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SCIENTIFIC ARTICLE
Toxicology

Cytotoxicity and the bioconversion strategy of *Aristolochia* spp.

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ABSTRACT

Aristolochia plants are notable from an ethnopharmacological viewpoint, but the relevance of these species for medicinal purposes has been debated because of their inherent toxicity. The convergence of these contrasting realities can be readily achieved using bioconversion methods, which have been shown to be useful tools for numerous applications, including the detoxification of biomass. In this context, methanolic extracts of leaves from *Aristolochia triangularis* and *Aristolochia gibertii*, as well as the feces of *Battus polydamas* larvae fed with leaves from these plants, were prepared, and their cytotoxic activities were evaluated on a human fibroblast cell line (GM07492). The leaf extracts were found to be cytotoxic, leading to reductions of 42.1 and 33.8% on cell viability, respectively, while the fecal extracts were considered inactive. In addition to evidencing the cytotoxicity of *A. triangularis* and *A. gibertii*, these findings demonstrated a potential bioconversion strategy for obtaining aristolochiaceous extracts with reduced toxicity using the larvae of a specialist phytophagous insect, thus renewing expectations in relation to the pharmacological importance of *Aristolochia* spp. The results were also ecologically relevant, as *B. polydamas* larvae were found to be able to detoxify compounds from host plants.

Keywords: Aristolochiaceae; bioconversion; insects; toxicity; detoxification.

INTRODUCTION

Despite being time-consuming, the natural product chemistry approaches continue to be the most promising for the discovery of new drugs. This statement remains valid even when the efficiencies of these strategies are compared with those of advanced tools, such as combinatorial chemistry and high-throughput screening of synthesized compounds (CHAGAS-PAULA et al., 2015).

However, the initial selection of a bioactive extract with minimal to no toxicity is a bottleneck to success in drug discovery programs (MCGAWL et al., 2014). Hence, extract libraries are usually initially submitted to toxicity testing, for example cytotoxicity assays, which are also used for other purposes, such as the search for anticancer drugs and the samples found to be toxic are classified as nonpriority (MCGAWL et al., 2014). Although the merit of this approach is widely recognized, caution should be exercised when applying it for three related reasons: i) it is essentially reductionist, ii) extracts are highly complex matrices and their potential should not be underestimated based on toxicological screening results, and iii) even biomasses/samples that are inherently toxic and/or inactive may show promise after being processed or engineered using tools such as bioconversion methods and strategies to generate natural product-like libraries (LÓPEZ et al., 2007; LIU; YU, 2010).

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The Aristolochiaceae family is an example of why caution should be observed before drawing an immediate conclusion regarding the pharmacological significance of certain plants. Species of this family of flowering plants, particularly those of the *Aristolochia* genus, contain a great number of bioactive compounds, making them notable within the global ethnopharmacological context (LOPES et al., 2001; HEINRICH et al., 2009). However, their medicinal potential conflicts with results of several toxicological studies, which demonstrated the cytotoxicity and mutagenicity of its representative chemical constituents as well as of herbal preparations containing such plants (LOPES et al., 2001; DECHBUMROONG et al., 2018; HAN et al., 2019). These findings have stimulated debate regarding the relevancy of *Aristolochia* spp. for medicinal purposes and have encouraged the development of approaches to overcome this issue (NOGUEIRA; LOPES, 2013a,b; DECHBUMROONG et al., 2018).

Among the various methods, bioconversion, which strictly differs from biotransformation and involves the use of living organisms (COLLINS; KENNEDY, 1999), has attracted great interest, as it has proven to be a very useful tool for numerous applications, including access to new compounds, structural modification and biomass detoxification (PALMQVIST et al., 2000; LIU; YU, 2010; NOGUEIRA; LOPES, 2013a,b). The greatest attention has been given to the utilization of microorganisms for these purposes, but the potential of insects, including the Swallowtail butterfly *Battus polydamas*, has also been verified (VENISETTY; CIDDI, 2003; NOGUEIRA; LOPES, 2013a,b; RAMOS, 2013).

Thus, the objective of this work was to evaluate the *in vitro* cytotoxicity of *Aristolochia triangularis* and *Aristolochia gibertii* before and after metabolization of leaf biomasses of these species by larvae of a specialist phytophagous insect, *B. polydamas*, in order to propose a potential bioconversion approach to obtain aristolochiaceous extracts with reduced toxicity, contributing to overcome challenges that have limited the use of the birthwort family for therapeutic purposes.

MATERIAL AND METHODS

Plant materials and insects: collection and identification

The plant materials and insects were collected at the Medicinal Plants Garden of the Federal University of Grande Dourados, Dourados (MS), Brazil, during April 2016. *A. triangularis* Cham. and *A. gibertii* Hook. were identified by Dr. Joelcio Freitas and voucher specimens (MBML 53232 and MBML 53233, respectively) were deposited at the herbarium of Museu de Biologia Prof. Mello Leitão (MBML) in city of Santa Teresa, Espírito Santo, Brazil. *Battus polydamas* was identified by MSc. Paulo Ricardo Barbosa de Souza. The authorization IBAMA number was 51842 and the access registers CGEN/MMA numbers were AC96E87 and A1F6637.

Larval rearing and collection of fecal material

Battus polydamas larvae of different instars, which were fed in the laboratory with fresh leaves from *A. triangularis* or *A. gibertii* during the first half of April 2016, were reared in cages [30 × 30 × 40 cm (w × h × l)] under semicontrolled conditions: artificial light during the natural photoperiod and ambient humidity and temperature. The fecal materials excreted by the insects were collected every 48 h, air-dried for 15 days and stored at room temperature until extraction.

Extraction steps

The feces of the *B. polydamas* larvae fed with *A. triangularis* and *A. gibertii* leaves (60.0 g and 8.6 g, respectively) and leaves from these two plants (25.6 and 10.0, respectively) were individually and exhaustively extracted by maceration with methanol (HPLC grade, Vetec). After the simple filtration steps, the obtained methanol solutions were concentrated under reduced pressure to give the extracts of the feces [FE-1: 6.82 g (11.4%); FE-2: 0.92 g (10.7%)] and leaves [LE-1: 4.92 g (19.2%); LE-2: 1.28 g (12.8%)].

Cytotoxic activity and data analysis

The cytotoxicity of the methanol extracts of *B. polydamas* feces and *A. triangularis* and *A. gibertii* leaves were evaluated using the liquid extraction method (International Standard ISO 10993-5, 2009). GM07492 cells were seeded in a 96-well plate, maintained in culture medium enriched with fetal bovine serum (FBS) and antibiotics, and incubated for 24 h at 37 °C in a humidified atmosphere containing CO_2 (5%) and atmospheric air (95%). The extract samples were individually prepared at a concentration of 20 mg/mL using Dulbecco's modified eagle medium (DMEM) as a diluent and allowed to stand for 24 h at 37 °C. After

this time, the culture medium of the wells was replaced with 100 µL of culture medium containing either the extracts, the negative control or the positive control, or vehicle, and the cells were maintained under ideal cultivation conditions for 24 h. Subsequently, the wells were washed with 150 µL phosphate buffered saline (PBS) (1×) and 50 µlL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each. The 96-well plate was again incubated for 4 h at 37 °C, after which 100 µL of isopropanol was added to each well to solubilize the formazan crystals. Finally, the quantity of dissolved formazan crystals was determined spectrophotometrically and the optical density values measured at 570 nm were converted into the perceptual of cell viability in relation to a negative control. DMEM + FBS + antibiotics and DMEM + DMSO 25% were used as the negative and positive controls, respectively. The calculations of the reduction in cell viability were performed following the recommendations described in International Standard ISO 10993-5 (2009).

RESULTS AND DISCUSSION

Monolayers of GM07492 cells were individually treated with 100 µL of culture medium containing 2 mg of one of the extracts to be tested: LE-1, FE-1, LE-2, or FE-2. Relative cell viability values of 57.9, 85.8, 66.2 and 94.2% were obtained for the samples, respectively (Fig. 1), corresponding to 42.1, 14.2, 33.8, and 5.8% reductions in the cell viability as determined using liquid extraction assay.

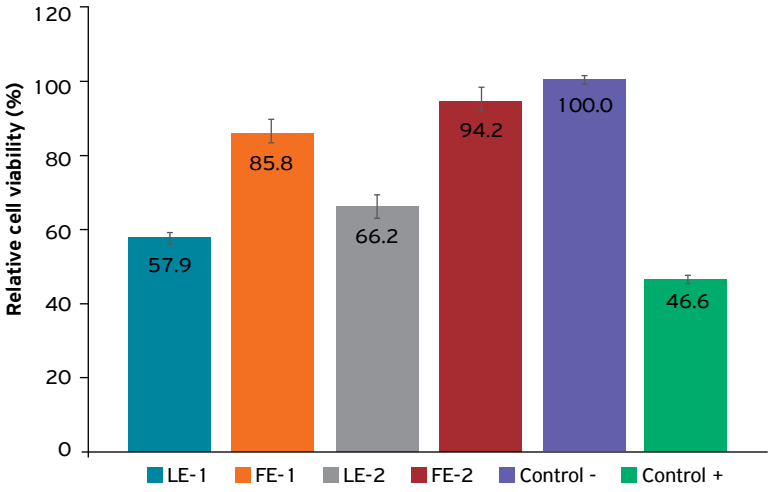

Figure 1. Relative cell viability values. The graph shows the comparison of cell viability between extracts of leaves and feces in relation to the negative and positive controls. LE-1 and LE-2: extracts of leaves from *A. triangularis* and *A. gibertii*, respectively. FE-1 and FE-2: extracts of feces of *B. polydamas* larvae fed on *A. triangularis* and *A. gibertii* leaves, respectively.

A reduction of more than 30% in cell viability can be interpreted as a cytotoxic effect (International Standard ISO 10993-5, 2009). Therefore, the LE-1 and LE-2 extracts were cytotoxic at the concentration assessed, with the former presenting significantly greater toxic action than the later ($p \leq 0.00$). In previous studies, extracts from *A. triangularis* were shown to cause mortality in *Artemia salina* larvae, as well as being cytotoxic in an *Allium cepa* bioassay and towards KB cells (MONGELLI et al., 1996; 2000; SILVA et al., 2019). Conversely, the toxicological properties of *A. gibertii* had not been previously described in literature.

The cytotoxicity of LE-1 and LE-2 reflected their chemical compositions, since both plants selected for this work can produce compounds that are known to be cytotoxic. Nearly fifty compounds have been reported to occur in *A. triangularis*, among which only aristolactam I and aristolochic acids I, II, C and D, all of which are cytotoxic, were detected in leaves of this species (SILVA et al., 2019; MICHL et al., 2016). In contrast, a total of twelve chemical constituents, including the cytotoxic lignans cubebin, (-)-hinokinin, and (-)-kusunokinin, have been isolated from *A. gibertii* leaves (MARCHESINI et al., 2009).

As expected, the fecal extracts were not considered cytotoxic, which supported the hypothesis of this study that bioconversion by *B. polydamas* larvae would be an efficient way to obtain Aristolochiaceous extracts with minimal to no toxicity. Consequently, the method developed in this research could renew expectations regarding the potential of the controversial *Aristolochia* spp.

These results also had chemical-ecological relevancy, since they suggest that *B. polydamas* larvae detoxify the chemical constituents of their host plants. Phytophagous insects have developed a variety of strategies to overcome the chemical barriers imposed by plants (EDWARDS; WRATTEN, 1981; OPTIZ; MÜLLER, 2009; RAMOS, 2013). A particularly effective way of dealing with this issue is to metabolize the chemical constituents via a variety of metabolic pathways or with the help of

endosymbiotic microorganisms (EDWARDS; WRATTEN, 1981; OPTIZ; MÜLLER, 2009; RAMOS, 2013). Similar detoxification pathways have been observed in Lepidoptera, whose larvae feed on plants belonging to several botanical families (RAMOS, 2013). The toxic compounds in the plants are generally transformed into more polar and less toxic derivatives, mainly by demethylation, hydroxylation and glycosylation reactions (RAMOS, 2013).

Although virtually nothing is known about the toxicity of most bioconversion products, *B. polydamas* larvae are known to be able to metabolize aristolochic acids (AAs), aristolactams (ALs), lignans and diterpenes (NOGUEIRA; LOPES, 2013a,b; NOGUEIRA, 2014). For the metabolism of AAs, all chemical transformations mentioned above have already been observed, although the glycosylated derivatives still lack an unambiguous structural determination (PRIESTAP et al., 2012). A single study on the metabolism of ALs has been published in the literature, which verified that the ALs I and II were oxidized into AAs I and II, respectively (URZÚA et al., 2013). The metabolism of dibenzylbutyrolactone lignans by *B. polydamas* larvae produced (−)-(8*S*,8*R*')-(3,4-methylenedioxy)-(3',4')-(dimethoxy)-9'-O-β-glucopyranosyl-lignan-9-oic acid and (−)-(8*S*,8*R*')-[3,4:3',4'-bis(methylenedioxy)]-9'-O-β-glucopyranosyl-lignan-9-oic acid, whereas the labdane diterpene (−)-(5*R*,8*R*,9*S*,10*R*,13*R*)-8-hydroxy-labdan-15-oic acid was bioconverted into (4*S*,5*S*,8*R*,9*S*,10*R*,13*R*)-8,18-dihydroxy-labdan-15-oic acid, (4*S*,5*S*,7*R*,8*S*,9*S*,10*R*,13*R*)-7,8,18-trihydroxy-labdan-15-oic acid and (−)-(4*S*,5*S*,8*R*,9*S*,10*R*,13*R*)-8-hydroxy-labdan-15,18-dioic acid (NOGUEIRA; LOPES, 2013a,b; NOGUEIRA, 2014).

CONCLUSIONS

Extracts of *A. triangularis* and *A. gibertii* leaves were found to be cytotoxic to GM07492 cells, whereas the extracts of the feces of *B. polydamas* larvae fed on leaves of these plants were inactive. Thus, the bioconversion strategy utilized in this work was shown to be effective for the detoxification of aristolochiaceous foliar biomasses, renewing expectations of the pharmacological relevance of *Aristolochia* spp. The reduction in toxicity observed after the digestion of leaves from these two plants by *B. polydamas* larvae also had chemical-ecological implications, as it demonstrated that these insects may have their own strategies to overcome the chemical barriers imposed by their host plants.

AUTHORS' CONTRIBUTIONS

Conceptualization: Nogueira, C.R.; Silva, J.D.A.; Vieira, M.C.; Cardoso, C.A.L.; Amaral, A.C. **Data curation:** Nogueira, C.R.; Silva, J.D.A.; Carvalho, R.A.; Amaral, C.S.T., Amaral, A.C. **Formal analysis:** Nogueira, C.R.; Silva, J.D.A.; Carvalho, R.A. **Funding acquisition:** Nogueira, C.R.; Vieira, M.C.; Cardoso, C.A.L.; Amaral, C.S.T.; Amaral, A.C. **Methodology:** Nogueira, C.R.; Silva, J.D.A; Vieira, M.C.; Cardoso, C.A.L.; Carvalho, R.A.; Amaral, C.S.T.; Amaral, A.C. **Writing – review & editing:** all author contributed equally.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

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CONFLICTS OF INTEREST

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

ETHICAL APPROVAL

Not applicable.

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