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ECOGENOTOXICITY EVALUATION OF SOIL OF AREA IMPACTED BY INVASIVE ALIENS SPECIES *Hovenia dulcis* Thumberg BY *Eisenia fetida* (Savigny, 1826) ASSAY

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Abstract: The species *Hovenia dulcis*, popularly known as the Japanese raisin tree, is considered an invasive alien species which can invade a variety of new habitats and establish new populations. Despite being quite prevalent in southern Brazil, environmental impacts are still poorly known, including those on the soil. The aim of the current paper was to evaluate the possible ecogenotoxic effects of the soil sampled in area with and without *H. dulcis* on *Eisenia fetida*. Soil samples were collected, and the experiments delineation for two toxicity tests were conducted: acute toxicity test and Comet assay. The results indicate the absence of ecogenotoxic potential of *H. dulcis* chemical compounds in E. *fetida* organism. Further studies should be performed using other organisms of the edaphic fauna to better evaluate the potential effects of such chemical compounds present in soil with Japanese raisin tree.

Keywords: biomonitoring, edaphic fauna, environmental toxicology, Japanese raisin tree.

Resumo: A espécie *Hovenia dulcis*, conhecida popularmente como Uva-do-Japão, é considerada uma espécie exótica que pode invadir uma variedade de *habitats* e estabelecer novas populações. Apesar de bastante prevalente no sul do Brasil, os impactos ambientais ainda são pouco conhecidos, inclusive no solo. Portanto, o objetivo do trabalho foi avaliar os efeitos ecogenotóxicos de amostras de solo coletadas em área com e sem *H. dulcis* no organismo modelo para fauna edáfica, *Eisenia fetida*. Foram coletadas amostras de solo e realizado dois testes de toxicidade: teste de toxicidade aguda e ensaio Cometa. Os resultados indicaram a ausência de potencial ecogenotóxico dos compostos químicos de *H. dulcis* no organismo *E. fetida*. Outros estudos devem ser realizados com diferentes organismos de solo para melhor avaliar os potenciais efeitos desses compostos químicos presentes no solo com Uva-do-Japão.

Palavras-chave: biomonitoramento, fauna edáfica, toxicologia ambiental, Uva-do-Japão.

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INTRODUCTION

Over the past few decades, invasive alien species (IAS) have been considered key drivers

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of human induced global environmental change, affecting ecosystem services, economy and public health (PEJCHAR; MOONEY, 2009; SIMBERLOFF, et al., 2013). Among IAS, plants (hereafter IAPS) represent a large group which has colonized new areas through well-known introduction pathways (intentionally and unintentionally) (PYŠEK, et al., 2011). The success of many IAPS in introduced areas has been explained by its high invasiveness associated with some performance-related traits (VAN KLEUNEN, et al., 2010), causing negative environmental impacts, such as decrease of local abundance and diversity, reduction of native plant fitness, as well as the increase of microbial activity and nutrient levels in the soil (PYŠEK, et al., 2012; VILÀ, et al., 2011).

In Brazil, 482 IAS have already been registered of which 209 are plants (I3N BRASIL, 2022). The Japanese rain tree (*Hovenia dulcis*) is a deciduous tree, native to East Asia (China, Japan, Korea, Thailand, and Vietnam) (KOPACHON, et al., 1996). Currently, it has been introduced in different regions as an ornamental tree (HYUN, et al., 2010), including southern Brazil (DECHOUM, et al., 2015). Over the past 40 years, *H. dulcis* has been intensively cultivated in this region, and acquisition of seedlings in seed nurseries and flower shops indicates an intense trade (BUTTENBENDER; ALMERÃO, 2018). Its main uses are as forestry products (e.g., wood production, windbreak, and ornamental), although honey production (where the bees are main pollinators) and forage (leaves and infructescence) for farm animals are common (CARVALHO, 1994). Apparently, cultivated individuals represent a key factor for the species' expansion on the invaded areas and are the most likely introduction vector in this region. Due to its high ecological plasticity, *H. dulcis* may invade areas in different successional stages, from open degraded areas to those forests with closed canopy (DECHOUM, et al., 2015). While some studies indicated changes in the structure and composition of plant communities (LAZZARIN, et al., 2015; SCHMIDT, et al., 2019) and in beta diversity of aquatic fauna (BIASI et al., 2020) the negative environmental impacts are still poorly known, including alleged impacts to soil community. Recently, some results indicated the impact of leaves and infructescence of *H. dulcis* on ant community in a subtropical Atlantic Rainforest in southern Brazil (PODGAISKI, et al., 2022). The Japanese raisin tree, as other plant species, produces allelochemicals (a subset of secondary metabolites, which are not required for metabolism) (LE, et al., 2018) which may influence the development of other native species (WANDSCHEER, et al., 2011; DE MATTOS, et al., 2018). At the same time, the effects of these chemical compounds on edaphic fauna are fully unknown. Thus, the aim this study was to evaluate possible toxic effects of *H. dulcis* chemical compounds on the biomonitor species *Eisenia fetida.*

MATERIAL AND METHODS

Study area and soil sampling

The study area is located in the Mata Paludosa Biological Reserve (hereafter REBIO Mata Paludosa), a Protected Area (PA) located in the municipality of Itati, state of Rio Grande do Sul (29°30'16"S; 50°07'02"W). Currently, this PA has an area of 271,87 acre, created in 1998 to conserve transition areas between the hillside and lowlands belonging to Atlantic Forest Biome, especially remnants of Mata Paludosa (forest formed on very humid soils, interspersed with wetland vegetation). Also, REBIO Mata Paludosa harbor a diverse flora and fauna, including endemic and endangered species (SEMA, 2010). The Japanese raisin fruit has been cultivated in this region since 1970. The uses of *H. dulcis* added to some biological (number of seeds) and ecological (fauna-mediated seed dispersion) attributes explain why this PA (and surroundings) has been successfully colonized. This species may form dense stands with high growth rates produces a patchy density distribution in invaded areas where it becomes dominant species (DECHOUM, et al., 2015). On the other hand, there are vicinity areas (2-3 meters) where *H. dulcis* was not capable to invade (2-3 meters). The REBIO Mata Paludosa has different trails located in distinct areas and, one of these, is Boi Trial (29°30'50.0"S 50°07'40.0"W) which connects two parts of the PA. This trail has 2 km long through which several stands of *H. dulcis* are observed mixed to native species.

The soil samples were collected in September 2019 in two sub-areas of Boi trial (300 m x 100 m each) with *H. dulcis* (HD+) and without the presence of *H. dulcis* (HD-). In both sub-areas, 20 soil (excluding litter) samples with 20 cm deep were collected using a metal shovel. The samples were stored in plastic bags, duly labeled and refrigerated with permanent ice, and then transported on the same day to the Ecogenotoxicology Laboratory of La Salle University, Canoas-RS. The soil humidity and pH were evaluated according to ABNT NBR 15537-2014.

Cultivation conditions

To carry out the toxicity tests, individuals of *Eisenia fetida* were purchased from a commercial supplier. Individuals were acclimatized in large plastic boxes (containing moist soil supplemented with organic material necessarily pesticide-free) in the laboratory during five weeks before test. After the acclimatation period, adults (identified by the presence of clitellum) were used in following experiments.

Preparation of Tropical Artificial Soil (TAS)

In order to perform the toxicity tests, a control soil was initially prepared, named tropical artificial soil (TAS), according to the recommendations of the toxicity test standards (OECD, 1984; ISO, 1998). The TAS is a composition of 70% of washed industrial fine sand, 20% of white clay (kaolin) and 10% of ground and dried *Shagnum* moss (bryophyte), both sieved. Before mixing, the sand was washed with distilled water and then over-dried in a stove during 24h at 105 °C - 110 °C. Soil humidity and pH were evaluated according to ABNT NBR 15537-2014. A positive control (TAS+) was prepared adding 750 mg/kg of boric acid to the TAS to validate the Comet assay and assess the sensitivity of individuals. Boric acid has been recommended as reference substance in some standardized tests in OECD soil (NIEMEYER, et al., 2018). Therefore, TAS and TAS+ were negative and positive control groups, respectively. Control items are used to evaluate the proper performance of the test system, thus validating the executed experiments (OECD, 2018). A negative control is an item for which the test system should give a response in relation to basal damage associated to animals handling, while a positive control induces a known change in the endpoint measured and fall within the dynamic (quantifiable) range of the test (here, mortality, height loss and DNA damage).

Acute Toxicity Test

The acute toxicity tests were performed in accordance with ISO 11268-1 (ISO, 1993) and ISO 11268- 2 (ISO, 1998). For each treatment, three samples of 200 g of soil (triplicates) were used, in which 10 individuals were exposed. The plastic containers were identified, and their lids perforated. Before being introduced into soil replicates, individuals were left to empty the intestinal contents for 1 h (purging) on filter paper and weighed in batches of 10 individuals. Here, two parameters were evaluated: mortality and weight loss percentages. Soil humidity and pH were constantly adjusted in according to ABNT NBR 15537-2014. During the 14 days of the test, the individuals were reared without food and under constant lighting (to maximize the contact with the soil) and room temperature (Min. 16.26 ° C and Max. 26.74 ° C; mean = 21.51 ºC). Mortality and weight were recorded on the 7th and 14th days, by applying mechanical stimuli to individuals, to assure they had died. The mortality and weight loss percentages obtained were compared between treatments. The mortality in the negative control should not exceed 10 per cent at the end of test as condition for the validation test (OECD, 1984).

Comet Assay

After the acute toxicity test, the six individuals survived of each treatment were exposed to 4 ml of extrusion liquid (5% ethanol, 2.5 mg / ml EDTA, 10 mg / ml of the mucolytic agent guaicol glyceryl ether, adjusted to pH 7.3), for 5 minutes in a heparin vacuum collection tube. The individuals were removed from the tubes and the material was resuspended and centrifuged for 15 minutes at 2500 rpm. The supernatant was removed, and 3 ml PBS was added for washing, being centrifuged again to isolate the *pellet*.

The DNA damage analysis was performed through the Comet assay as described by Tice, et al., (2000). Briefly, coelomocytes were embedded in 0.75% low melting agarose and spread onto a microscope glass slide pre-coated with 1.5% agarose. Slides were treated in lysis buffer for 1 h. The slides were then incubated for 20 min in freshly prepared alkaline buffer (pH > 13) and subjected to electrophoresis for 15 min at constant voltage (25 V; 0.90 V/cm) and current (300 mA). The slides were then stained with silver nitrate solution and scored under blind code for analysis.

Celoma cells were analyzed to assess the presence of comet-like nuclei. In the blades, the comets to be analyzed were searched from top to bottom (vertically), maintaining the impartiality. Measures close to the margin and comets at the blade ends were avoided. Two slides per individual were analyzed with 50 cores in each slide, adding up to 100 cores per individual.

In the visual analysis, the slides were observed in a standard optical microscope, at a 40X magnification. Comets were classified into five damage classes described previously. Two parameters were used in the visual analysis: Damage Index and Damage Frequency. The Damage Index (DI) was calculated as the sum of the product of the class with the number of comets of each class. Damage Frequency (DF) was calculated as the percentage of all damaged comets (class 1 to 4) in relation to the total number of comets counted.

Statistical Analysis

The normality of variables was evaluated by the Kolmogorov–Smirnov test. The statistical analyses of differences in mortality percentage, weight loss percentage and DNA damage measured by Comet assay were carried out using the non-parametric Kruskal-Walli's test. The critical level for rejection of the null hypothesis was a two-tailed P value of 5%. All analyses were performed using the GraphPad Prism 5.1 software.

RESULTS AND DISCUSSION

Biological invasions are considered a direct driver of biodiversity loss and there are many examples of significative negative environmental impacts (PYŠEK, et al., 2020). Due to its high ecological plasticity the Japanese raisin tree is an IAPS that has established populations in some are of southeastern South America (Argentina, Uruguay, Paraguay and Brazil). Some studies have indicated its different negative environmental impacts, especially those in the structure and composition of local plant communities (DECHOUM, et al., 2015; SCHMIDT, et al., 2020). Despite the influence of the allelochemicals compounds of *H. dulcis* upon seed germination of some plant species (RIBEIRO, et al., 2019) have been documented, the knowledge about influence of these compounds on edaphic fauna is poorly understood.

Overall, tested soil samples with and without *H. dulcis* (HD+ and HD-, respectively) did not cause lethality on individuals across all experiment, evaluated in 7th and 14th exposure day (Table 1). Only in positive control (TSA+), mortality exceeded 10% in 14th day. Also, individual's weight loss percentage did not differ considerably between treatments with exception of positive control in which individuals lost approximately 31% of their total weight after of 14 exposures days. Individuals in both treatments (HD+ and HD-) had similar results in relation weight loss percentage compared to negative control due food restriction during the test (Table 1). Studies assessing mortality and weight loss parameters to evaluate acute toxicity in any species of edaphic fauna exposed to IAPS (or exotic plant species) are scarce. Ortiz, et al., (2015) observed ecotoxicity of *Pinus elliottii* in *E. fetida* by avoidance behavior test, where the soil samples under pine plantations showed a significant difference from the other studied areas. Liu, et al., (2020) compared the influence of extracts from native species and those from IAPS on growth and respiration rates of *E. fetida*, showing significantly decrease in IAPS treatment.

Table 1. Acute toxic responses showing mortality (%) and weight loss (%) of earthworms exposed to soil without *Hovenia dulcis* (HD-) and soil with presence de *Hovenia dulcis* (HD+).

	Mortality (%)		Weight loss (%)	
Groups	**7th**	**14th**	**7th**	**14th**
TAS	0	3.33 ± 5.77	7.54 ± 3.52	11.22 ± 4.24
HD-	0	0	6.60 ± 5.37	6.53 ± 6.75
HD+	3.33 ± 5.77	0	11.70 ± 0.17	13.83 ± 8.41
TAS+	3.33 ± 5.77	23.33 ± 11.55**	15.61 ± 9.66	31.70 ± 12.74*

Mortality (%) and weight loss (%) was evaluated on the 7th and 14th days of exposure. Negative control = soil artificial tropical (TSA). Positive control = soil artificial tropical with boric acid (TSA+). **Difference in relation all groups in 14th ($P<0.01$); *Difference in relation HD- in 14th ($P<0.05$); Mean ± standard deviation. Kruskal-Wallis test ($P<0.05$).

There are some studies about the impact of IAPS on soil community. A review of the literature revealed a positive influence of IAPS on earthworm community (COYLE, et al., 2017). In our study, HD+ group did not differ significantly in relation to weight lost during exposure time. Rapidly growing species, such as *H. dulcis*, have edible leaves, rich in nutrients with high concentrations of nitrogen and phoshorus (KAZAKOU, et al., 2009; SZEFER, et al., 2016), larger leaf area, lower resistance and lower carbon concentration (ARAGO'N, et al., 2014; KAZAKOU, et al., 2006), which result in higher decomposition rates (SZEFER, et al., 2016). Therefore, there tends to be an increase in the availability of nutrients in the soil in places with *H. dulcis*, again benefiting invasive species and facilitating their establishment (DAVIS, et al., 2000; HUGHES; DENSLOW 2005). Kourtev, et al., 1999 observed significantly higher earthworm densities in areas colonized by IAPS (*Berberis thunbergii* and *Microstegium vimineum*). Also, soil pH, available nitrate and net potential nitrification were significantly higher in these areas.

DNA damage in *E. fetida* has been an important biomarker to evaluate some types of abiotic environmental perturbations (ZHANG, et al., 2015). However, little is known about IAPS influence in DNA damage induction on edaphic organisms. Liu, et al., (2020) observed increase in oxidative and DNA damage biomarkers in *E. fetida* when grown under root and leaf extracts from *Ageratina adenophora*, *Bidens pilosa* and *Erigeron annuus*. In our study, the Comet parameters, such as Damage Index (DI) and Damage Frequency (DF) were scored for evaluating DNA damage of coelomocytes cells (Figures 1 and 2). The results showed that the organisms exposed to the HD+ group presented less DNA damage in coelomocytes when compared to positive control group (P<0.05).

Figure 1: Damage index on coelomocytes of *Eisenia fetida* exposed to different treatments. The column represents the mean observed in 6 individuals of each replicate (triplicate). * P <0.05 (ANOVA test). Negative control = soil artificial tropical (TSA); Positive control = soil artificial tropical with boric acid (TSA+); HD- (soil without *Hovenia dulcis*) and HD+ (soil with *Hovenia dulcis*).

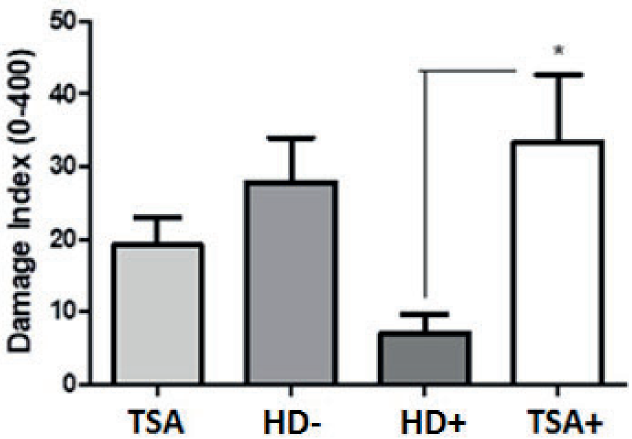
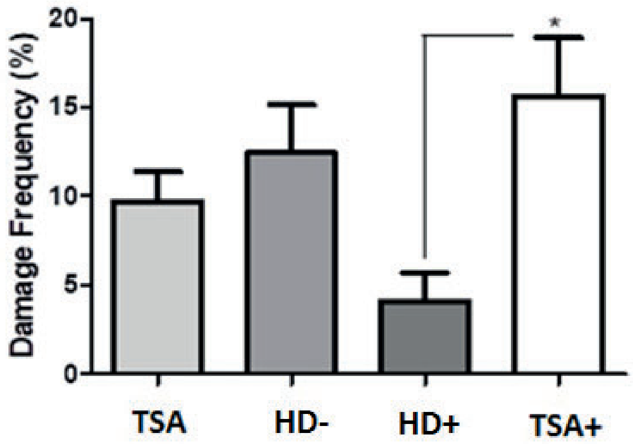


Figure 2: Damage frequency on coelomocytes of *Eisenia fetida* exposed to different treatments. The column represents the mean observed in 6 individuals of each replica (triplicate). * P <0.05 (ANOVA test). Negative control = soil artificial tropical (TSA); Positive control = soil artificial tropical with boric acid (TSA+); HD- (soil without *Hovenia dulcis*) and HD+ (soil with *Hovenia dulcis*).



Basic research providing information about *H. dulcis* toxicity and its action in system biology are limited. In HepG2 cells, ethanolic extract of branches from *H. dulcis* inhibited angiogenesis through suppression of VEGFR2 signaling and HIF-1α expression (HAN et al., 2017); and the aqueous *H. dulcis* seeds extracts reduced lipid accumulation in oleic acid-induced steatosis (KIM et al., 2016). Ethanolic extract of fruits from *H. dulcis* suppressed lipopolysaccharide (LPS)-stimulated inflammatory responses in Raw 264.7 cells (PARK et al., 2016). However, ethyl acetate and n-butanol *H. dulcis* ethanolic extract fractions induces mutagenicity, at the evaluated concentrations, in mitochondrial and genomic DNA in *Saccharomyces cerevisiae* strain, mediated by oxidative lesions (ARAÚJO, et al., 2021). One *in vivo* and *in vitro* study, investigated the antioxidant activity of the calli and leaf extract of *H. dulcis* found positive results (RIBEIRO, et al., 2015). One of the important roles of antioxidants in the biological system is to protect macromolecules from damage, including proteins, lipid and DNA (BECKER, et al., 2004).

Studies have shown that flavonoids are the main group of secondary metabolites produced by *H. dulcis* (HASE, et al., 1997; JI et al., 2002). Flavonoids are phenolic substances isolated from a wide range of vascular plants, acting in a sum of biological actions in mammals such as: antioxidant, antibacterial, antiviral, analgesic, antiallergic, hepatoprotective, cytostatic, apoptotic, among others (CHUNG, et al., 2010; HODEK, 2002).

Antioxidant compounds as myricetin, quercetin, ampelopsin (dihydromyricetin), taxifoline and others are among the most common flavonoids found in the invasive species *H. dulcis* (DE GODOI, et al., 2020). Myricetin causes antigenotoxic, antioxidant and hypoglycemic effects, whereas quercetin benefits glucose and lipid metabolism when tested in rodents with diabetes (DUTHIE, et al., 1997; NGO; KHOO, 2000; PENG, et al., 2017). Ampelopsin has hepatoprotective function and taxifoline is described as having an antioxidant role (HASE, et al., 1997; LEE, et al., 2007). Therefore, antioxidant action of the compounds present in *H. dulcis* may be protecting the genetic material of the individuals against attack by oxygen free radicals, causing positive effects on *E. fetida* when compared to genotoxic substance (positive control).

CONCLUSIONS

The *Hovenia dulcis* has been considered a major environmental problem, especially in the southern region of Brazil, where it has become increasingly prevalent. Overall, the acute toxicity test and Comet assay in *Eisenia fetida* were able to reveal no toxic effects in individuals exposed to soil collected in regions with *Hovenia dulcis*. The data here reported represent the first study evaluating toxicity in edaphic fauna exposed this invasive alien species. Even so, this result should be viewed with caution, with other experiments being carried out with the model species and with other species to examining how plant–soil edaphic fauna interaction can contribute to explanations related to exotic plant species problem.

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