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Original Article

Effects of combined treatment of cadmium and oxytetracycline on the terrestrial isopod *Porcellio laevis*

Efeitos do tratamento combinado de cádmio e oxitetraciclina no isópode terrestre *Porcellio laevis*

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Abstract

The influence of pharmaceutical residues and heavy metals on living organisms has received global attention. The present study assessed the interactive effect of antibiotic residues and heavy metals in soil, as contaminated food with cadmium (Cd) and oxytetracycline (OTC) on the isopod *Porcellio laevis*. It was fed on fresh plant leaves contaminated with different concentrations of cadmium, Cd+OTC1000 ppm, Cd+OTC2000 ppm and Cd+OTC3000 ppm for 4 weeks. The changes in the feeding patterns, protein, lipid peroxidation (LPO), catalase activity (CAT), and total free amino acids (TFAA) were recorded. There were significant differences in the obtained results where Cd reduced the egestion ratio (ER) however, OTC enhanced this ratio. Biochemical analysis illustrated that combination between OTC and Cd inhibits the toxic effects of Cd at low concentration (1000 ppm), while at high concentration (3000 ppm) raise the toxicity. Detailed studies are required for further understanding of the interaction between OTC and heavy metals, and also its impact on soil animals and for improving soil risk evaluation.

Keywords: isopoda, pharmaceutical residues, heavy metals, feeding patterns, biochemical responses.

Resumo

A influência de resíduos farmacêuticos e metais pesados em organismos vivos tem recebido atenção global. O presente estudo avaliou o efeito de resíduos de antibióticos e metais pesados no solo, como alimentos contaminados com cádmio (Cd) e oxitetraciclina (OTC), sobre o isópode *Porcellio laevis*, o qual foi alimentado com folhas frescas de plantas contaminadas com diferentes concentrações de cádmio, Cd + OTC1000 ppm, Cd + OTC2000 ppm e Cd + OTC3000 ppm, por quatro semanas. As mudanças nos padrões de alimentação, proteína, peroxidação lipídica (LPO), atividade da catalase (CAT) e aminoácidos livres totais (TFAA) foram registradas. Houve diferenças significativas nos resultados obtidos, em que o Cd reduziu a taxa de excreção (ER), no entanto o OTC aumentou essa proporção. A análise bioquímica mostrou que a combinação entre OTC e Cd inibe os efeitos tóxicos do Cd em baixa concentração (1.000 ppm), enquanto, em alta concentração (3.000 ppm), aumenta a toxicidade. Estudos detalhados são necessários para uma maior compreensão da interação entre OTC e metais pesados, e seu impacto sobre os animais do solo, bem como para melhorar a avaliação de risco do solo.

Palavras-chave: isópode, resíduos farmacêuticos, metais pesados, padrões de alimentação, respostas bioquímicas.

1. Introduction

Pharmaceutical residues are chemical compounds, reaches water ways and soil, influencing human health and non-target animals causing severe problems (Volção et al., 2020). Additionally, some plants can transfer these chemicals from the soil to both human and animals through food chains (Tiwari et al., 2017; Nasr et al., 2020). Veterinary drugs can be introduced by various means to the ecosystem (Bártíková et al., 2016), such as soil fertilization using manure of animals.

Oxytetracycline (OTC) is a major one of the tetracycline, extensively used worldwide in veterinary medicines and feed additives. It cannot be completely absorbed in their intestine and up to 75% of OTC administered to animals will enter the environment by excretion and pollute the soil (Bao et al., 2013; Leston et al., 2014; Li et al., 2015). With the growth of intensive animal and poultry breeding programs and the large use of manure fertilizers, increasing the ecotoxicological effects of antibiotics

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on the surrounding environment (Thiele-Bruhn, 2005; Hammesfahr et al., 2008; Liu et al., 2009).

OTC inhibits bacteria, actinomycetes, and total microorganism populations (Wang and Zhang, 2009); decreases urease, sucrose phosphatase, as well as hydrogen peroxidase activity (Yao et al., 2010); reduces root and shoot elongation in wheat and chinese cabbage (Jin et al., 2009); and causes genotoxicity to earthworms (Dong et al., 2012).

Cadmium is known for its non-corrosive nature and one of the non-essential metals. Cd may antagonize essential trace elements and causes competition with nutrient elements by blocking binding sites of transport and storage proteins (Wright and Frain, 1981). It is an extremely toxic trace element to the ecosystem that mainly comes from industrial processes and phosphate fertilizers (Liu et al., 2007, 2013) and animal feeds are generally contain higher heavy metals (Kumar et al., 2013; Wang and Wei, 2013 and Adamse et al., 2017). Thus, excessive levels of heavy metals are often passed to the soil and environment in feces. It reaches high levels in agricultural soil, and is easily absorbed by plants. These metals cannot be degenerated chemically or biologically; hence they form serious threats to the surrounding environment, food safety, and consequently human health (Olawoyin et al., 2012).

Terrestrial isopods (woodlice) are important detritivores in soil macrofauna (Schultz, 1982). They greatly contribute to the breakdown of leaf litter and are an intrinsic part of the decomposition process of organic matter, which recycles essential nutrients for the soil and maintains its fertility (Wieser, 1979; Hassall et al., 1987). The terrestrial isopods might be useful for monitoring bio-accumulation of such contaminants and can serve as bio-indicators of heavy metal pollution (Paoletti and Hassall, 1999). Pollution of soil in which these animals live and feed may decrease their number that consequently influences the flow of matter and energy through the food webs (Drobne, 1997). Additionally, presence of antibiotic and heavy metals in low concentrations for long periods results metal and antibiotic resistant bacteria (Zhang et al., 2005; De Souza et al., 2006; Park et al., 2009; Gao et al., 2015).

The combined effects of antibiotics and heavy metals on the feeding patterns need more investigations. Therefore, the current study aimed to assess the combined toxicity of OTC and Cd on *Porcellio leavis* in laboratory.

2. Materials and Methods

2.1. Sampling sites

Specimens of *Porcellio leavis* were collected randomly from mango orchard at Assiut University farm, Egypt, during November 2017.

2.2. Experimental design

The collected isopods were maintained in plastic containers for one week in the laboratory; they were fed on mango leaves. Animals were starved for 48 hours before experiment to allow the contents of the gut to be evacuated. They were checked for ovigerous females at

the beginning of the experiment but it was not possible to know if females will give future juvenile or not. All isopods in the experiment were within the same size and weight range, so it was assumed to be the same age.

Cadmium solution 80 ppm and Oxytetracycline concentrations, 1000, 2000 and 3000 ppm were prepared and kept in the fridge at 4°C. Every 15 isopods were placed in plastic boxes and divided into five groups. The experiment consists of control, cadmium treatment, T1 (Cd +1000 ppm OTC), T2 (Cd +2000 ppm OTC), and T3 (Cd +3000 ppm OTC) groups. The plastic boxes were supplied with a plaster substrate to maintain humidity necessary for the animal life. There were three replicates of control and each treatment.

Dry leaves of mango were fragmented into small pieces (1 cm²), every week; the fragmented leaves (2±1 g) were sprayed with the treatment solutions (9 ml), which prepared from 80 ppm Cd mixed with OTC solutions (1000, 2000 and 3000 ppm). The leaves supplied to control groups were sprayed with 9 ml of distilled water.

The experiment lasted 4 weeks. Dead individuals were removed daily along the experiment period. Every week, the animals were weighed and transferred to clean boxes and supplied with freshly sprayed food with treatment concentrations. The remaining food and fecal pellets were separated and weighed. Feces were collected, weighed weekly and stored in plastic bags to avoid coprophagy.

2.3. Feeding parameters calculations

At the end of the experiment, food (mango leave), animals and fecal pellets weights were used to calculate feeding parameters such as, food consumption, assimilation and egestion ratios and assimilation efficiency. These parameters were calculated weekly by the Equations (1, 2, 3, and 4) according to Abd El-Wakeil (2001, 2005) as follows:

$$CR = (W_f.wt - W_r.f.wt) = W_{isop} * day \quad (1)$$

$$AR = [(W_f.wt - W_r.f.wt) - Fp.wt] / W_{isop} * day \quad (2)$$

$$AE = [(W_f.wt - W_r.f.wt) - Fp.wt] / (W_f.wt - W_r.f.wt) * 100 \quad (3)$$

$$ER = Fp.wt / W_{isop} \quad (4)$$

Where, CR: consumption rate, AR: assimilation rate, AE: assimilation efficiency, ER: egestion rate, W_f.wt: food weight, W_r.f.wt: remaining food weight, W_{isop}: animal weight and Fp.wt: fecal pellets weight.

2.4. Biochemical analysis

The randomly chosen gut and heptopancreas of four woodlice from each experimental group were weighed and homogenized in ice-cold 50 mM K-phosphate buffer pH 7.4 to get 0.1% homogenate solution, centrifuged at (8000 g for 20 min) and supernatant of homogenates were used directly for the evaluation of biochemical parameters. Total protein concentration was determined

using assay kits supplied by (spectrum diagnostics, Egypt) and expressed as mg/ml. The activity of catalase (CAT) was determined based on its ability to decompose H_2O_2 to H_2O and O_2 according to (Beers Junior and Sizer, 1952). Lipid peroxidation was indirectly measured according to the method of Ohkawa et al. (1979) (thiobarbituric acid reactive substance, TBARS test). Lipid peroxidation (LPO) was done by the reaction of lipid radicals and oxygen to form peroxy radicals (Powell, 2000). Total free amino acids also are known as Ninhydrin positive substances were estimated by the Ninhydrin method using leucine as standard (Moor and Stein, 1954).

2.5. Data analysis

The obtained data were statistically analyzed using SPSS software (Version 20) and Excel (Office 2010). After data was checked for normality, analysis of Variance (ANOVA) was used to test the differences between means of experimental groups followed by Duncan test to detect the distinct variances between means. While for the non-normality of the data, the non-parametric Kruskal-Wallis test was used to test the differences. Repeated measures analysis of variance was used to analyze the weekly changes in feeding parameters.

3. Results

3.1. Impact of Cd and OTC on mortality during the experiment

The average mortality of the isopod per week was represented in Table 1. Statistical analysis did not show significant differences in mortality among different groups ($\chi^2 = 2.98$, $P=0.561$) and also among weeks ($\chi^2 = 1.119$, $P=0.772$).

3.2. Impact of Cd and OTC on the feeding parameters in different treatments

Feeding behavior of *P. leavis* was influenced by concentration of pollutants. Feeding parameters of isopods in different groups was not significantly differs except egestion ratio was high significantly affected.

Highly significant differences were noticed in all feeding parameters among weeks of study period. Consumption ratio showed highly significant difference in the interaction among treatments and weeks (Table 2).

Obviously, the highest consumption ratio was observed in the control animal group. Non-significant decrease was observed in ingested Cd or Cd combined with different concentrations of OTC (Figure 1A). In contrast, the assimilation ratio recorded non-significant increase in animals fed on Cd-treated leaves then decreased in treatment combination with different concentrations of OTC compared to control (Figure 1B). Also assimilation efficient showed, non-significant increase in animals fed on Cd and decreased gradually with high concentrations of OTC compared to the control (Figure 1C). Similarly, the control animal group recorded the highest egestion ratio whereas there was a statistically significant decrease in animals fed on Cd, and combined Cd with 1000 ppm OTC (Figure 1D).

3.3. Impact of Cd and OTC on the feeding parameters of *P. leavis*

There was increase in CR, AR and AE, in the control group along the period of the experiment. The CR of the Cd treatment was fluctuating during the first three weeks then sharply increased in the fourth week, while each AR and AE in the Cd group gradually increased with time of exposure. The CR and AR of the T1 group recorded the highest increment in the third week and slightly decreased in the fourth week, while AE increased by time of exposure. In T2 group, all feeding parameters (CR, AR, and AE) decreased in the first weeks, and then upsurge occurred in the fourth week. In T3 group all parameters CR, AR, and AE attained the highest values in the third week and then decreased in the fourth week. ER in the control group showed the highest value in the second week and decreased with time. In the treated groups (Cd, T1, T2, and T3), ER showed high peak in the first week then recorded significant decrease in Cd, T2, and T3 and a non-significant decrease in T1 (Figure 2).

3.4. Impact of Cd and OTC on the biochemical parameters

A significant decrease was noticed in the concentration of total protein in animals fed on Cd sprayed leaves, while

Table 1. Means \pm standard deviations (SD) of mortality in each treatment groups every week.

Treatment		Average dead animals per week				Total
		1	2	3	4	
Control	Mean \pm SD	1 \pm 0	2 \pm 1	0.33 \pm 0.58	1 \pm 1	1.08 \pm 0.9
Cd	Mean \pm SD	0.67 \pm 0.58	0.67 \pm 0.58	1 \pm 1	1.33 \pm 0.58	0.92 \pm 0.67
T1	Mean \pm SD	1.33 \pm 0.58	1.67 \pm 0.58	0.33 \pm 0.58	1 \pm 1	1.08 \pm 0.79
T2	Mean \pm SD	1 \pm 0	0 \pm 0	0.67 \pm 0.58	1 \pm 1.73	0.67 \pm 0.89
T3	Mean \pm SD	1 \pm 1	0.67 \pm 0.58	1.67 \pm 1.15	0.33 \pm 0.58	0.92 \pm 0.9
Total	Mean \pm SD	1 \pm 0.53	1 \pm 0.93	0.8 \pm 0.86	0.93 \pm 0.96	

Control (Distilled water), Cd (Cadmium treatment), T1 (Cd +1000 ppm OTC), T2 (Cd +2000 ppm OTC), and T3 (Cd +3000 ppm OTC)

Table 2. Two-way multivariate analysis of variance (MANOVA) for feeding parameters of *P. leavis* treatment groups (P<0.05 is significant; P<0.01 is highly significant).

Source		Sum of Squares	Degree of freedom (Df)	Mean Square	F-Statistic	p Value
Treatments	Cr	0.000772	4	0	0.733	0.575
	Ar	0.000918	4	0	1.035	0.401
	AE	553.6037	4	138.401	0.961	0.44
	Er	0.000984	4	0	4.091	0.007
Week	Cr	0.004509	3	0.002	5.708	0.002
	Ar	0.00948	3	0.003	14.243	<0.001
	AE	8258.305	3	2752.768	19.107	<0.001
Treatment * Week	Er	0.001286	3	0	7.129	0.001
	Cr	0.007817	12	0.001	2.474	0.016
	Ar	0.004699	12	0	1.765	0.089
Error	AE	1063.291	12	88.608	0.615	0.817
	Er	0.001104	12	0	1.53	0.154
	Cr	0.010532	40	0		
	Ar	0.008874	40	0		
	AE	5762.737	40	144.068		
	Er	0.002406	40	0		

Df: degree of freedom (n-1). F: F-statistic. p value: Significance level. *The significance between treatments and weeks.

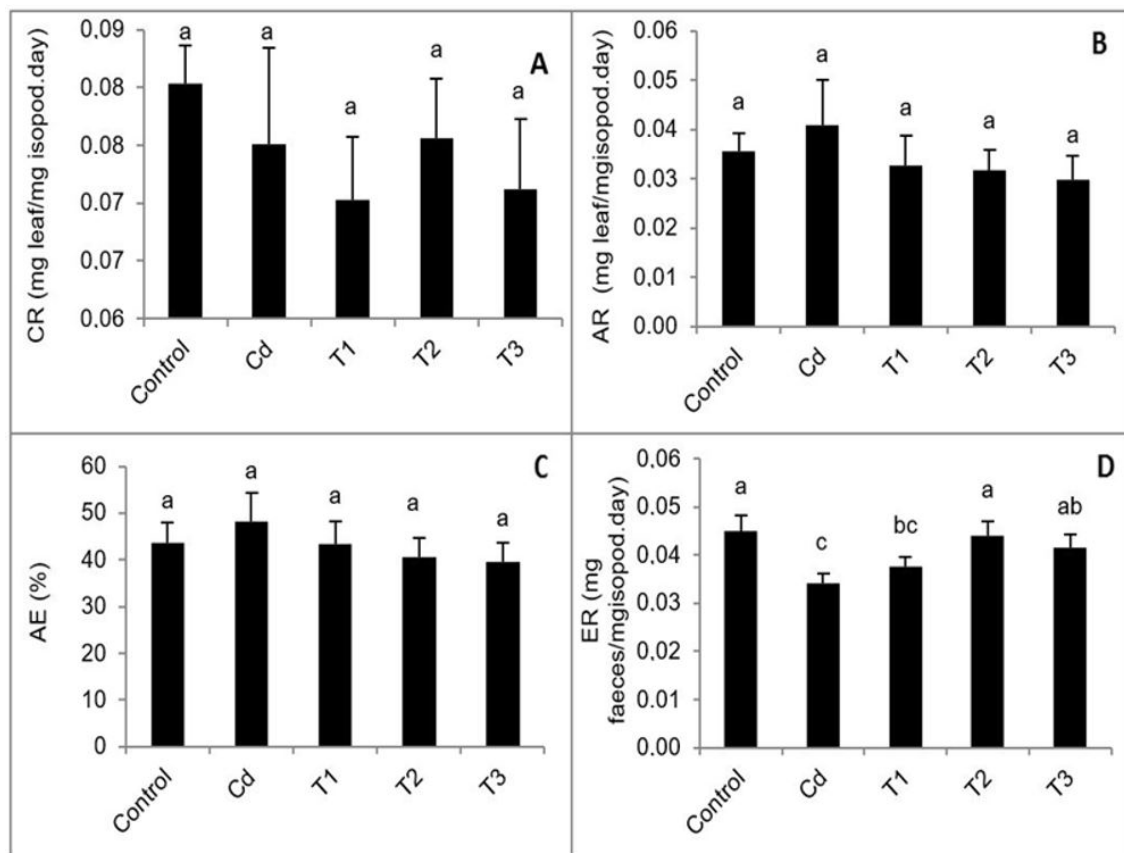


Figure 1. Difference between the means of feeding parameters; (A) consumption rate (CR), (B) assimilation rate (AR), (C) assimilation efficiency (AE), (D) egestion rate (ER) of control and treated isopod groups. Bars show mean ± SD (similar characters mean no significant difference).

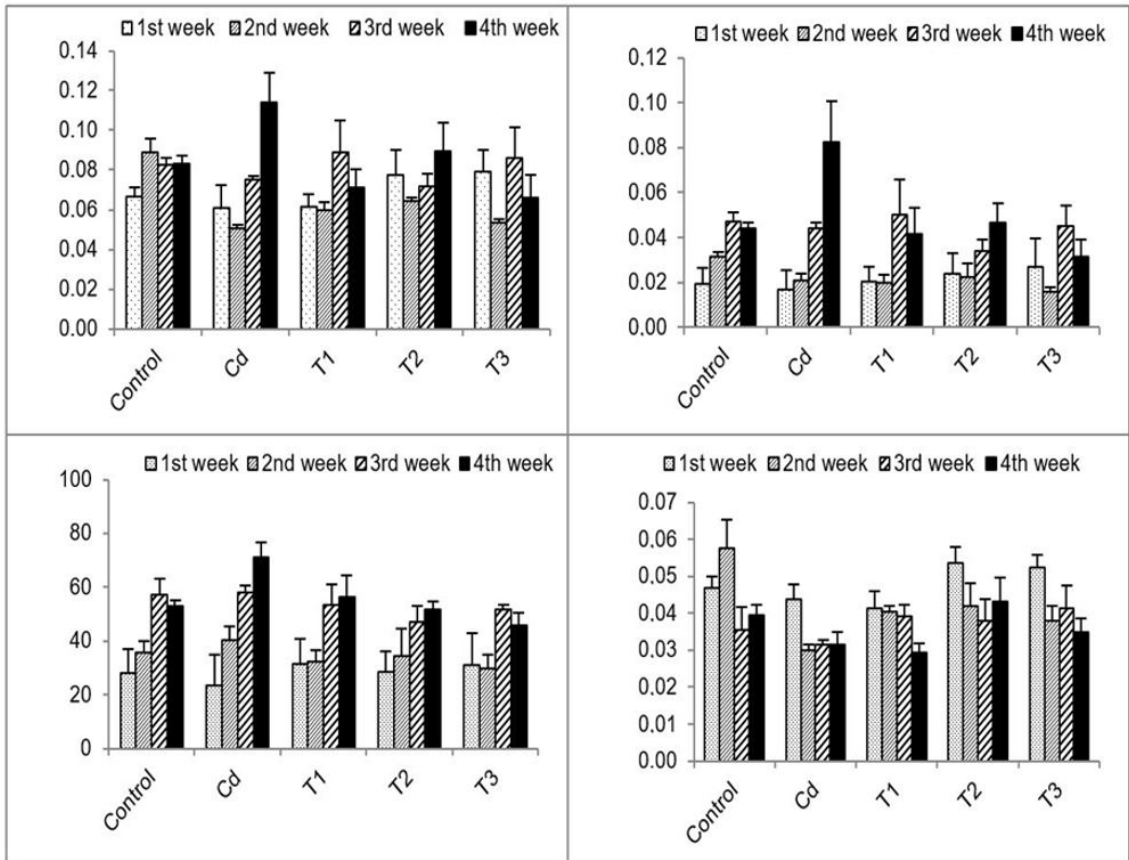


Figure 2. Histogram showing mean \pm SD of Consumption Ratio (CR) (mg leaf/mg isopod. Day), AR (mg leaf/mg isopod. Day), AE % and ER (mg faeces/mg isopod. Day) of isopod *Porcellio leavis* in different weeks of study period.

no significant increase was found in T1 group and there was a significant increase in T2 and T3 groups compared with the control. Lipid peroxidation showed high significant increment in animals fed on Cd sprayed leaves; moreover, there was no significant difference recorded change in lipid peroxidation in T1, T2, and T3 groups compared with control. Catalase activity showed a high significant increase in the Cd group comparing to control, while no significant decrease was noticed in the T2 group and no significant differences were observed for catalase activity in T1 and T3 groups. A significant increase in total free amino acids was noticed in Cd, T2, and T3; however, no significant difference was found in T1 compared to control group (Figure 3).

4. Discussion

The present study indicated that neither single nor combined OTC with heavy metal (Cd) caused mortality for isopods during the experiments. Remains of the dead animals were observed that, led to the suggestion that the main cause of death was due to the cannibalism not according to toxicity. This could be due to accumulation of

Cd in cells of mid-gut and digestive gland (Nasr et al., 2020) for short-term exposure (Rost-Roszkowska et al., 2020).

The single and combined impacts of Cd and OTC induced an irregular decrease in consumption ratio of food for *P. leavis*, where food consumption is indicative of the overall condition of an organism (De Coen and Janssen, 2003). This result may be due to the toxic effect of Cd that led *P. leavis* to reduce its consumption of food where Cd may influence the gut epithelium (Rost-Roszkowska et al., 2020). The observed excessive decrease in food consumption ratio in T1 group may be due to *P. leavis* reduced consumption of contaminated food; where declined feeding rate was recorded after exposure of isopods to metals, biocides or veterinary drugs in a dose-dependent manner (Novak et al., 2012, 2013; Volco et al., 2020).

Decreased assimilation ratio and assimilation efficiency in Cd combined with different concentrations of OTC because *P. leavis* consumed less contaminated leaves in all treatments. The increment of assimilation efficiency might be explicated by the slower passage of food material through the animal's gut, which consequently decreased fecal production. As the longer time the food stays in the gut the higher the assimilation of food and this might

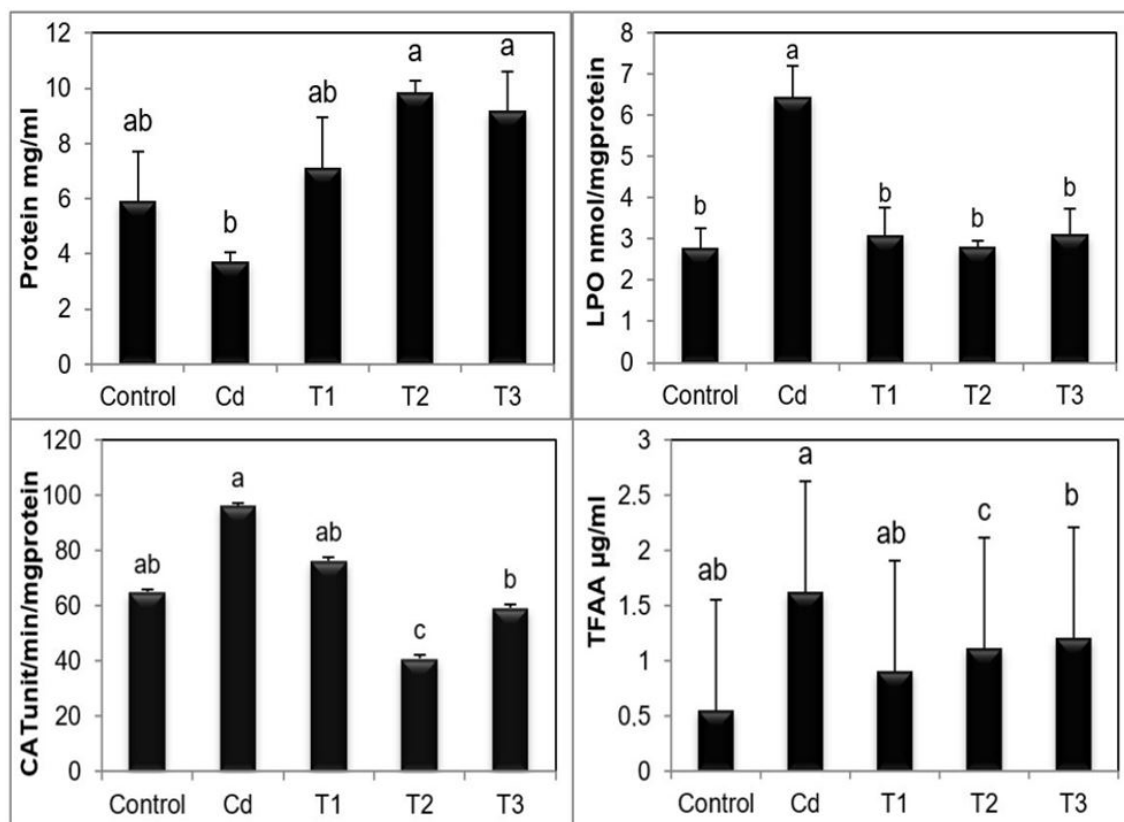


Figure 3. Concentration of biochemical parameters: Total protein, Lipid peroxidation (LPO), Catalase activity (CAT) and Total free amino acids (TFAA) in *P. leavis* in different treated groups control, Cd, T1, T2, and T3. Values are the mean of three replicates (n=3) ± SE. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b,c,d) were significantly different (p<0.001).

be associated with food characters, like leaf hardness and nutrients availability. Previous studies showed that terrestrial isopods displayed high assimilation efficiencies in animals fed on leaves with high levels of contamination more than animals fed on uncontaminated leaves (Drobne and Hopkin, 1995; Loureiro et al., 2006). The upsurge of consumption rate, assimilation rate and assimilation efficiency in isopod, *P. leavis* fed on a diet with Cd only (conc. 80 ppm) makes the animal more obese than the control and may affect its vitality and movement. This means that the pollution of Cd may increase obesity in soil isopods.

Antagonistic effect of OTC on heavy metal was observed through increasing OTC concentrations; this was more visible in assimilation ratio and assimilation efficiency of food. OTC may chelate heavy metal, and consequently can decrease the toxicity of metal (Kong et al., 2006). A similar result was obtained from earthworms treated with oxytetracycline and Pb (Gao et al., 2014). In this study, the significance antagonistic effect of OTC and Cd appears after 2 weeks of exposure. Zhu et al. (2006) stated that combined toxicity might be similar, stronger, or weaker than single toxicity; it depends on various factors such as concentrations, exposure times and the constituents of the mixtures.

Egestion ratio is an ecologically related parameter because fecal production occurs as a direct outcome of litter fragmentation, included in the primary step in the leaf decomposition process furthermore, it was grinded and digested to be easily decayed by fungi and bacteria than fallen decay leaves, thereby accelerating leaves decomposition. In this study egestion ratio was decreased in animals fed on contaminated food with Cd then increased till reached the control value in Cd combined with OTC. The decrease of ER may be attributed to the movement of the gut which could be affected by the toxicity of Cd furthermore the increase in ER may a result of the antagonistic effect of OTC on Cd.

The highly significant decrease in total protein concentrations of animals fed on contaminated food with Cd; may be attributed to the reduction in general metabolic activity (Obuid-allah et al., 2015). The increase of total protein concentrations in animals fed on Cd combined with OTC (1000 and 2000 ppm) and began to retreat in Cd combined with 3000ppm OTC. This result may explain that adding OTC to food contaminated with Cd may antagonists the toxic effect of Cd and increase the metabolic activity of the animal, while high concentrations of OTC may

influence the gut micro flora (Ihnen and Zimmer, 2008; Volcão et al., 2020).

In the present study, a significant increase in total free amino acids in animals fed on Cd contaminated diet only, this can be presumed to be an indication of the acute effect of the used concentration of cadmium on those tissues. The decline in total protein content and the simultaneous increase in free amino acid in the digestive tract of *P. leavis* may indicate the activation of protein catabolism to counteract the toxic effects of cadmium (Reddy and Bhagyalakshmi, 1994); where the breakdown of protein is a functional response to deal with the extra energy requirements to cope with Cd stress. De Smet and Blust (2001), illustrated that proteolysis is a sign of using protein in energy production during cadmium stress. Furthermore, Rost-Roszkowska et al. (2020) reported that Cd induces cell lysis.

The significant increase of TBARS content, in the tissue of gut and hepatopancreas of *P. leavis* was in animals fed on Cd-sprayed leaves, may indicate that cadmium toxicity was linked to lipid peroxidation because Cd produces excessive reactive oxygen species including superoxide radicals (Obuid-allah et al., 2015) Rijstenbil (2000) observed increase of LPO caused by UV-A radiation in marine invertebrate *Ditylum brightwellii*. On the other side, the non-significant differences in groups fed on Cd combined with OTC, may be due to OTC antagonists the toxic effect of Cd preventing the loss of membrane integrity and cell degeneration.

Catalase (CAT) is an anti-oxidative enzyme, which has been involved in protection against H₂O₂. High significant increase was noticed in CAT activity in animals fed on Cd alone. This increment could be considered as a kind of defense mechanism of the cells in order to offset the oxidative stress induced by increased H₂O₂. This happened in many invertebrate species as response to pollution (Stephensen et al., 2000). Previous studies reported that Cd had adverse effects on cellular/subcellular levels of earthworms (Panzarino et al., 2016; Yang et al., 2012; Zhou et al., 2016).

In conclusion, decreasing of CR, AR, and AE observed in *P. leavis* fed on Cd combined with OTC at high concentrations. For a long period of exposure (4 weeks) the moderate concentration of OTC (2000 ppm) may antagonist the toxic effect of Cd enhancing the above feeding parameters. Treatment of woodlice only with Cd containing diet inhibited the concentration of total protein, increased LPO, CAT activity and TFAA. In contrast combination between Cd and OTC in the diet increased the concentration of the total protein and decreased LPO followed by inhibition of CAT activity. This combination of Cd and OTC increased the amount of TFAA in gut and hepatopancreas tissues. More investigations are required to indicate the effect of antibiotics and heavy metals pollution and their effects on soil animals.

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