Focus on Sciences **Original Article**

Nov 2017, Volume 3, Issue 4

Evidence of the Genotoxic Effect of Aloin on Earth Worms Eisenia Andrei

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DOI: 10.21859/focsci-03041467

Submitted: 09.13.2017
Accepted: 09.27.2017

Keywords:
SP-8203
Alloin
Genotoxicity

Abstract

Introduction: The aim of this work is to investigate the genotoxic effect and related gene expression in the worms Eisenia andrei exposed to four sublethal concentrations of aloin crude extracts (10, 50, 100 and 200 g/kg soils) for 7 and 14 days.
Methods: First, worm's growth after the exposure periods was assessed. Micronucleus (MN) frequency in worm's coelomocytes was evaluated for the potential genotoxic effect of aloin. The gene expressions regulations were evaluated with the genes involved in DNA repair: the P21 and topoisomerase, The Lysosomal/cytoplasm ratio was scored as general biomarker of cytotoxicity.
Results: Our results demonstrate a significant increase in the MN frequency along the aloin increasing concentrations associated with a significant reduction in worm's growth. Similarly Lysosomal/Cytoplasm ration was highly affected by aloin particularly at higher concentrations and after 14 days of exposure. The gene expression pattern of P21 and Topoisomerase was markedly up-regulated as low concentrations and down–regulated at higher concentrations.
Conclusions: Our data should be particularly considered in view of the toxic effect of aloe vera wastes mainly at industrial level.

INTRODUCTION

Aloe vera is a plan native to Africa and Mediterranean. This plan becomes very popular and cultivated worldwide mainly due to its medicinal benefits. The production of aloin, which is the majority antraquinone in the aloe vera leaf, is a procesus occurs naturally and that constitutes a mixture of two diastereoisomers aloin A and aloin B. In addition to these compounds, other compounds including aloenin, aloenin B, and isoaloesin have been related to the biological properties of Aloe vera extracts the Plants of aloe vera containing aloin and aloe emodin are largely used in folk medicine and recently in many products as food supply and cosmetics. Thus, some studies have evaluated the toxicological properties of these compounds rendering very controversial conclusions. Some studies suggested that aloin can not be considered as mutagenic using an in vitro assay [1]. More recently, [2] reported the cytotoxic and the gentotoxic effect of aloin on humain skin fibroblasts. Among the most recent properties studied, the effects of aloin derivatives against some human breast cancer cell lines [3] have been suggested. Other activities found for these compounds include several antimicrobial properties [4] and some oxidant and antioxidant properties on free radical-induced DNA breaks [5]. Earthworms may represent a good allternative for ecotoxicological testing in terrestrial environment, since they are excellent indicators of soil fertility. They are also considered as cosmopolitan and found in many different types of soils and above all, they are vulnerable to soil contaminants [6]. An assortment of data available for comparison executed on Eisenia species, have been demonstrated as substantial toxicity test results [7]. The DNA alteration may occurs in organisms exposed to aloin and its derivates either directly or through the production of active metabolites [8]. Indeed, following metabolism by phase I and II enzymes, the main operating mode of metabolites is excretion [9]. However, the activation of chemicals to reactive intermediates, which are due to the process of biotransformation can finally, caused toxicity, mutagenicity, or carcinogenicity [10]. The Micronucleus frequency (MN) has become one of the standard technique for evaluating DNA damage because of its simplicity, sensitivity, speed, and economy [11]. The MN applied to earthworms has been widely used to assess the genotoxicity of heavy metals, pesticides [6], and multi-contaminated field soils [11]. This work aims to evaluate the effect of four

sublethal concentrations of aloin crude extracts on growth and DNA damage in the earth worm Eisenia Andrei and to investigate the gene expression regulation of genes involved in DNA repair mechanism; P21 and topoisomerase using quantitative reverse-transcription PCR (qRT-PCR).

METHODS

Soil Test and Aloe Vera Leaf Latex

The artificial soil test was performed as described in the OECD guidelines for the testing of chemicals [12]. 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 outer leaf rind, and collecting it [13]. 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 andaloin B at a total amount of 12.5 g.Kg^{-1}latex.

Animals

Earthworms of the species E. andrei [14] were raised as described in the OECD guidelines [12]. Organisms were selected with a homogeneous age structure from a synchronized culture. Adult worms with clitellum of similar size and weight (400 to 500 mg) were utilized in the experiments.

Exposure Procedure

Earthworms were displaced directly from the culture medium to the experimental soils to avoid the stress of moving. Earthworms were placed in polyethylene pots with soil and were maintained with constant light and 35% humidity at 20±1°C during the exposure period (7 and 14 d) [14]. Ten animals were introduced into pots. The experiment was under controlled conditions and three replicates per treatment were carried out.

Growth

Growth was determined as% of fresh weight variation and after 7 and 14 days of exposure 10 worms per experimental group was used. The results were treated with the following formula: % of fresh weight variation = (Wi-W0)/W0
W0 is the weight on day 0; Wi is the weight on day 7 or 14.

MN Frequency Determination

The MNi frequency was determined with the extraction of coelomocytes by ethanol [11, 15, 16]. Then, the earthworms were incubated in saline solution (0.85 mg/ml NaCl at 4 °C) and two animals were mixed with 4 ml of cold extrusion medium (ethanol, EDTA, and mucolytic agent guaiacol glycerol ether). After we added to the earthworms 2 ml of Hanks' balanced salt solution (HBSS) (Sigma H6648). The cell suspension was centrifuged to remove mucus and the cells. In the coelomocytes of worms exposed to aloin, the determination of the MNi frequency was established following the method described by [11]. Briefly, the cells were mixed with a DNA-specific fluorescent probe DAPI (4',6-diamidino-2-phenylindole) (ICN Biomedicals Inc., USA) and for each sample two thousands cells with intact cytoplasm were calculed with inverted photomicroscope (Zeiss Axiovert100M).

Gene Expression

Total RNA was extracted from worms according to [17] with TRI-Reagent (Sigma-Aldrich). The quality of RNA was controlled by UV spectroscopy and TBE agarose gel electrophoresis. The mRNA abundance of the genes encoding P21 and topoisomerase were evaluated in multiplex Taqman assays according to [18] and [19. Relative expression data were geometrically normalized to18S rRNA (AB558505.1), actin (DQ286722.1), and ribosomal protein riboS13 (BB998368.1) [20]. A triplex Taqman assay was use as described by [19]. qRT-PCR was performed with four biological replicates and three technical replicates. Statistical analyses were carried out on the group mean values using a random reallocation test [21]. The relative expression stability of the three reference genes was estimated in our experimental conditions using GeNorm [22].

Statistical Analysis

The non-parametric Mann–Whitney U-test was used to compare the data from earthworms exposed to aloin concentrations with data from the control soil (without aloin)-exposed worms.

RESULTS

Effect of Aloin on Growth

About growth, no worm mortality was noted during the exposure period in any of the test soils. After 14 days, we observed the maximum weight increase in control soil and it was 13.51 ±5.41% (Fig.1). In body weight no significant change was noted for worms in soils treated with the lowest aloin concentration when compared to controls. However, we observed a significant decrease in the weight of worms exposed to C2, C3 and C4 aloin concentrations being more pronounced after 14 days of exposure. The highest body weight lost was observed in worms exposed to C4 after 14 days of exposure with up to 19.49 ± %6.14 when compared to control.

Effect of Aloin on Lysosomal/Cytoplasmic Volume Ratio

The L/C volume ratio was affected in worms exposed to all the concentrations of aloin except the lowest one compared to control; in particular, the greatest L/C increase was noted for animals exposed to C4 and was more pronounced after 14 days (172.22 ± %11.22 increase compared to control) (Fig. 2). This result suggests a loss of cytoplasm due to improved lysosomal activity in the cells in the chloragogen tissue of the earthworms exposed to aloin.

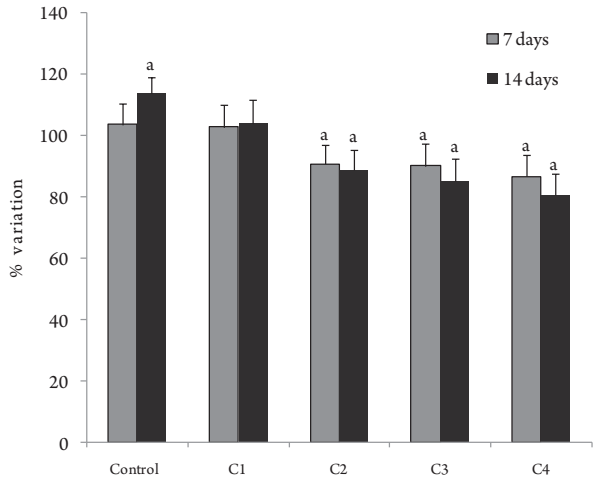

Figure 1: Growth Rate of E. andrei after Exposure of Worms for 7 d (Grey Columns) and 14 d (Black Columns to Aloin Crude Extract (10, 50, 100 and 200 g/kg soils). Data, Expressed as Percent Change with Respect to Values Measured at the Start of the Incubation (time 0), Represent the Mean ± SD of At least Ten Replicates. a: Indicates Statistically Significant Differences Respect to Control, b: Indicates Statistically Significant Differences Respect to the Same Condition after 7 Days Exposure ($P < 0.05$ Mann-Whitney U-test).

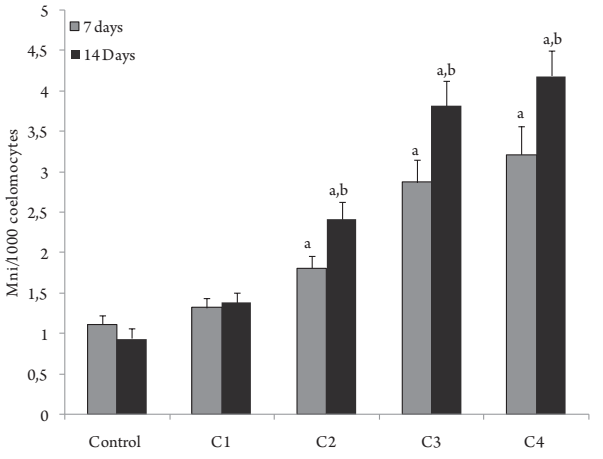

Figure 3: Aloin Exposure Effects on Micronucleus Frequency MN in EISENIA Andrei Colemocytes. Worms were exposed for 7 and 14 Days to Aloin Crude Extract (10, 50, 100 and 200 g/kg soils). Data Expressed as MN Frequency per 1000 cells (n = 10). a: Indicates Statistically Significant Differences Respect to Control, b: Indicates Statistically Significant Differences Respect to the Same Condition After 7 Days Exposure ($P < 0.05$ Mann-Whitney U-test).

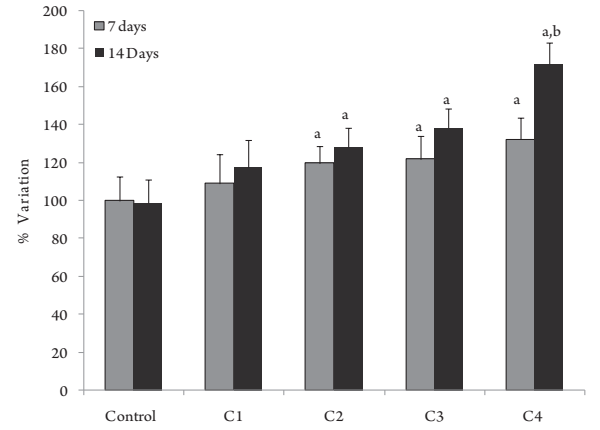

Figure2: Lysosomal/Cytoplasmic Volume Ratio in the Chloragogenous Tissue of E. Andrei after Exposure of Worms for 7 and 14 d to to Aloin Crude Extract (10, 50, 100 and 200 g/kg soils). Data, Expressed as Percent Change with Respect to Control Values, Represent the Mean ± SD of At least Ten Replicates. a: Indicates Statistically Significant Differences Respect to Control, ($P < 0.05$ Mann-Whitney U-test)

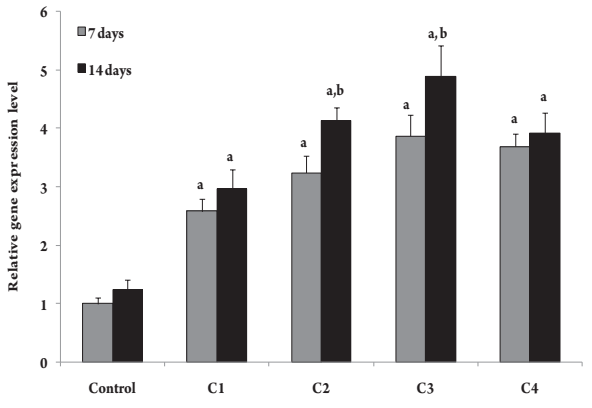

Figure 4: QPCR Data of p21 Target. Gene Expression was Performed Respect to the Control Condition; and was Normalized against Actin, 18S and Ribo-S13. a: Indicates Statistically Significant Differences Respect to Control, b: Indicates Statistically Significant Differences Respect to the Same Condition after 7 Days Exposure *$P < 005$ Threshold Cycle Random Reallocation Test According to Pfaffl et al. (2002), n = 4. Worms were Exposed for 7 and 14-Day to Aloin Crude Extract (10, 50, 100 and 200 g/kg soils).

Effects of Aloin Exposure on Genotoxicity Biomarkers

The effects of the aloin crude extract in the coelomocytes on DNA damage were evaluated by the MNi test (Fig. 3). No significant effect was observed in animals exposed to the lowest aloin concentration after 7 and 14 days. However exposure to C2, C3 and C4 for 7 and 14 days significantly increased the MNi frequency compared to control; we found the highest frequency, 4.19%, in worms exposed to C4 after 14 days. Interestingly, in worms exposed to C3 and C4 the MNi frequency was more pronounced after 14 days of exposure when compared to those depicted after 7 days of exposure. The analysis of the expression of two selected genes which encode proteins involved in DNA repair: P21 and topoisomerase, was realized by mean of qRT-PCR of worm total body transcripts using 18S rRNA, actin, and protein-ribo-S13 as reference genes (Figs. 4 and 5). A significant enhancement was observed in P21 mRNA in worms exposed to C1, C2, C3 and C4 after 7 days of exposure, compared to Control.

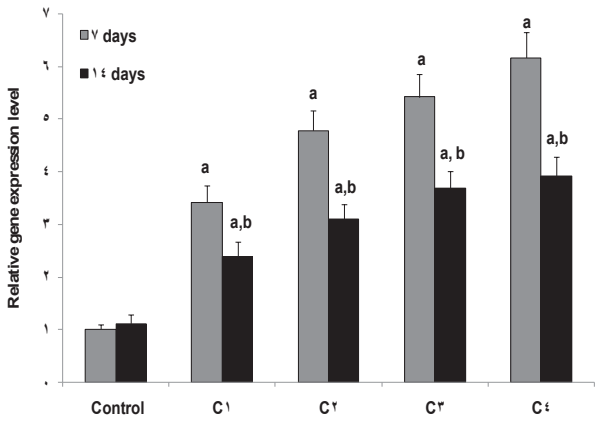

Figure 5: QPCR Data of Topoisomerase Target. Gene Expression was Performed Respect to the Control Condition (Soil 6); and was Normalized against Actin, 18S and Ribo-S13. a: Indicates Statistically Significant Differences Respect to Control, b: Indicates Statistically Significant Differences Respect to the Same Condition after 7 Days Exposure *P < 005 Threshold Cycle Random Reallocation Test According to Pfaffl et al. (2002), n = 4. Worms were Exposed for 7 and 14-Day to Aloin Crude Extract (10, 50, 100 and 200 g/kg soils).

After 14 days of exposure, this increase was maintained, and the difference in mRNA abundance was significantly higher than that observed after 7 days in worms exposed to C2 and C3. The same result was noted for topoisomerase for animals exposed to all of the aloin concentrations (C1-C4) after 7 days when compared to control. The highest expression levels of the topoisomerase gene were depicted in organisms exposed to C4 after 7 days. However and despite the sustained up-regulation pattern, topoisomerase gene expression was decreased after 14 days of exposure when compared to the same conditions after 7 days of exposure.

DISCUSSION

Chemical analyses of heavy metals cannot consider issues as mixture toxicity and the environmental conditions influencing chemical bioavailability. Biological systems are the target for the action of pollutants, and thus give information which cannot be retrieved from chemical analyses [23]. Thus, they could be utilized as diagnostic tools for integrated environmental assessment [24]. This underlines the importance of evaluating biological responses as an integral part of toxic and risk measurement [25]. For a correct assessment of soil contamination, the use of bioassays to quickly screen exposure and to show potential effects of contaminants is mandatory. Due to their life-style, earthworms are the first to be exposed to soils contaminants and, therefore, an environmental pollutant occurs earlier and their harmful effects are more evident than in other organisms [6, 26, 27]. To our knowledge, this is the first study that aims to investigate the effects of aloin exposure on DNA alterations in a species that is relevant to soil ecology. In addition, no studies have explored the transcriptional regulation of genes that may be involved in DNA repair mechanisms in the response to aloin exposure. Here we report the sub-chronic effects of increasing aloin concentrations on the biological responses of a non-target organism, the earthworm E. andrei. We first evaluated the growth response of worms and found a decreasing trend in all conditions expect in control. The maximum inhibitory effect of animal growth (in term of total body weight) was observed in animals exposed to C4 after 14 days. At the cellular level, le L/C volume ratio in worm's coelomocytes was assessed. Our data provided clues about the negative effect of aloin on this biomarker in a dose dependent manner. L/C volume ratio is reported to be a very sensitive cellular marker for evaluating the effects of environmental contaminants on living organisms [6, 11]. Stressed cells affected by noxious molecules react by metabolizing cellular macromolecules and organelles. When the elevated activity of the lysosomal vacuolar system is counterbalanced by an elevation in the protein synthesis rate, this is considered as an augmented turnover of the altered cellular elements. Therefore, the main effect of contaminant will be higher energy expenses. Conversely, if the contaminant level is extended affecting protein synthesis itself, the cells change of conformity to become catabolic with the loss of some of their cytoplasm following to disordered autophagic activity. Increasing in the volume ratio lysosomal /cytoplasmic is an early indication of such a condition [28]. Our findings revealed that this ratio was improved only in the cells of the chloragogenous tissue of organisms challenged to aloin crude extract being vey pronounced in worms exposed to C4. These results are in trend with the earthworm weight data; worms challenged with C4 exhibited a significant inhibition of growth rate (Fig. 1). In this work, the stability of genomic

Table 1: Q-PCR Primers and Taqman Probes

Gene name	Probe	Sense Primer	Antisense Primer
18S	CGCCGACAGAGTGC-CATCGACGAA	AATTCCGATAACGAACGAGAC-TCT	GCCACTTGTCCCTCTAAGAAGT-TA
b-Actin	AGTCCGGGCCATCCATCGTC-CACA	GGATCAGCAAGCAGGAGTACG	TGGTCATTGATAATGGAGG-CACTT
RiboS13	TCGCATGGTGTCGCTCAGACC-CGT	TCACAGATTGGTGTTATCCTTC-GA	GCAAGACCCTTAGCCTTCAGG
P21	ACTGGTCCATCAG-CGTCGATGCCA	AAAGGAGAATCTGTTTCGAG-GGAA	GGAACTGTGTGCTTGTCATC-TTC
Topoisome-rase	ACGGCCACATTCCTCACAGAG-GGT	AGCAGCAGTCGATCTTCACG	GCGGCAGCTTATAGTGGAAGA

Given are: Gene ID, NCBI Gene Identifier; Taqman Probe, Sense Primer and Antisense Primer Sequences. All Sequence are given 5' to 3'. Legend: 18S (AB558505.1), -Actin (DQ286722.1), RiboS13 (BB998368.1), P21 (CO058650.1), Topoisomerase (JZ124176.1).

DNA of E. andrei was significantly affected by the exposure to aloin in a concentration depend way. Indeed, the micronuclei frequency of coelomocytes was significantly higher in organisms challenged with the four applied concentrations when compared to control. The highest alteration was recorded in the highest concentration (C4). These alterations were probably due to the exposure to aloin and its derivates as well as to the metabolites that could be more toxic. Aloe vera latex contains numerous of biologically active compounds, especially anthraquinones. Various in vivo and in vitro tests have been realized to assess the cytotoxicity, genotoxicity, and carcinogenicity of the chemical components contained within the latex, particularly aloin, aloe-emodin, danthron, and emodin [29]. The incidence of genotoxic effect of anthraquinones in a set of in vivo and vitro assay systems [30-32]. One key mechanism of anthraquinone genotoxicity is that these compounds may promote the production of bioactivated metabolites that may yield to DNA damages [33]. As mentioned above in the chloragogen tissue of the earthworms exposed to the higher aloin concentrations an increase in the volume ratio Lysosome/Cytoplasme a consequence of the lysosomal vacuolar system activity was observed and this may clarify, at least partly, the high genotoxic effect induced in worms exposed to highly aloin contaminated soils.

Our data related to the gene regulation of the expression of two target genes, P21 and topoisomerase implicated in DNA repair [34, 35] provided indication about the occurrence in DNA of a transcriptional regulation metabolism in aloin exposed worms. The topoisomerases promote the sequential breakage and returning of single DNA or double helices strands, respectively, and take part pivot function in the genome stability [36]"9V. The significant up regulation were reported in worms exposed to the aloin concentrations. Moreover, the level of gene expression was significantly decreased after 14 days respect to 7 days but maintained up regulated when compared to control. The significant increase in the expression of the two genes implicated in DNA repair may in partly explicate the absence of significant MNi stimulation in animals exposed for 7d to the aloin lowest concentration. However, at 14 d the aloin causes DNA damage overwhelms the repair system and an increase in MNi is evident especially in the cells of the worms exposed to the higher aloin concentrations. The repair of DNA lesions provoked by toxic agents may vary between different species and tissues and the degree of repair are specific to the lesion [34]. Generally, there is modest information of DNA repair regulation including their time kinetics for the earth worms that are an ecological relevance. This is the first study, to our knowledge, integrating DNA repair gene expression, genotoxicity and cytotoxicity regulation in earthworms exposed to aloin. The results of this research highlight the risk due to the incorporation of aloe vera wastes and mainly aloin exudates in the agricultural soils. The data provided clues for the first time about the occurrence of alterations at cellular/at tissue and at total organism levels of worms exposed to an increasing aloin concentrations, thus highlighting a possible mechanism of action of the toxic chemicals. The genotoxic effects induced in earthworms exposed to aloin were also discussed and, the participation of genes related to DNA repair in the molecular response was demonstrated for the first time. Our result should be carefully considered in view of the re-use of aloe vera wastes and their incorporation in soils especially their effect on soil organisms, thus soil bio-fertility.

This work was supported by funds from the Ministry of higher Education, Scientific Research and Technology of Tunisia "Research Unit UR13AGR08".

ACKNOWLEDGMENTS

There is no acknowledgment for the present study.

CONFLICTS OF INTEREST

There is no conflict of interest.

FUNDING

There is no funding for the present study.

AUTHOR S' CONTRIBUTIONS

Fatma LANOUAR: Experiment, data analysis, paper redaction
Iteb BOUGHATTAS: Experiment, data analysis
Marouen MKHININI: Data analysis
Noureddine BOUSSERHINE: Experiment conception, paper redaction
Mohamed BANNI: experiment conception, data analysis, paper redaction

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