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TOXICOLOGICAL EVALUATION BY *IN-VITRO* ASSAY OF AQUEOUS EXTRACT OF *ARISTOLOCHIA GIGANTEA* MART. *ET* ZUCC. LEAVES

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ABSTRACT

Aristolochia gigantea Mart. *et* Zucc. (Aristolochiaceae) is an ornamental herbaceous plant used in folk medicine for its abortifacient, emmenagogue, and anti-snake bite properties. This study evaluated the genotoxic and cytotoxic activities of aqueous extracts of leaves of *A. gigantea*, by means of the Ames test (*Salmonella typhimurium* strains TA97, TA98, TA100, and TA102), SOS Chromo test (*Escherichia coli* PQ35 and PQ37), Inductest [*Escherichia coli* WP2s(λ) and RJF013] and a cytotoxicity assay using human embryo kidney epithelial cells (HEK cell line). The extracts showed no genotoxic activity, as evaluated through the bacterial test of SOS induction and cytotoxic activity on the HEK cell line. On the other hand, the extracts showed cytotoxic activity, suggesting that they contained compounds that are toxic to eukaryotic organisms but not to prokaryotic organisms.

KEYWORDS: *Aristolochia gigantea*, Aristolochic acid, Genotoxicity, Cytotoxicity.

Abbreviations: AA, aristolochic acid; BPB, bromophenol blue; DAUNOR, daunomycin or daunorubicin; DMEM, Dulbecco's Minimal Essential Medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HEK, Human embryo kidney epithelial cell; MNNG, N-methyl-N'-Nitro-N-nitrosoguanidine; 4-NQO, 4-nitroquinoline 1-oxide; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; Tris, Tris (hydroxymethyl) amino methane.

INTRODUCTION

The therapeutic use of medicinal plants has increased in recent decades. Many of these plants contain mutagenic and/or carcinogenic compounds (Glück et al. 2018; Prinsloo et al. 2018).

Members of the genus *Aristolochia* contain terpenoids, lignoids, alkaloids and alkamides, phenolic derivatives, and other compounds (Kuo et al. 2012). This chemical diversity contributes to several pharmacological activities.

Aristolochia gigantea Mart. et Zucc. (Aristolochiaceae), popularly known as papo-de-peru, jarrinha, mil homens, and angelicó, is an ornamental herbaceous plant native and endemic to Brazil (Reflora, 2022). *Aristolochia* species have been used to regulate fertility (Kumar et al. 2012), to treat malaria (Mathew et al. 2021), lack of appetite, fevers, and skin ulcers (Ricardo et al. 2017), against snakebite (Giovannini and Howes 2017; Upasani et al. 2018), and in many other traditional uses (Kuo et al. 2012). Several studies have investigated the therapeutic actions described for species of *Aristolochia*: anti-*Helicobacter pylori* (Gadhi et al. 2001), antidiabetic (Sulyman et al. 2016; El Omari et al. 2019), anticancer (Akindele et al. 2014; Das and Kumar 2018), wound healing (Bolla et al. 2019), anti-inflammatory (Desai et al. 2014; Wang X. et al. 2018), and antibacterial (Venkatadri et al. 2015).

Some active compounds in members of *Aristolochia* are mutagenic (Zhang et al. 2004; Attaluri et al. 2010; Poon et al. 2015), carcinogenic, hepatotoxic, or nephrotoxic (Bourhia et al. 2019a,b; Chan et al. 2019; Nault and Letouzé 2019; Zhang et al. 2019). The properties of these plants have been attributed to aristolochic acid (AA), which was not detected in *A. gigantea*, the species investigated in this study (Leitão and Kaplan 1992; Han et al. 2019).

As far as we are aware, no study has examined the toxicological effects of an extract of *A. gigantea* leaves. The present study evaluated the genotoxic and cytotoxic activities of

aqueous extracts of leaves of *A. gigantea*, using the Ames test, the SOS Chromo test, Inductest, and HEK cell cytotoxicity assays.

MATERIAL AND METHODS

Chemicals

Dulbecco's Minimal Essential Medium (DMEM), penicillin, streptomycin, Catalase, superoxide dismutase, and bromophenol blue were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, Brazil). Ethidium bromide was supplied by Serva (Heidelberg, Germany). Hanks' balanced salt solution (without Ca^{2+} and Mg^{2+}), EDTA (ethylenediaminetetraacetic acid), and agarose were obtained from Gibco BL (Gaithersburg, MD, USA). All other reagents were purchased from Merck (Brazil) and were of analytical grade.

Plant Material and Extract Preparation for the Bioassays

Leaves of *A. gigantea* were collected from the Botanical Garden of Rio de Janeiro (RJBG), under the deposit number RB: 301.112/ section 34-flowerbed B. A crude aqueous extract was prepared by extracting 7 g of dried and powdered leaves with 100 ml of boiling distilled water. The extract was filtered and maintained in the dark at room temperature until used for the genotoxicity assays. After that, the extract was kept under refrigeration, which result in a precipitate. The extract was centrifuged and both the precipitate and the supernatant were separated, filtered, solubilized in 5% acetic acid and evaluated in the cytotoxicity assays. All extracts were prepared immediately before each experiment, and the samples were filtered on Millipore filters (0.22 μm).

Bacterial Strains

Salmonella typhimurium strains TA97 and TA98 (which detect frameshift mutations) and TA100 and TA102 (which identify base-pair substitutions) were kindly provided by B. N. Ames, University of California, Berkeley, CA, USA. The *Escherichia coli* K-12, SOS Chromo test, strains PQ35 and PQ37 (as described by Quillardet and Hofnung 1985), were obtained from P. Quillardet, Institut Pasteur, Paris, France. The *E. coli* B/r WP2s(λ) (*trpE, uvrA*) and RJF013 strains were obtained from A. G. Miguel, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. RJF013 is a spontaneous ampicillin-resistant mutant derived from *E. coli* strain SR714 (*trpE, uvrD3*) (Rossman et al. 1984).

Mutagenicity Assay

The mutagenic studies were performed as described by Maron and Ames (1983). Bacteria in the stationary growth phase (0.1 ml) were placed in tubes containing different concentrations of aqueous leaf extract (7%) of *A. gigantea* (2.2, 4.4, 8.8, 17.5, and 35 mg/ml) with strains TA97, TA98, TA100, and TA102. Exponential-phase cultures of *S. typhimurium* strains TA97, TA98, and TA100 were grown in LB medium [1% bacto tryptone, 0.5% bacto yeast extract (Difco), and 1% NaCl plus ampicillin (20 µg/ml); Miller and Michaels 1992] and TA102 was grown in LB medium, plus ampicillin (20 µg/ml) and tetracycline (10 µg/ml) for 15 h. Briefly, 100-µl aliquots of a 7% aqueous extract of *A. gigantea*, at different concentrations, were added to 100 µl of each bacteria strain and incubated at 37 °C for 20 min without shaking. After this procedure, 2 ml of molten top agar supplemented with L-histidine/D-biotin (1:10) was added to a Petri dish containing Vogel-Bonner E medium (Vogel and Bonner 1956). Negative (DMSO) and positive [H_2O_2, Daunomycin, and 4-nitroquinoline 1-oxide] controls were included per plate for strains TA97, TA98, TA100, and TA102 in each assay.

A compound was considered positive for mutagenicity when the number of revertants was at least double the spontaneous yield.

Galactosidase Induction Assay

The SOS chromotest was performed as described by Quillardet and Hofnung (1985). It is a simple bacterial colorimetric assay for genotoxicity, based on measuring the induction of *sfiA*, a gene controlled by the general repressor of the SOS (DNA repair) system in *E. coli*. Expression of *sfiA* is monitored employing *lacZ*, the structural gene for *β*-galactosidase.

Escherichia coli strains PQ35 and PQ37 were grown in LB medium to the exponential phase. After overnight incubation at 37 °C, the presence of a blue ring around the zone of inhibition indicates that the compound can induce the *sfi-A* gene (Quillardet and Hofnung 1985). Both negative (sterile distilled water) and positive (hydrogen peroxide) controls were used.

In this assay, the *sfiA* gene-linked β-galactosidase activity is determined to measure the induction of the SOS repair system. A significant increase in the β-galactosidase activity in relation to the control was considered a positive result.

Lysogenic Induction Assay

The Inductest is based on the induction of prophage λ in *E. coli*; in this assay, most of the treated lysogenic cells undergo a dramatic change, i.e., they lyse and release free phages (Moreau et al. 1976). Strains WP2s(λ) and RJF103 of *E. coli* B/r were used for the lysogenic induction assays, with a protocol similar to the qualitative Inductest developed by Moreau et al. (1976). Strains WP2s(λ) and RJF103 grown in LB medium were incubated overnight at 37 °C with shaking to the log growth phase. An aliquot of 0.1 ml WP2s(λ) was added to 0.3 ml RJF103, and after addition of 2.5 g top agar was plated with LB medium with and without ampicillin (20 µg/ml).

The results were considered positive when the number of plaque-forming units/plate was twice the spontaneous rate (Rossman et al. 1984). Both negative (sterile distilled water) and positive (MNNG) controls were used.

Mammalian Cell Culture

Human embryo kidney epithelial cells (HEK cell line, BCRJ No. CR017) were purchased from the Rio de Janeiro Cell Bank. Cells were routinely grown in DMEM supplemented with 10% FBS and antibiotics, and maintained in 10% CO_2 at 37 °C, using standard cell culture techniques.

Cytotoxicity Assay

For the cytotoxicity assay, cells were plated onto 35 mm-diameter culture dishes (Nunc) in DMEM/10% FBS, and 24 h later, the growth medium was renewed and crude aqueous *A. gigantea* extract added in different fractions and concentrations. Adherent cells were collected daily by *in situ* fixation with 5% TCA, and their number was determined by protein staining with bromophenol blue (BPB). Fixed cells were stained for 30 min with 1% BPB in 1% acetic acid, rinsed three times with water, and the stain extracted for 15 min with 10 mM unbuffered Tris base. The absorbance of extracted BPB was determined at 570 nm (Microplate reader, BioRad).

Statistical Analysis

The results from *in-vivo* experiments were evaluated by Student's *t*-test, using GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Genotoxicity of *A. gigantea* in *E. coli* and *S. typhimurium* strains

The absence of a mutagenic response by plant extracts against *S. typhimurium* bacterial strains in the Ames assay is a positive step toward determining the safety of plants used in traditional medicine. The aqueous leaf extract of *A. gigantea* showed no mutagenic activity (Table 1), as it did not increase the number of histidine-independent bacteria (Ames et al. 1975). Previous results showed that aristolochic acid (AA) was mutagenic to strains TA100 and TA98 (Zhang et al. 2004); TA100 and TA1537 (Robisch et al. 1982); TA98, TA100, and TA 1537 (Hwang et al. 2012); TA100 (Schmeiser et al. 1984; Pfau et al. 1990); and TA100 and TA1538 (Meinl et al. 2006). However, AA was not mutagenic to strains TA98 (Schmeiser et al. 1984), TA98, TA1535, or TA1538 (Robisch et al. 1982), or had only a weak effect on strains TA100 (Bianucci et al. 1993), TA98 (Pezzuto et al. 1998; Gotzl and Schimmer 1993), and TA1537 (Pfau et al. 1990). The present study found no mutagenic activity to strains TA97, TA98, TA100, and TA102, which could be attributed to the absence of AA in *A. gigantea* (Leitão and Kaplan 1992; Han et al. 2019).

Similar results were obtained when another bacterial mutation assay was used, the SOS Chromo test and Inductest (Table 2). This assay could be a useful complement to the Ames test, due to its simplicity and speed (results were obtained within a few hours); requirement for only a single bacterial strain, allowing the detection of genotoxic chemicals that are inactive in the Ames test; and finally, because it is possible to discriminate false-positive results for both tests.

The absence of a genotoxic effect on the *E. coli* strains treated with the aqueous extract of *A. gigantea* did not increase the β-galactosidase activity of *E. coli* (PQ35 and PQ37) and did not induce prophage lysing in a lysogenic strain of *E. coli* [WP2s(λ) and RJF103], in contrast to several studies that stated that all *Aristolochia* species have genotoxic properties.

Table 1: Evaluation of mutagenic potential of *Aristolochia gigantea* and controls by Ames test.

Group	TA97	TA98	TA100	TA102
DAUNOR	_	+	_	_
MNNG	+	_	+	+
4-NQO	_	+	+	_
H_2O_2	+	+	_	+
PSA	_	_	+	_

DMSO	_	_	_	_
A. gigantea	_	_	_	_

DAUNOR: daunomycin (40 µg/plate); MNNG: N-methyl-N'-Nitro-N-nitrosoguanidine (0.2 µg/plate); 4-NQO: 4-nitroquinoline 1-oxide (20 µg/plate); H_2O_2: hydrogen peroxide (3M); PSA 10%; DMSO: dimethylsulfoxide 100%. (+) Revertants histidine dependents of "Salmonela typhimurium" increased around compound tested.

Table 2: Genotoxic evaluation of *Aristolochia gigantea* and controls by Inductest and Chromotest.

Group	WP2s(λ)+ RJF103	PQ35	PQ37
4-NQO	+	+	+
UV-C	+	+	+
Saline	_	_	_
A. gigantea	_	_	_

4-NQO: 4-Nitroquinoline 1-oxide *(20µg/plate); UV-C 10 J/m^2; Saline: NaCl 0.9%. (+) Number of phage plaques increased or blue ring present around the compound tested.*

In-vitro* Evaluation of the Cytotoxic Potential of *A. gigantea

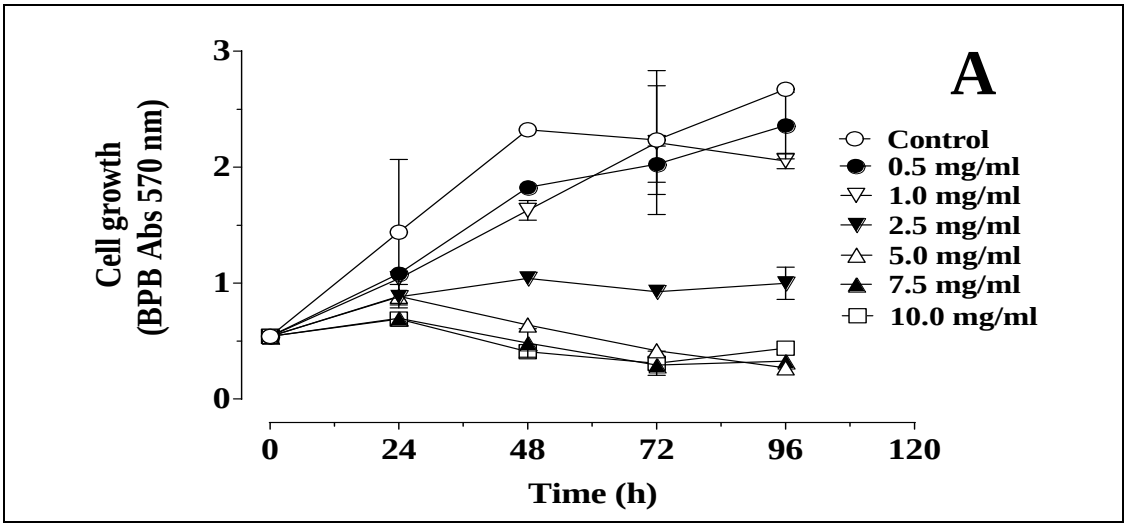
These evaluations were performed with the HEK cell line because the cells are a potential target for substances present in members of *Aristolochia*. The exponential growth of this cell line was followed for four days in the absence and presence of *A. gigantea* aqueous extract, as shown in Figs. 1A, 1B, and 1C.

All fractions of the *A. gigantea* aqueous extracts showed cytotoxic activity, which differed in each fraction. Fig. 1A shows the cytotoxic effect of the total extract at concentrations above 1.0 mg/ml, in contrast to the supernatant (Fig. 1B) and the precipitate (Fig. 1C) fractions, which showed a cytotoxic effect at concentrations above 0.5 mg/ml and 50 µg/ml, respectively. After 96 h of exposure, IC_{50} values of 1.98 mg/ml (total extract), 0.63 mg/ml (supernatant fraction), and 0.04 mg/ml (precipitate fraction) were determined.

Remarkably similar results were obtained by Ruffa et al. (2002) with a methanol extract of *A. macroura* against human hepatocellular carcinoma cells (Hep G2 cells) and by Mongelli et al. (2000) with a dichloromethane extract of *A. triangularis* that showed toxicity to human oral epidermoid carcinoma cells (KB cells). However, methanol, ethyl acetate, and petroleum ether extracts of *A. elegans* tested on cultures of KB (nasopharyngeal carcinoma), HCT-15 COLADCAR (colon carcinoma), and UISO-SQC-1 (squamous cervix carcinoma) cells were

considered non-cytotoxic, as the IC_{50} values of the organic extract were higher than 30–100 µg/ml (Popoca et al. 1998). The high IC_{50} values observed for the total aqueous extract and the supernatant fraction with HEK cells suggest that these extracts show a low *in-vitro* toxicity for these cells. However, the precipitate fraction showed higher toxicity, although lower than that observed by Ruffa et al. (2002). Hadem et al. (2014) found an *in-vivo* chemopreventive effect of *A. tagala* against hepatocellular carcinoma, through enhancing the antioxidant status by a free radical-scavenging mechanism.

Nephrotoxic and carcinogenic activities have been attributed to aristolochic acid from species of *Aristolochia*. Oral administration of *A. manshuriensis* Kom in rats destroyed renal functions and induced anemia, resulting in loss of body weight. In addition, tumors were induced in the kidney, stomach, bladder, and subcutaneous tissues after 51 mg/kg to 1029 mg/kg was administered for periods of up to 30 weeks (Wang L. et al. 2018). A single dose of less than 4 g/kg of a decoction of rhizomes of *A. paucinervis* can be safe. However, repeated oral administration of 1 or 1.5 g/kg/day for 28 days induced significant disturbances of serum parameters and histopathological injuries (Bourhia et al. 2019a). The findings of a toxicological study of roots of *A. baetica* L. were remarkably similar (Bourhia et al. 2019b). However, AA has not been detected in *A. gigantea* (Leitão and Kaplan 1992; Han et al. 2019). In addition to cytotoxic activity against HEK cells, the absence of AA indicated that *A. gigantea* may have an anticancer action.



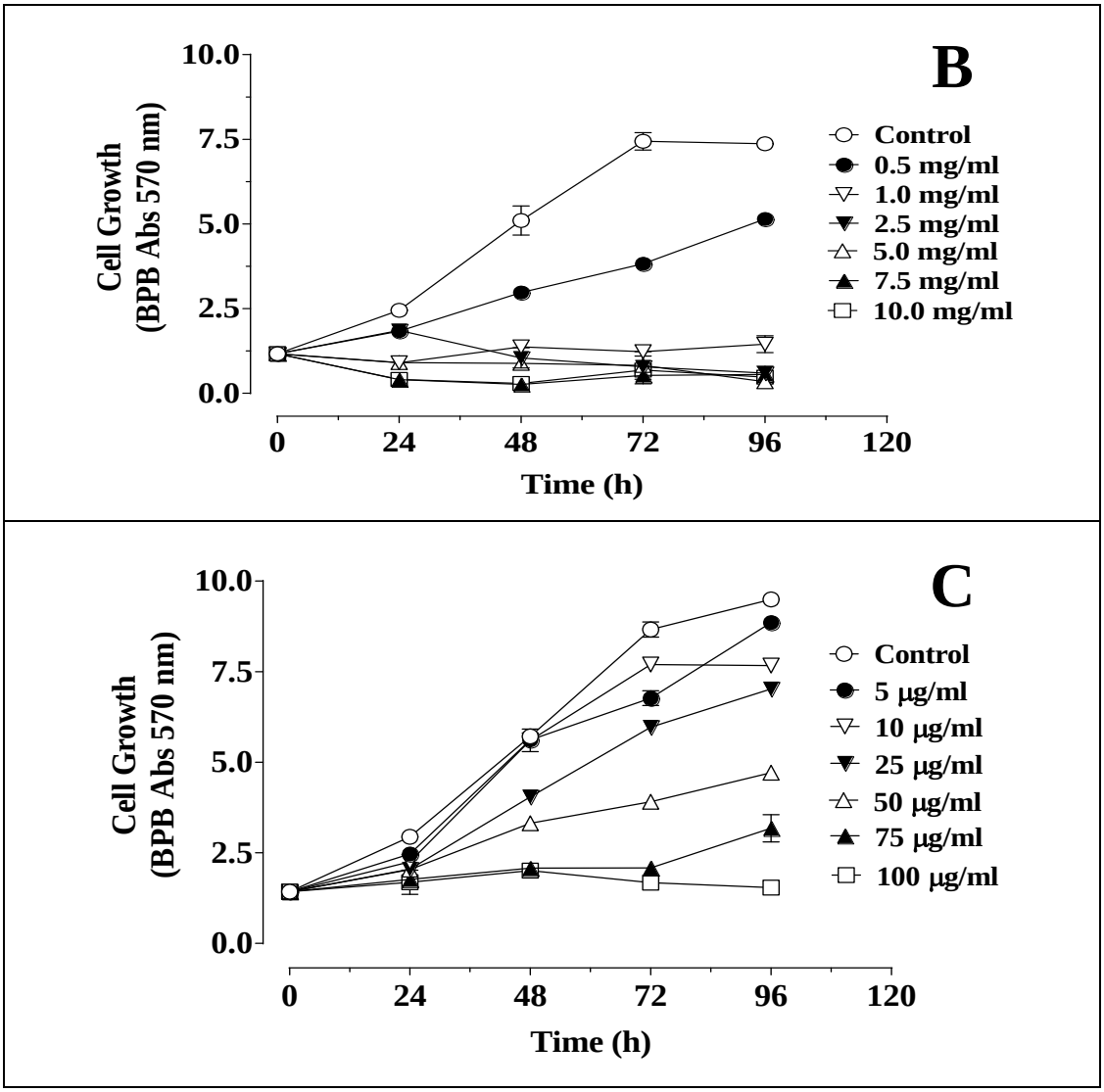


Fig. 1: Growth kinetics of HEK cells in the presence of the total extract (A), the supernatant fraction (B), and the precipitate fraction (C) of a crude aqueous extract (7%) prepared from dried leaves of *Aristolochia gigantea*. Exponentially growing cells in DMEM/10% FBS. Cells were collected at the time shown, fixed with 5% TCA, and their mass determined by absorbance at 570 nm after staining with bromophenol blue and extraction with 10 mM Tris.

CONCLUSIONS

According to our results, the aqueous extract of leaves of *A. gigantea* showed a dose- and time-dependent cytotoxic activity in eukaryotic organisms. It acted on kidney cells but not on a prokaryotic organism, and did not exhibit mutagenic activity.

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Authors' Contribution This work is part of KAL's doctoral thesis. CLSL and AS guided and contributed to the planning of KAL's doctoral thesis. KAL and SSL wrote the manuscript. KAL, ABS, and AACL performed the assays with prokaryotes. KAL, GATL, NPAN, and MGPC performed the assays with eukaryotes. AS and MGPC collaborated in writing the text and revised the manuscript. All the authors approved the submission.

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