



## Toxicological properties of an aqueous extract of *Aristolochia triangularis* leaves, using the brine shrimp lethality and *Allium cepa* bioassays

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**ABSTRACT:** The aqueous extract of *Aristolochia triangularis* leaves showed activity against *Artemia salina* larvae, with an LD<sub>50</sub> of 370.6 µg/mL. In experiments with *Allium cepa* seeds, the extract caused a 51.26% reduction in the germination index, inhibited mean root growth, and was cytotoxic at concentrations of 668 and 2,000 µg/mL. Its antioxidant activity was additionally assessed in this research and the ferric reducing antioxidant power value was found to be 391.2 µM/g. Such health-beneficial property can be attributed partly to the total phenolic content, spectrophotometrically determined as 52.67 mg/g. The occurrence of cytotoxicity suggested caution when consuming teas from *A. triangularis* leaves for medicinal purposes, and equally reveals the need for further studies to investigate their adverse effects.

**Key words:** cipó-mil-homens, infusion, toxicity.

## Propriedades toxicológicas de um extrato aquoso de folhas de *Aristolochia triangularis* utilizando os bioensaios de letalidade com *Artemia salina* e *Allium cepa*

**RESUMO:** O extrato aquoso de folhas de *Aristolochia triangularis* mostrou atividade sobre larvas de *Artemia salina*, com um DL<sub>50</sub> de 370,6 µg/mL. Em experimentos com sementes de *Allium cepa*, o extrato provocou uma redução de 51,26% no índice de germinação, inibiu o crescimento radicular médio e foi citotóxico nas concentrações de 668 e 2.000 µg/mL. Sua atividade antioxidante foi adicionalmente avaliada nesta pesquisa e o valor de poder redutor de íons férrico foi de 391,2 µM/g. Tal propriedade benéfica para a saúde pode ser parcialmente atribuída ao conteúdo de compostos fenólicos totais, espectrofotometricamente determinada como 52,67 mg/g. A ocorrência de citotoxicidade sugeriu cautela ao consumir chás de folhas de *A. triangularis* para propósitos medicinais, e igualmente revela a necessidade por estudos adicionais para investigar os efeitos adversos deles.

**Palavras-chave:** cipó-mil-homens, infusão, toxicidade.

## INTRODUCTION

*Aristolochia* spp. (*Aristolochiaceae*), widely used in homeopathy and traditional medicine, contains numerous bioactive compounds, and are chemically characterized by the predominance of lignoids, terpenoids, alkaloids, alkalamides, flavonoids, and nitrophenanthrene derivatives (LOPES et al., 2001).

*Aristolochia triangularis* Cham., a woody liana, is 1 of the 92 known Brazilian *Aristolochia* species (BARROS et al., 2015). It is among the most cited plants within the ethnobotanical sector

of the South Region of Brazil, and has been used for treating several disease conditions such as diabetes, rheumatism, infections, and wounds and skin diseases (HEINRICH et al., 2009; TROJAN-RODRIGUES et al., 2012; PEREIRA et al., 2018; VIBRANS et al., 2018).

Based on its ethnopharmacological uses, extensive studies investigating the therapeutic potentials and biosafety of this species have been carried out and reported. The dichloromethane extract of *A. triangularis* roots have been shown to possess an anti-inflammatory efficacy similar to that of phenylbutazone (MUSCHIETTI et al., 1996).

Conversely, organic extracts from aerial parts of this plant have been shown to exhibit significant cytotoxicity and DNA interaction (MONGELLI et al., 2000). Other evidence indicated the cytological effects of infusions from *A. triangularis* branches and decoctions from secondary growth stems, on the cell cycle of *Allium cepa* L. meristems (SCHVARTZMAN et al., 1977; AMAT et al., 2002), and the anti-mycobacterial activity of hydroalcoholic drinks prepared from *A. triangularis* (OLIVEIRA et al., 2007). In addition, a recent study demonstrated that the methanol extract from the rhizomes and roots of this species and some compounds isolated from it had promising bioactivities against microbial pathogens, in certain cases comparable or better than those of existing commercial drugs (ampicillin and chloramphenicol) (PEREIRA et al., 2018).

*A. triangularis* stems, rhizomes, leaves, and roots have been chemically examined, and a total of 47 non-extractable (by steam distillation) compounds described (RÜCKER & LANGMANN, 1978; RÜCKER et al., 1981; LOPES et al., 1990; PRIESTAP et al., 1990; LEITÃO et al., 1991; LIN et al., 1997; MICHL et al., 2016; PEREIRA et al., 2018). Among them, only the aristolactam I and aristolochic acids I, II, C and D have been reported in leaves so far (MICHL et al., 2016).

Cytotoxicity and mutagenicity studies indicated that more attention be paid to the consumption of *Aristolochia* spp., as evidenced by the toxicity of its representative chemical constituents, particularly those from aristolactams and aristolochic acids (MICHL et al., 2016). Incidentally, these phenanthrene derivatives have been implicated as the main active and toxic components in herbal preparations containing *Aristolochia*, and account for the extensive literature on this species (LOPES et al., 2001; MICHL et al., 2016). However, there are fears that such a reductionist view can be detrimental to understanding the true pharmacological significance of these plants; therefore, stimulating more detailed investigations (LOPES et al., 2001).

Overall, it can be considered that studies specific to the aqueous extracts of *Aristolochia* spp. are relatively scarce. Hence, the main objective of this study was to evaluate the toxicological properties of the aqueous extract of *A. triangularis* leaves, using the brine shrimp lethality and *A. cepa* bioassays. Its ferric reducing antioxidant power, total phenolic, and total tannin content were secondarily evaluated in this research, for the only purpose of generating preliminary chemical data and mapping a possible biological activity for this extract.

## MATERIALS AND METHODS

### General experimental procedures

All chemical reagents and solvents were of analytical grade, purchased from Synth<sup>®</sup> or Sigma-Aldrich<sup>®</sup>, and used without further purification. Distilled water was obtained from a distiller, and ultrapure deionized water (resistivity of 18.2 MΩ) from a Milli-Q<sup>®</sup> system (Songpa-gu, South Korea). The UV absorptions were measured on a UV-Vis digital spectrophotometer from Global Trade Technology. Freeze drying was carried out using a lyophilizer (CHRIST, ALPHA 1-2 LD, plus) (Spain). An ACB labor water bath (Germany) was used for temperature control. The Binocular E100 (Nikon) microscope was used to read slides.

### Plant material

Plant material (leaves) was harvested from the Medicinal Plants Garden of the Federal University of Grande Dourados, Dourados, MS, Brazil, in November 2016, and identified as *A. triangularis* Cham. by MSc. Joelcio Freitas. A voucher specimen (MBML53232) was deposited at the herbarium of Museum of Biology Prof. Mello Leitão (MBML), in the city of Santa Teresa, Espírito Santo State, Brazil. Authorization IBAMA number 51842. Access register CGEN/MMA number A1F6637.

### Infusion preparation

To prepare the infusion to 2% (w/v), *in natura* leaves of *A. triangularis* (30.0 g) were selected and processed using a preliminary mechanical operation. In brief, the leaves were section-divided, immersed in pre-heated distilled water to 95 °C, and the resulting mixture allowed to rest for 15 min at room temperature, before being filtered through analytical filter paper. Two thirds of the aqueous solution obtained was frozen and lyophilized, successively, to give the freeze dried aqueous extract (FDAE; 1.93 g; 6.43%), and the left over was stored at 4 °C for the phytochemical analyses and bioassays.

### Toxicity and genotoxicity assays

#### Acute toxicity test in *Artemia salina*

The brine shrimp (*A. salina*) lethality test was performed using the Meyer method (MEYER et al., 1982). Briefly, *A. salina* cysts were incubated under artificial light (60 W) and aeration for 48 h, in a synthetic sea salt solution (20 g/L), at pH 8, after adjusting with NaHCO<sub>3</sub>. FDAE solutions in 5 concentrations (67, 401, 668, 1,336, and 2,000 µg/mL) were prepared for this bioassay. Saline solution and

rutin were used as negative and positive controls, respectively. Each concentration level was evaluated in triplicates with 10 larvae each. The death counts were obtained after 24 h of larvae-solution contact, and the acute toxicity was expressed as LD<sub>50</sub>.

#### *Allium cepa* bioassay

Tests involving *A. cepa* were performed following the protocol proposed by MATSUMOTO AND MARIN-MORALES (2005), with modifications. The FDAE was screened at the concentrations of 67, 401, 668, and 2000 µg/mL, with distilled water and the aqueous solution of the trifluralin herbicide (0.84 ppm) being the negative and positive controls, respectively. For each treatment, 60 seeds of *A. cepa* (Baia Periforme variety; ISLA®) were placed in a Petri dish with germitest paper. Dishes containing the seeds were treated with FDAE solutions and maintained in a BOD incubator at 25±2 °C with a photoperiod control (12 L:12 N) for 96 h. The visible radicle was measured with a Digimess® digital caliper passed 96 h after of the germination onset. The parameters used to define the toxicity effects of the extract were the percentages of Relative Seed Germination (RSG), Relative Root Growth (RRG) and Germination Index (GI) which were calculated using the formula described by HOEKSTRA et al. (2002) and WALTER et al. (2006). Roots 1.5 cm long were placed on a Carnoy's solution [ethanol and acetic acid (3:1 v/v)] for 24 h, washed 3x with distilled water, hydrolyzed with 1 N HCl at 60 °C for 10 min in a water bath, and washed again with distilled water. After another round of washing, they were stained in Schiff's reagent using Feulgen's method (ALEXANDER et al., 1950) for 1 h 30 min. After staining, root meristems were covered with a coverslip and slightly smashed in a drop of 45% acetic carmine. For each treatment, 5 slides were prepared and photographed using the Binocular E100 (Nikon) optical microscope at a 400x magnification, such that 1,000 cells were checked per slide, resulting in a total of 5,000 cells. The cytotoxic potential was based on mitotic index (MI), and cell death index (CDI). The chromosomal alterations index (CAI) was evaluated by counting chromosome alterations (nuclear budding, C-metaphase, chromosomal loss, multipolar anaphases, chromosomal breaks, lobed nuclei and chromosomal bridges), and micronuclei were used to calculate mutagenicity indices (MTI). Cytotoxicity and genotoxicity potentials were calculated using the formula described by FRANCISCO et al., 2018.

#### Statistical analyses

The Kruskal-Wallis test and Dunn's post test were used to compare negative control and

treatment groups in the cytotoxicity and genotoxicity assay, and the analysis of variance (ANOVA) test and Tukey post test were used for the tests involving the Relative Seed Germination (RSG), Relative Root Growth (RRG) and Germination Index (GI) ( $\alpha=0.05$ ). All analyses were conducted using the R statistical software (R DEVELOPMENT CORE TEAM, 2018). LD<sub>50</sub> value was derived using the technique described by MEYER et al., 1982, which consists of obtaining a straight line by means of logarithmic transformation, followed by linear regression analysis.

#### *Ferric reducing antioxidant power (FRAP)*

The antioxidant potential of FDAE was evaluated in 5 concentrations (67, 401, 668, 1,336, and 2,000 µg/mL), in triplicate, using the FRAP method (EMBRAPA, 2006). Two hundred and seventy microliters of distilled water was initially added in to each test tube containing 90 µL of the test solution or distilled water (blank), followed by 2.7 mL of the FRAP reagent. The samples were homogenized in a vortex, incubated in a water bath at 37 °C for 30 min, and their respective absorbencies measured at 595 nm. Aqueous solutions of ferrous sulfate heptahydrate were used to construct a calibration curve in the concentration range of 500-2,000 µM, and the FRAP value was expressed as µM Fe/g.

#### *Phytochemical analyses*

##### *Total phenolic compounds*

The total phenolic content was determined following the method described by DJERIDANE et al. (2006). Briefly, 100 µL of the infusion (2% w/v) was added to 1,000 µL ultrapure water and 500 µL Folin-Ciocalteu's reagent (1/10) in water. After 1 min, 1,500 µL Na<sub>2</sub>CO<sub>3</sub> (20% w/v) was added. The final mixture was homogenized, and incubated for 2 h in the dark. The absorbance was read using a spectrophotometer (FENTO 700 PLUS) ( $\lambda=760$  nm). Gallic acid from Sigma-Aldrich (USA) was used as a standard at concentrations ranging from 10-100 µg/mL. The results were expressed as mg GAE/g (GAE: gallic acid equivalent).

##### *Tannin content*

The condensed tannins were quantified by the vanillin-HCl method (BROADHURST & JONES, 1978; AGOSTINI-COSTA et al., 1999) and expressed in mg of catechin by g of FDAE. Briefly, an aliquot of 4 mL of a methanol solution of vanillin (4%) and HCl (8%) was heated in a water bath at 30 °C for 30 min. To preheated aliquot was added 1 mL of infusion to 2%, the mixture obtained was maintained at 30 °C for

another 20 min, and then their absorbency measured at 500 nm. Catechin was used to plot a calibration curve at the concentrations ranging from 2.5-40 µg/mL and estimate the condensed tannin content in the infusion of *A. triangularis* leaves.

## RESULTS AND DISCUSSION

FDAE caused mortality on *A. salina*, with an LD<sub>50</sub> of 370.6 ± 47.3 µg/mL, being classified as moderately toxic (MEYER et al., 1982; AMARANTE et al., 2011). Although the occurrence of aristolochic acids and 1 aristolactam was hitherto described in leaves of an *A. triangularis* specimen cultivated at the botanical garden at Dresden University of Technology, Dresden, Germany (MICHL et al., 2016), the compounds responsible for the larval mortality observed in our study remain unknown. However, studies verifying the toxicity of FDAE highlight the need for careful consumption of teas from *A. triangularis* leaves, independent of their metabolomic profile.

To investigate the risks associated with the consumption of the infusion of *A. triangularis* leaves, the mutagenic, cytotoxic, and genotoxic activities of FDAE were equally evaluated by the *A. cepa* bioassay. The percentages of Relative Seed Germination (RSG), Relative Root Growth (RRG) and Germination Index (GI) are presented in table 1. Relevant statistical differences in GI were observed between the negative control and the concentration 2,000 µg/mL. Because a 51.26% reduction was observed in the GI of *A. cepa* seeds, the toxicity of FDAE was confirmed, besides the existing evidence of their allelochemical potential. At all concentrations the RSG of the treated groups was statistically different from that of the negative control groups.

It can, therefore, be affirmed that the FDAE inhibits root growth at all concentrations.

Concordantly, the MI obtained after FDAE treatment of *A. cepa* seeds indicated cytotoxic action on the cell cycle at 668 and 2,000 µg/mL (Table 2). Antimitotic action was initially reported for a decoction of secondary growth stems from *A. triangularis*, with MI exhibiting a statistically relevant value in relation to the controls (AMAT et al., 2002). For the CDI, CAI and MTI no significant difference was observed between the treatment and negative control groups. The genotoxic and mutagenic effects were not observed at any concentration of FDAE when compared with the negative control group (Tables 1 and 2). Though not statistically significant, the main genetic alterations found were chromosome bridges, chromosomal loss, nuclear budding, and lobed nuclei.

These results further confirm the need for special attention by the World Health Organization (WHO) in discouraging the use of *Aristolochia* for medicinal purposes (MICHL et al., 2016; JADOT et al., 2017). Acquiring extensive knowledge on the therapeutic potential and risks of consuming *A. triangularis* will require further studies, specifically on pharmacological biosafety, as this species is one of Brazil's most cited plants in the ethnopharmacological sector.

Although the FDAE had some level of toxicity, it exhibited a considerable ferric reducing power, with a FRAP value of 391.1 ± 1.2 µM/g (FERNANDES et al., 2016). This is in line with already existing studies demonstrating that plant extracts from members of the *Aristolochia* genus possess antioxidant properties. Such species include: *Aristolochia bracteata*, *Aristolochia bracteolata*, *Aristolochia brasiliensis*, *Aristolochia clematitis*, *Aristolochia giberti*, *Aristolochia indica*, *Aristolochia*

Table 1 - Relative Seed Germination (RSG), Relative Root Growth (RRG) and Germination Index (GI) of the aqueous extract from *A. triangularis* leaves upon the cell cycle of *A. cepa* meristems.

TR (µg/mL)	RSG%	RRG %	GI (%)
NC	100.00±0.00	100.00±0.00	100.00±0.00
2000	76.19±0.00*	63.97±25.57	48.74±19.49*
668	79.34±0.00*	75.81±30.23	60.17±24.00
401	82.54±0.00*	88.57±30.00	72.11±24.76
67	82.54±0.00*	97.73±23.99	80.66±19.80
PC	76.19±0.00*	67.54±19.31	51.46±14.71*

TR: Treatments; PC: Positive control (Trifluralin 0.84 ppm); NC: Negative control (deionized water); \*Significantly different in relation to the negative control.

Table 2 - Median of mutagenic, genotoxic and cytotoxic potentials of the aqueous extract from *A. triangularis* leaves upon the cell cycle of *A. cepa* meristems.

TR ( $\mu\text{g/mL}$ )	MI %	CAI %	MTI %	CDI %
NC	79.51 2.15	0.10 0.22	0.00 0.05	0.00 0.02
2000	60.51 1.58*	0.15 0.19	0.49 1.03	0.00 0.02
668	63.42 6.82*	0.14 0.17	0.00 0.00	0.00 0.02
401	70.67 2.17	0.05 0.19	0.00 0.08	0.00 0.06
67	70.42 5.32	0.05 0.12	0.00 0.02	0.00 0.05
PC	44.76 12.12*	6.28 2.34*	2.80 0.92*	0.41 1.14*

TR: Treatment; MI: Mitotic index; CAI: Chromosomal alteration index; MTI: Mutagenicity index; CDI: Cell death index; PC: Positive control (Trifluralin 0.84 ppm); NC: Negative control (distilled water); \*Significantly different in relation to the negative control.

*krisagathra*, *Aristolochia longa*, *Aristolochia ringens*, and *Aristolochia tagala* (VELÁZQUEZ et al., 2003; THIRUGNANASAMPANDAN et al., 2008; ANGALAPARAMESWARI et al., 2009; ANILKUMAR et al., 2014; BENMEHDI et al., 2017; MEROUANI et al., 2017; OBONGA et al., 2017).

The FDAE antioxidant potential observed can be attributed partly to the presence of tannins, and mainly to the high total phenolic content, since quantitative phytochemical analyses revealed the content of these compounds in FDAE as  $0.02 \pm 0.01$  and  $52.67 \pm 0.13$  mg/g, respectively. These values are in conformity with what was expected for an *Aristolochia* sp., provided that species belonging to this genus are rich sources of flavonoids, particularly diglycosides, and other phenolic chemical constituents, mainly phenylethyl and phenanthrene derivatives, phenylpropanoids, lignoids, alkaloids, and alkaloids (LOPES et al., 2001; NOGUEIRA & LOPES, 2012).

## CONCLUSION

The aqueous extract of *A. triangularis* leaves exhibited toxicity and cytotoxicity in the *A. salina* and *A. cepa* bioassays, while simultaneously exhibiting a ferric reducing ability. The toxicological properties observed, indicate the need to be more cautious in the consumption of infusions from *A. triangularis* leaves, for medicinal purposes.

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## DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

JDAS, CRN, SCHV, and CALC designed the study and executed the experiments: extract preparation, quantitative chemical analyses, and bioassays; MCV cultivated and processed the plant; ABG, BAC, LFV, and LFVF performed the *A. cepa* assay and the statistical analyses. The authors contributed equally to the manuscript.

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