 221-226.
[27]. Lindgren D, Vincent L, and Krohne H (1955) The khapra beetle, Trogoderma granarium Everts. Hilgardia *24*, 1-36.
[28]. Liu H, Qin S, Wang Y, Li W and Zhang J (2008) Insecticidal action of Quinomycin A from Streptomyces sp. KN-0647, isolated from a forest soil. World Journal of Microbiology and Biotechnology *24*, 2243-2248.
[29]. Mamun M and Shahjahan M (2011) Effect of some indigenous plant extracts on the germination of wheat seeds. Bangladesh Journal of Agricultural Research *36*, 733-739.
[30]. Morin N, Lanneluc I, Connil N, Cottenceau M, Pons A M and Sablé S (2011) Mechanism of bactericidal activity of microcin L in Escherichia coli and Salmonella enterica. Antimicrobial agents and chemotherapy *55*, 997-1007.
[31]. Nair K and Desai A (1972) Some new findings on factors inducing diapause in Trogoderma granarium Everts (Coleoptera, Dermestidae). Journal of stored products research *8*, 27-54.
[32]. Nair K and Desai A (1973) Studies on the isolation of diapause and non-diapause strains of Trogoderma granarium Everts (Coleoptera, Dermestidae). Journal of Stored Products Research *9*, 181-188.
[33]. Piskunova I (2017) Maturation and Functional Analysis of Microcin C-Like Compounds (Doctoral Thesis, Moscow).
[34]. Scheff D S, Arthur F H, Myers S W and Domingue M J (2020) Efficacy Determination of Commercial Deltamethrin-Treated Storage Bags on Trogoderma granarium Everts Adults and Larvae. Agronomy *10*, 814.
[35]. Simons A, Alhanout K and Duval R E (2020) Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. Microorganisms *8*, 639.
[36]. Singh A, Chand P, Vishwakarma R and Singh C K (2017) Khapra beetle (Trogoderma granarium Everts): A food security threat. Bulletin of Environment, Pharmacology and Life Sciences *6*, 1-6.

[37]. Stibick R (2007) New pest response guidelines. USDA-APHIS-PPQEmergency and Domestic Programs, Khapra Beetle.
[38]. Wilches D, Laird R, Floate K and Fields P (2019) Control of Trogoderma granarium (Coleoptera: Dermestidae) using high temperatures. Journal of economic entomology *112*, 963-968.
[39]. Xie M, Cheng H and Zhao J (1992) Use of liuyangmycin to control yellow mite, Polyphagotarsonemus latus (Acari: Tarsonemidae) infesting green pepper. Chinese Journal of Biological Control *8*, 29-32.