

Biochemical and transcriptomic evaluation of the toxic effects of aloin contaminated agricultural soils on the earth worm *Eisenia andrei*.

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Abstract— In the present study, we investigated the response of oxidative stress markers and related gene expression in worms exposed to *Aloe vera* crude exudates (aloin) contaminated agricultural soils for 7 and 14 days. Four sublethal concentrations ranging from 10, 50, 100 and 200 g/kg soils (corresponding to 0.125, 0.625, 1.25 and 2.5 g aloins.Kg⁻¹ soils) were tested. Acetyl cholinesterase activity was evaluated to assess the potential neurotoxic effect of aloin. Lysosomal membrane stability (LMS) was evaluated after the exposure periods in worm's coelomocytes as indicator of cytotoxicity. Our data indicated a significant increase in the antioxidant enzyme activities termed as catalase (CAT), and glutathione-S-Transferase (GST) and caused a pronounced increase of malonedialdehyde accumulation (MDA). Similarly, LMS was highly affected by aloin particularly at higher concentrations and after 14 days of exposure. Cat and gst, gene expression levels showed a significant increased pattern in animals exposed for 7 and 14 days to the aloin concentrations compared to the control condition. ACHE activity was inhibited in animals exposed to C2, C3 and C4 particularly after 14 days of exposure. Our data provide new insights into the cytotoxicity effect of *aloe vera* crude exudates on the earthworm *Eisenia Andrei*; one of the principal components of agricultural soil biofertility and sustainability.

Keywords— *Aloin* crude exudates, Toxicity, *Eisenia Andrei*, Agricultural soils, sustainability.

I. INTRODUCTION

Aloe vera is a plant that has been used as a constituent of several prescriptions in the conventional medicine worldwide [1,2,3]. Many biological activities like, immunostimulative activity, antiviral and antibacterial as well as antioxidant activity [4,5] have been reported in this plant extracts. Moreover, *Aloe vera* extracts, rich in polyphenols has increased its use as food supply [1,5] and

therefore related agricultural surfaces dedicated to its cultivation increased substantially over the past decade.

Aloin is the main anthraquinone (10-glucopyranosyl-1,8-dihydroxy-3-Hydroxylmethyl-9(10H)-anthracenone) in *Aloe vera* shoots, which occurs naturally as a mixture of two isomers aloin B and aloinA. In addition to these compounds, other compounds including isoaloesin, aloenin B and aloenin have been described to be involved in the biological properties of *Aloe vera* extracts [6]. Aloin is related to *aloe emodin*, which lacks a sugar group but maintain aloin's biological properties [7].

Usually and for human consumption purposes, aloin content in food based products such as juices must be very low, thus during the preparation process aloin is separated due to its noxious properties. Indeed, aloin was reported to provoke pathological modifications and changes the composition of microbiota in the large intestine of rats [8]. Moreover, [] demonstrated that aqueous extract of *Aloe vera* was able to affect DNA alteration and to generate reactive oxygen species in a dose-dependent manner.

Earthworms are usually used as test animals in terrestrial ecotoxicology due of their burrowing habits. Earthworms highly influence and control soil processes, thus maintaining the soil structure and maintain the dynamics of organic matter. Moreover, these organisms have been used so far as indicators of environmental changes [10, 11]. Among the earthworms, the *Eisenia* species has been widely employed for biomonitoring soil's quality [12,13]. A panel of toxicity tests have been performed using these species, generating a great variety of data covering behavioral [14], cellular [15] and molecular [16] aspects.

This investigation aims first, to evaluate the cytotoxicity effects of contaminated agricultural soils with four sublethal concentrations of aloin throw the evaluation of the anti-oxidant stress enzymes activities CAT and GST, the malonedialdehyde (MDA) accumulation and the lysosomal membrane stability (LMS) on the earthworm

Eisenia andrei. Neurotoxicity of aloin was tested using acetylcholinesterase enzyme activity (ACHE). Second, we investigated the gene expression pattern of the glutathione S-transferase (*gst*) and catalase (*cat*) genes using quantitative reverse-transcription technique.

II. MATERIAL AND METHODS

2.1. Soil test and Aloe vera leaf latex.

The agricultural soil test was obtained from an agricultural local farming [10]. Leaves from Aloe vera (var. *barbadensis*) were obtained from 5 years aged plants supplied by a local production activity. Briefly, Aloe vera leaf latex was obtained by stripping away the outerleaf rind, and collecting it [17]. The obtained latex was further added to soils to achieve final concentrations of 10, 50, 100 and 200 g/kg soils corresponding to 0.125, 0.625, 1.25 and 2.5 g aloins.Kg-1 soils. Chemical analysis of Aloe vera latex revealed the presence of aloenin, aloenin B, isoaloesin, aloin A and aloin B at a total amount of 12.5 g.Kg-1 latex.

2.2. Animals

E. andrei specimens [18] were cultured as according to OECD guidelines [19,20]. Worms were chosen from a synchronized culture with a homogeneous age structure. Adult animals with clitellum of similar size and weight (400 to 500 mg) were employed in the experiments.

2.3. Worm's Exposure

Worms were removed from the culture medium to the experimental soils. Earthworms were kept during the exposure period (7 and 14 d) in 3-L containers with constant light and 35% humidity at $20 \pm 1^\circ\text{C}$ (20). Each condition was conducted in triplicate and a total of 10 worms per condition were randomly utilized for the biomarkers analysis. After the 7-or 14-day exposure period, the animals were placed on moist filter paper for 24 h to allow gut clearance before molecular and biochemical analyses.

2.4. LMS determination

The LMS was assessed in coelomocytes as described by Sforzini et al [21,22,23]. Slides were scored at 630 \times magnification using an inverted photo-microscope (Zeiss Axiovert 100M) equipped for fluorescence microscopy with a rhodamine emission filter.

2.5. Biochemical analysis

2.5.1. Antioxidant enzyme activity

Worms (0.5–1 g; n=10) were homogenized in ice-cold extraction buffer (pH 7.5) containing 250 mmol.L⁻¹ sucrose, 50 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, and 1 mmol L⁻¹ DTT at a 1/4 w/v ratio. Proteins in

the S9 fraction were quantified according to the Bradford method [24].

The GST activity was measured in DG cytosol by the method of Habig et al. [25]. The CAT activity was determined according to Clairbone's method [26].

2.5.2. MDA determination

Worms (0.5–1 g; n=10) were homogenized in two volumes of 20mM Tris-HCl (pH 7.4) and 0.1% mercaptoethanol buffer. The homogenate was centrifuged at 18,000 \times g at 4 $^\circ\text{C}$ for 20 min. MDA level was evaluated as described by Gérard-Monnier et al. [27].

2.6. Gene expression

Total RNA was extracted from the worms using acid phenol-chloroform precipitation according to Chomczynski and Sacchi [28] using TRI-Reagent (Sigma-Aldrich). The abundance of the mRNA of the genes encoding glutathione-S-transferase and catalase were evaluated in multiplex Taqman assays according to Negri et al. [29] and Banni et al., [30]. cDNA (25 ng RNA reverse-transcribed to cDNA) was amplified in a CFX384 Real-Time PCR detection system (Bio-Rad Laboratories) using iQTM Multiplex Power mix (Bio-Rad Laboratories).

cDNA was amplified in the presence of 1X iQTM Multiplex Power mix using 0.3 μM of each primer and 0.1 μM of each probe in a final volume of 10 μL . Gene expression data were geometrically normalized against ribosomal protein riboS13 (BB998368.1) an invariant actin isotype (DQ286722.1) and 18S rRNA (AB558505.1) (29). Probes, sense primers and antisense primers are reported in table 1.

Statistical analyses were performed on the group mean values using a random reallocation test [31]. The LMS, enzymatic activity, and MDA content data are presented as the mean \pm SD of 10 samples. Statistica Software, version 6.0 (Statsoft. Inc. 2002) was used for statistical analysis. For multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed on data along with Tukey's test.

III. RESULTS

Figure 1 shows the effects of exposure to the increasing concentrations of aloin crude extracts for 7 and 14 days on coelomocyte LMS. The LMS was altered in all of the conditions compared to the control group being time and concentration dependent. The highest LMS destabilization was observed in animals exposed for 14 days to highest aloin concentration.

Lipid oxidative alteration was investigated by assessing MDA accumulation evaluated as thiobarbituric acid reactive species (TBARS) (Fig. 2). This biomarker increased significantly in all animals exposed to aloin for

7 and 14 days. Exposure to C2, C3 and C4 rendered a significant increase in MDA accumulation being significantly different over the two exposure periods. The highest MDA level was observed in animals exposed to C4 for 14 days.

The antioxidant activity of CAT and GST significantly increased in worms exposed to aloin after 7 days compared to control animals (Fig.3). This enhancement was maintained for CAT after 14 days being significantly different from the response depicted after 7 days. For GST activity, the significant increase was maintained after 14 days of exposure for animals exposed to C1, C2 and C3. However we observed a strong reduction in GST activity in worms exposed to C4 after 14 days exposure. The later reduction was even lower than the control value (71.74 ± 9.54 nmole/mn/mg proteins) with 36.55 ± 8.21 nmole/mn/mg proteins.

Transcriptomic data highlighted significant up-regulation in the mRNA levels of the *gst* and *cat* genes in worms exposed to aloin crude extracts compared to control animals (Fig. 4). For *cat* the maximum expression levels was observed after 14 days in animals exposed to C3 (4.89-fold increase). Regarding *gst*, the maximum was depicted after 7 days in worms exposed to C4 (6.17-fold increase). It is important to note that *gst* expression levels decreased after 14 days exposure compared to 7 days even if it remains significantly higher than control.

The response of AChE activity in earth worms exposed to increasing aloin concentrations is reported in figure 5. AChE activity was unchanged in animals exposed to C1 for the two exposure periods. However, AChE was significantly inhibited after 7 days and 14 days in worms exposed to C2, C3 and C4 when compared with control animals. Interestingly for C2 we noticed a recover in AChE activity after 14 days when compared to those after 7 days even if they remain inhibited respect to control. However, in animals exposed to C3 and C4 AChE activity significantly decreased after 14 days when compared to the values obtained in the same condition after 7 days.

IV. DISCUSSION

The utilization of different types of agricultural and agro-food industries' wastes through composting are important for environmental sustainability and restoring soil quality. However, in some cases a toxicity effects can be observed if persistent toxic molecules are introduced in the soils. The important increase of land surfaces dedicated to Aloe vera cultivation due to its important use in food, cosmetic and pharmacological applications rendered a consequent production of aloin, the principal anthraquinone present in this plant leaves. The later compound is usually re-introduced in agricultural soils.

Earthworms are increasingly considered as a good choice for ecotoxicological testing in terrestrial habitats due to their cosmopolitan characteristics, widespread in many soils, and are excellent indicators of land use and soil fertility. Due to their life-style, earthworms are the first to be exposed to soils contaminants and, therefore, environmental xenobiotics occurs earlier and its effects are more pronounced than in other organisms [32,16,10,11,15]. To our knowledge, this is the first report that aims to investigate the effects of aloin exposure on oxidative stress parameters in a earth earthworms.

In this study we report the sub-chronic and acute effects of aloin concentrations on the cellular responses of a non-target organism, the earthworm *E. andrei*. We first evaluated the LMS in worm coelomocytes and found that aloin negatively affected LMS in a dose dependent manner. LMS is known to be a sensitive cellular marker for the assessment of environmental contaminants effects on living organisms [11, 21].

Interestingly, the observed effect was maximal at the highest aloin concentrations. Thus, the anthraquinone seems to increase catabolic activity in worm's cells. Previous studies reported positive correlations between high-level ecotoxicological endpoints, such as and cell death and immune response and LMS [33]. From this point of view, it is of interest to point out that in marine mussels, a direct relationship between the scope for growth and LMS is well established. This may link LMS to population alterations [34]. The same effect was observed in earth worms exposed to increasing concentration of the herbicide 2-4-D where LMS decrease was correlated with a loss of body weight after 14 days of exposure [11]. Indeed, in the catabolic organisms, dietary energy is used mostly to rapidly turnover the cellular components damage by the toxic compounds and can be used only in a reduced manner for growth and reproduction [34].

In this study the time course response of the LMS showed a more pronounced toxic effect after 14 days of exposure when compared to the same experimental condition after 7 days of exposure. A completely opposite trend was observed in recent works after the exposure of *E. Andrei* from the same period of times to the herbicide 2-4D and to a polymetallic soils gradient [10,11]. This may support the highly toxic effect of aloin at the tested concentrations that empower over time probably due to its metabolites production.

Our data suggested also that exposure to aloin crude extract increased the level of oxidative alterations in the earthworms cells. This was assessed by evaluating the MDA content in animal's tissues after 7 and 14 days of exposure. 7 and 14 days treatment induced higher MDA accumulation being concentration dependent. In parallel,

exposure to aloin significantly increased the activities of CAT and GST in all the tested concentrations except for GST in animals exposed to the highest aloin concentration. Interestingly, gene expression analysis of the anti-oxidative stress genes *cat* and phase II biotransformation related gene *gst* in worms exposed to aloin showed significant up-regulation of these targets after 7 days. However, expression pattern of *gst* markedly decreased after 14 days when compared to 7 days even if it remains up-regulated when compared to control.

Oxidative stress can be induced by numerous stressors either by alterations in antioxidant defense mechanisms, or by over production of free radicals [35]. Our data provided evidences of physiological alterations in worm's cells that resulted in the accumulation of ROS. In addition to the direct production of ROS, other factors involved in aloin toxicity can be evoked. Indeed as a PAH, aloin may be bio transformed by organisms, and it or its metabolites may affect normal biotransformation process as GST activity was down regulated at higher aloin concentrations. Indeed, it has been suggested that cleavage of the glycosyl bonds by the intestinal microflora of humans and animals may results in the production of aloe-emodin[1,8-dihydroxy-3-(hydroxymethyl)anthraquinone] and other free anthraquinones and anthrones [36]. Moreover, the International Agency for Research on Cancer classified the whole leaf extract of aloe vera as possibly carcinogenic to humans (IARC Monographs, 2014). More recently, [37] reported the activation of aloe-emodin from aloe vera whole leaf extracts to biologically electrophiles compound and their involvement in the carcinogenicity of exposed organisms.

AChE activity of different worm species ranging from soil to marine ones has been found to be modulated by environmental contaminants other than organophosphate and carbamate insecticides, such as metals and hydrocarbon compounds [38,39,40,41]. Data reported in the present work showed a significant inhibition of AChE activity in worms exposed to C2, C3 and C4. This negative effect was significantly pronounced after 14 days of exposure when compared to 7 days. Although anthraquinones have not been so far involved in the neurotoxicity events of exposed organisms our data may suggest that the observed effect is rather due to the general toxic effect at the body level of exposed worms to aloe vera leaves exudates. Similar effect was observed in marine mussels exposed to the polyaromatic hydrocarbon benzo[a]pyrene at relatively higher levels [42].

V. CONCLUSION

The results of this research stress for the first time the risk due to the introduction of aloe vera exudates in

agricultural soils and their harmful effect on the earth worm *Eisenia Andrei*. Our data provided clues about the occurrence of alterations at cellular level of worms exposed to increasing aloin concentrations, thus highlighting a possible mechanism of action of the toxic compound through a relevant alteration of the activity of the lysosomal vacuolar system and a disorder in the antioxidant balance at protein and gene expression level. Finally a neurotoxic effect of the plant exudates was demonstrated. More in deep investigation of the single component of the tested exudates have to be performed to better understand the observed toxic effect.

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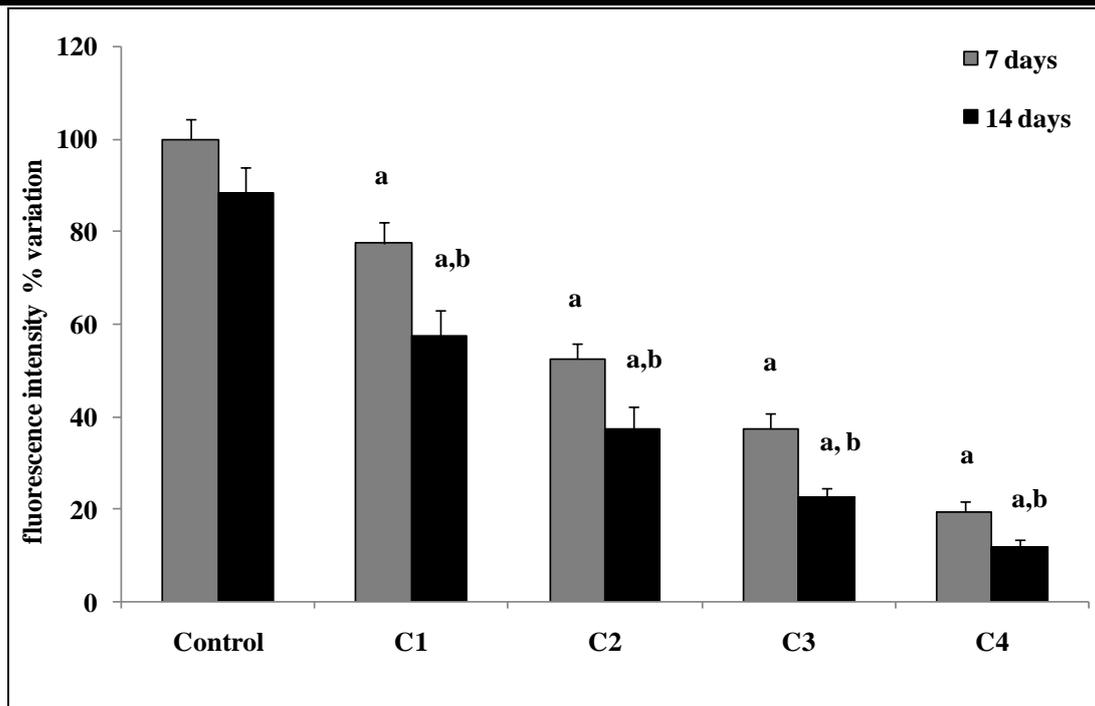


Fig1. Lysosomal membrane stability in *E. andrei* coleomocytes after exposure for 7 and 14 days to aloin crude extract (10, 50, 100 and 200 g/kg soils). Data expressed as % variation in fluorescence intensity ($n=10$), were analyzed by ANOVA + Tukey's post test. a: Statistically significant differences ($P<0.01$) in comparison with control condition. b: Statistically significant differences ($P<0.01$) in comparison with animals exposed to the same condition for 7 d.

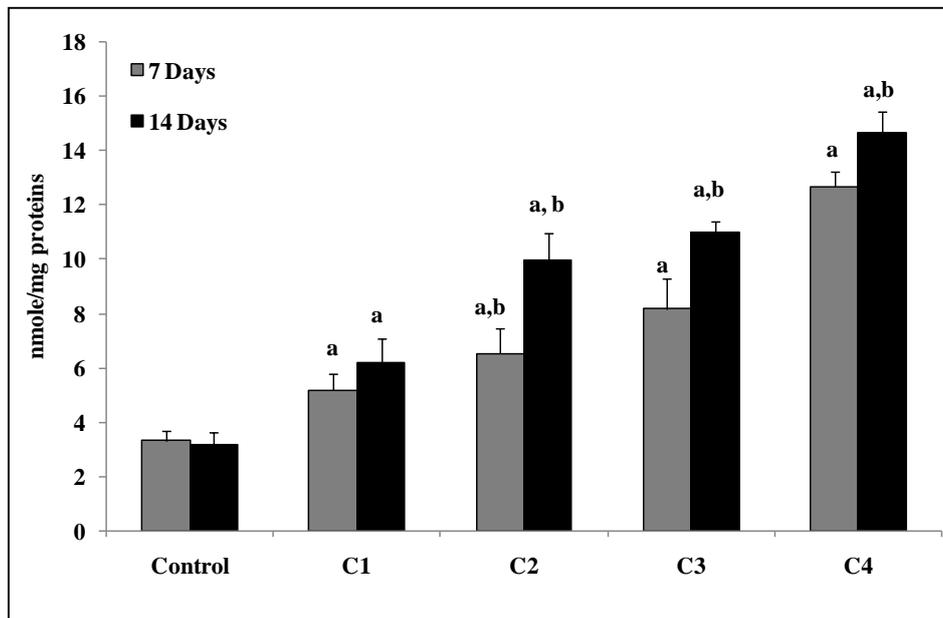


Fig 2. MDA accumulation in *E. andrei* exposed for 7 and 14 d to aloin crude extract (10, 50, 100 and 200 g/kg soils). Data, expressed as nmole/mg proteins ($n = 10$), were analyzed by ANOVA + Tukey's post test. a: Statistically significant differences ($P<0.01$) in comparison with control condition. b: Statistically significant differences ($P<0.01$) in comparison with animals exposed to the same condition for 7 d.

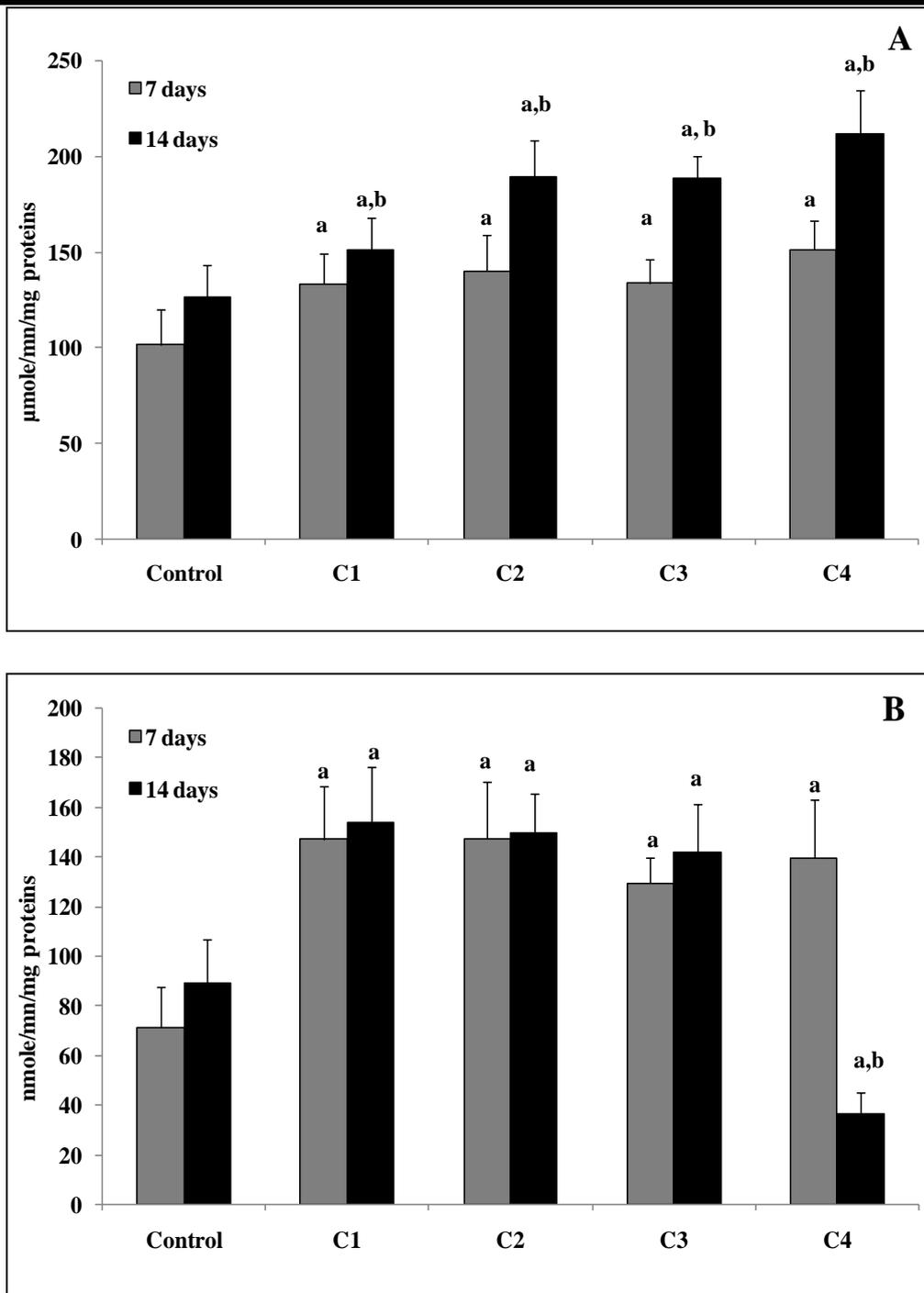


Fig 3: CAT (A) and GST(B) activities in *E. Andrei* exposed for 7 and 14d to aoin crude extract (10, 50, 100 and 200 g/kg soils). Data, µmole/mn/mg proteins for CAT and as nmole/mn/mg proteins GST (n=10), were analyzed by ANOVA + Tukey's post test. a: Statistically significant differences (P<0.01) in comparison with control condition. b: Statistically significant differences (P<0.01) in comparison with animals exposed to the same condition for 7 d.

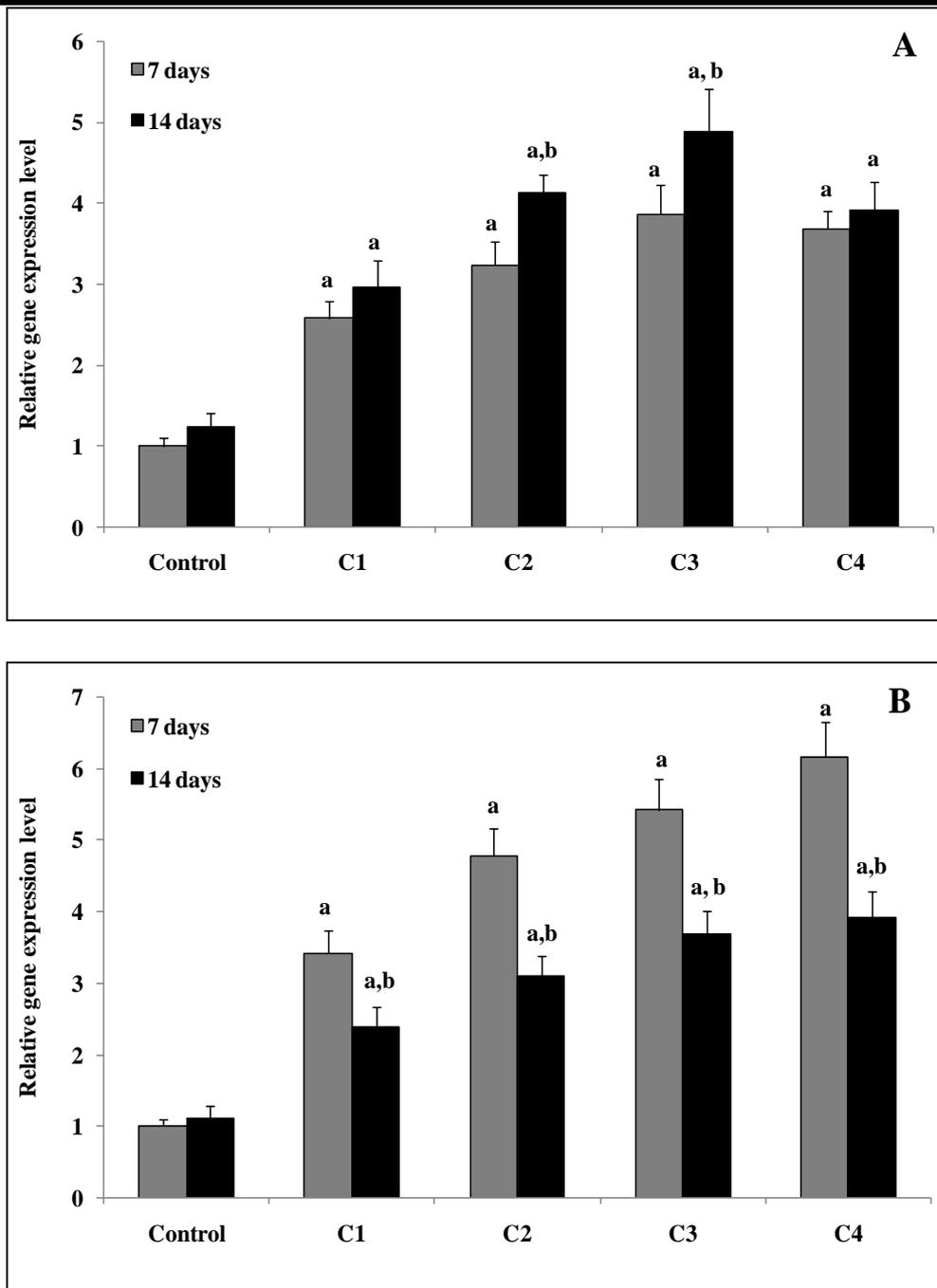


Fig 4. QPCR data of *cat* (A) and *gst* (B) targets in worms exposed for 7 and 14 d to aoin crude extract (10, 50, 100 and 200 g/kg soils). Gene expression was performed respect to the control condition (Soil without aoin supply); and was normalized against Actin, 18S and Ribo-S13. A: Significantly different from reference condition, b: Statistically significant differences in comparison with animals exposed to the same condition for 7 d. ($p < 0.05$) threshold cycle random reallocation test according to Pfaffl et al. (2002), $n=4$.

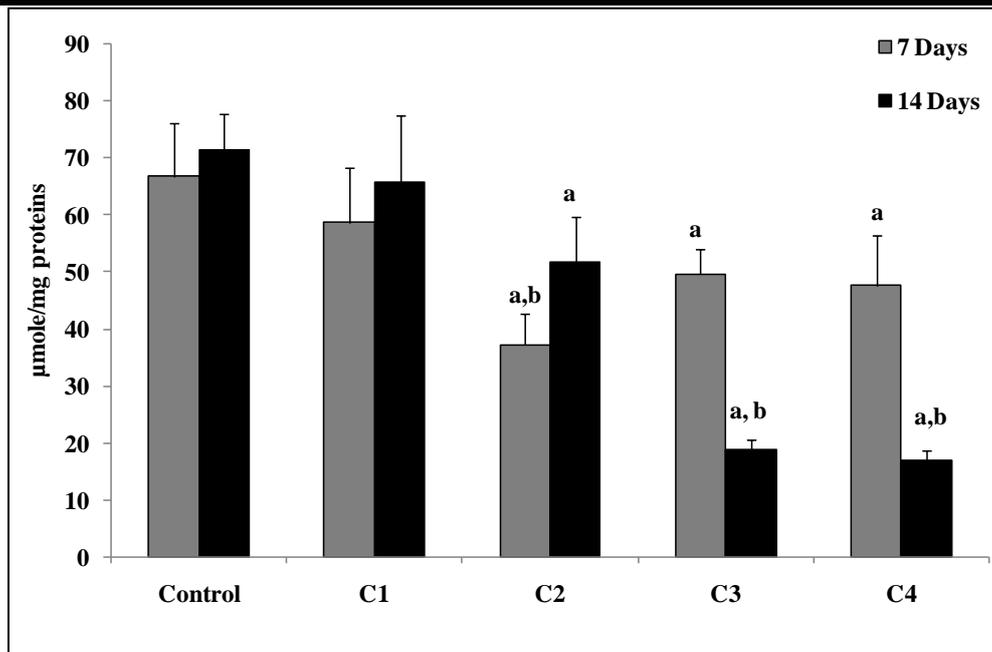


Fig5: AChE activity in *E. Andrei* exposed for 7 and 14d to alocin crude extract (10, 50, 100 and 200 g/kg soils). Data, $\mu\text{mole/mn/mg proteins}$ ($n=10$), were analyzed by ANOVA + Tukey's post test. a: Statistically significant differences ($P<0.01$) in comparison with control condition. b: Statistically significant differences ($P<0.01$) in comparison with animals exposed to the same condition for 7 d.

Table.1: Q-PCR primers and Taqman probes from Hattab et al. (2015)

Gene name	Probe	Sense Primer	Antisense Primer
18S	CGCCGACAGAGTGCCATCGAC GAA	AATTCCGATAACGAACGAGAC TCT	GCCACTTGTCCTCTAAGAAG TTA
β - Actin	AGTCCGGGCCATCCATCGTCC ACA	GGATCAGCAAGCAGGAGTAC G	TGGTCATTGATAATGGAGGCA CTT
RiboS1 3	TCGCATGGTGTGCTCAGACC CGT	TCACAGATTGGTGTATCCTTC GA	GCAAGACCCTTAGCCTTCAGG
gst	AGCGGAGTGCCTGACCACGAC CTC	GGTGTCCGATAGAATTCCTGC TAT	CTCCAGACCATTGTCTACAGC TAA
cat	TGCCTTGTCTCTTGCCGCCATC GT	CTCGATTTCGTCTTATTCTTCG CC	CTTGTATTGTTGAGTTGCTC GG