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Evaluation of the toxicity, cytotoxicity and antibacterial activity of *Tibouchina papyrus* (Pohl) Toledo (Melastomataceae)

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Abstract

The objective of this research was to perform a preliminary phytochemical study and investigate the toxicity, cytotoxicity and antibacterial activity of the species *Tibouchina papyrus* (Melastomataceae). The toxicity test was performed with the extracts against *Artemia salina*. The cytotoxic study was also performed against the following cell lines: promyelocytic leukemia (HL60), human colon carcinoma (HCT-116), breast carcinoma (MCF-7), prostate carcinoma (PC3), astrocytoma (SNB-19) and non-tumor cells (L929). Last, antibacterial activity was assessed through against *Staphylococcus epidermidis* (25923), *Staphylococcus aureus* (12228), *Escherichia coli* (25312) and *Pseudomonas aeruginosa* (27853). The phytochemical investigation indicated the presence of these metabolite groups: flavonoids, triterpenes, steroids and tannins. The methanolic extracts showed moderate antibacterial activity against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. *T. papyrus* extracts showed high cytotoxic activity against promyelocytic leukemia cell lines (80.43 to 93.31%). The other lines did not reveal good activity against the extracts tested.

Keywords: natural product; ethyl pheophorbide; β-amyrin; biological activity; promyelocytic leukemia.

Avaliação da toxicidade, citotoxicidade e atividade antibacteriana *de Tibouchina papyrus* (Pohl) Toledo (Melastomataceae)

Resumo

O objetivo dessa pesquisa foi realizar triagem fitoquímica e investigar a toxicidade, citotoxicidade e atividade antibacteriana da espécie *Tibouchina papyrus* (Melastomataceae). A toxicidade foi realizada realizado com os extratos frente à *Artemia salina*. O estudo citotóxico também foi realizado, frente às seguintes linhagens celulares: leucemia promielocítica (HL60), carcinoma de cólon humano (HCT-116), carcinoma de mama (MCF-7), carcinoma de próstata (PC3), astrocitoma (SNB-19) e células não tumorais (L929). Por fim, foi analisada a atividade antibacteriana frente às bactérias *Staphylococcus epidermidis* (25923), *Staphylococcus aureus* (12228), *Escherichia coli* (25312) e *Pseudomonas aeruginosa* (27853). A investigação fitoquímica indicou presença dos grupos metabólitos: flavonoides, triterpenos, esteroides e taninos. Os extratos metanólicos apresentaram atividade antibacteriana moderada frente à *S. aureus*, *S. epidermidis* e *P. aeruginosa*. Os extratos de *T. papyrus* apresentaram alta atividade citotóxica frente a linhagem de leucemia promielocítica 80,43 a 93,31%. As demais linhagens não apresentaram boa atividade frente aos extratos testados.

Palavras-chave: Produto natural; etil feoforbídeo; β-amirina; atividade biológica; leucemia promielocítica.

Introduction

Brazil boasts a wide diversity of plants with medicinal properties, many of which have not yet been studied under a biological or chemical point of view. The utilization of plants as therapeutic agents, or a model for new synthetic medications or even as the basis for the semi-synthesis of molecules or drugs, has been the subject of research in several studies of bioprospection and/or of access to the genetic heritage (Neto & Morais, 2003).

Out of the new drugs developed over the last 38 years (1981 to 2019, data related to the research performed), 1,881 new drugs were approved. The synthetic drugs correspond to 24.3% (463), followed by 18.9% (356) related to products derived from natural products, 18.4% (346) related to biological macromolecules and only 14 new drugs of sole botanic origin, corresponding to 0.8% of the total number. This low number of approval of botanic drugs is due to the fact that only recently have they begun being approved (Newman & Cragg, 2020).

The genus *Tibouchina* contains species with ornamental value, with some of them being used in popular medicine, such as: *T. asperior*, with diuretic and depurative activity (Vargas, Guidobono & Henriques, 1991); *T. ciliaris*, for gout control (Colorado et al., 2007); *T. grandiflora*, for the amelioration of wound scarring (Kuster, Arnold & Wessjohann, 2009; Scio et al., 2012); *T. granulosa*, with antinociceptive action (Barnaby, Reid & Warren, 2016; Sobrinho et al., 2017; Scio et al., 2012); *T. kingii*, with anti-inflammatory activity (Ramirez-Atehórtua et al., 2018; Jimenez et al., 2015); *T. pereirae*, for the treatment of renal diseases (Dias, Hamerski & Pinto, 2016); *T. semidecandra*, used in the treatment of headache symptoms and with scarring action (Sirat, Rezali & Ujag, 2010).

Among the several possibilities of running a toxicological study on plants to confirm their likely toxic action, some conditions need to be taken into account, such as: dose, time, frequency and administration route. The employment of simple organisms like *Artemia salina* is more common for the preliminary toxicity assays (Ferreira et al., 2017).

The species *T. papyrus* is endemic and its occurrence takes place in the Brazilian *Cerrado*, being particularly found in Serra dos Pirineus (Pirenópolis and Cocalzinho de Goiás, GO), Serra Dourada (Mossâmides, GO), Serra Negra (Piranhas, GO), Serra da Natividade (Natividade, TO) and Parque Bacaba (Nova Xavantina, MT) (Versiane, Santos & Romero, 2016). No studies on the biological or phytochemical activity of this species of the family Melastomataceae were found.

The goals of this research were: make the phytochemical trial and perform a study on the toxic, cytotoxic and antibacterial activities with the extracts of leaves and branches of *Tibouchina papyrus*.

Materials and Methods

The specimen was collected in Serra dos Pirineus (Goiás, Brasil) and identified *in loco* by Prof. Dr. Mirley Luciene dos Santos. The registration of the access to Genetic Heritage in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (National System for the Management of Genetic Heritage and Associated Traditional Knowledge, SisGen) was made under the number A35C504. The preparation of the exsiccate was made according to the method proposed by the herbarium of the State University of Goiás (HUEG), listed with the number HUEG 13795.

The extraction process used was the cold serial exhaustive maceration, with solvents in increasing order of polarity. The pulverized plant material (leaves and branches, separately) was kept in contact with the solvent for 4 days, with occasional shakings. The process was repeated 3 times, followed by filtration and solvent replacement. The solvents used were, respectively, hexane, ethyl acetate and methanol.

The cytotoxicity assay was performed against *Artemia salina* nauplii, following the methodology adapted from Rehman, Chohan, Gulnaz and Supuran (2005), in triplicate and in 3 independent series of repetitions.

For the anti-bacterial activity assay, we used the broth microdilution method, according to the protocol recommended by the *Clinical and Laboratory Standard Institute* (CLSI) for susceptibility tests by means of anti-microbial agent dilution in broth (CLSI, 2016).

The *in vitro* cytotoxicity assay was run by the Experimental Oncology Laboratory of the Federal University of Ceará (UFC). The analysis was made by means of the colorimetric method of conversion of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) salt to formazan, first proposed by Mossman (1983). The SNB-19 (astrocytoma), HCT-116 (human colon carcinoma), PC3 (prostate carcinoma), HL60 (promyelocytic leukemia) and MCF-7 (breast carcinoma) lines were supplied by the National Institute of Cancer (USA), whereas the L929 line (murine fibroblast) was supplied by Rio de Janeiro´s cell bank (BCRJ).

Toxicicity bioassay

For the preparation of the recipient, we used 3.6% saline solution, supplemented with yeast extract at 6 $mg.L^{-1}$, upon sterilization. The saline solution prepared was added to a decantation chute and then 30 mg of *A. salina* cysts was incubated. The system was then kept under constant aeration and lightning for 36 hours.

Ten nauplii were added to 96-well plates in 100 µL of saline solution and 100 µL of the extracts. Eight mg of the extracts was weighed, solubilizing in 2 mL of dimethyl sulfoxide (DMSO) at 5% (1900 µL) and Tween 0.02% (100 µL). The serial dilution began from the initial concentration of 4,000 $\mu g.mL^{-1}$ following to 2,000, 1,000, 500, 250, 125 and 62.5 $\mu g.mL^{-1}$. The viability controls were saline solution 3.6%, DMSO 5% and Tween 0.02% and the technique control was potassium dichromate in the initial dilutions of 100, 50, 25, 12.5 and 6.25 $\mu g.mL^{-1}$. The plates were kept at room temperature, constant lightning and after 24 hours, both the dead and the surviving nauplii were counted. The data were used for the calculation of the mean lethal concentration (LC_{50}), through the PROBIT method of statistical analysis.

Meyer et al. (1982) define the numbers for the classification of the samples in the toxicity assays against *A. salina* nauplii, considering as atoxic the samples that show LC_{50} >1,000 $\mu g.mL^{-1}$ and as toxic the ones that show LC_{50}< 1,000 $\mu g.mL^{-1}$. Nguta et al. (2012) confirmed and expanded this classification, whereby the toxic activity is considered as weak when the LC_{50} is between 500 and 1,000 $\mu g.mL^{-1}$, moderate when between 100 and 500 $\mu g.mL^{-1}$, strong when <100 $\mu g.mL^{-1}$ and not toxic when >1,000 $\mu g.mL^{-1}$.

Antibacterial activity

In order to run the experiment, the extracts were solubilized in dimethyl sulfoxide (DMSO) at 5% and diluted in Müller Hinton broth (MH) to yield a stock solution of 4,000 $\mu g.mL^{-1}$ concentration, which was further diluted for the concentrations of 2,000, 1,000, 500, 250 and 125 $\mu g.mL^{-1}$. The tests were followed by viability control of the micro-organisms in the absence of the extracts (MH + bacterial inoculum + DMSO 5%), sterility control (extracts + MH and MH) and technique control (antimicrobials chloramphenicol and gentamicin).

Chloramphenicol was used in the concentrations of 64, 32, 16, 8, 4, 2 and 1 µg.mL^{-1}. Gentamicin was used in the concentrations 8, 4, 2, 1 and 0.5 µg.mL^{-1}. The bacterial inoculum were prepared in sterile physiological solution with 0.9%, with the suspension of typical and isolated colonies, after 24 hours of growth in MH agar, with the turbidity corresponding to 0.5 of the McFarland scale, equivalent to the adjustment of the inoculum to the bacterium concentration of 10^8 UFC.mL^{-1}. Next, the suspension was diluted (0.1:9.9 mL of MH broth) in a way to yield the cell concentration of 10^6 UFC.mL^{-1}.

In 96-well U-shaped sterile microplates, 50 µL of the bacterial inoculum at 1.5 x 10^6 UFC.mL^{-1} and 50 µL of both the diluted extracts and the controls were placed. The plates were incubated for 24 hours, under temperature at 35 ºC. In order to make the reading, 25 µL of sodium resazurin at 0.01% was added to each well and after 30 minutes of incubation, the visual reading was made. The blue color was predominant, what indicates the inhibition of bacterial growth; the rose-red color indicates metabolic activity due to growth. The assay was made in triplicate and in three series of independent repetitions.

The antibacterial activity was classified according to the number obtained from the Minimal Inhibitory Concentration (MIC). For the extracts that showed values lower than 100 µg.mL^{-1}, antimicrobial activity was considered as good; from 100 to 500 µg.mL^{-1}, activity was moderate; from 500 to 1,000 µg.mL^{-1}, activity was weak; higher than 1,000 µg.mL^{-1}, the extract was considered as inactive (Hertz et al., 2002).

Cytotoxicity bioassay

The strains were cultured in RPMI 1640 medium and Dulbecco's Modified Eagle Medium - DMEM (L929), supplemented with 10% of bovine fetal serum and 1 % of antibiotics (penicillin 100 U.mL^{-1} and streptomycin 100 µg.mL^{-1}), and kept in an oven at 37 ºC and atmosphere containing 5% of CO_2. The samples were diluted in pure DMSO for the stock concentrations of 20 mg.mL^{-1}.

The cells were plated in the concentrations of 0.7 x 10^5 cells/mL (HCT-116 and L929), 0.1 x 10^6 cells/mL (SNB-19, MCF-7 and PC3) and 0.3 x 10^6 cells/mL (HL60). The samples were tested in the sole concentration of 100 µg.mL^{-1}. Doxorubicin was used as positive control in the concentration of 5 µg.mL^{-1}. The plates were incubated for 72 hours in an oven at 5% of CO_2, under temperature of 37 °C; at the end of this period, they were centrifuged and the supernatant was removed. Next, 100 µL of the MTT solution was added and the plates were incubated again for 3 hours. Absorbance was read, after precipitate dissolution with 100 µL of pure DMSO, in a plate spectrophotometer at 595 nm.

The means ± standard deviations of the means (SDM) were calculated from the inhibition rate of the cell growth of the three repetitions, using the *Graph Pad Prism* 6.01 software (Pires et al., 2011).

The intensity scale used to assess the cytotoxic potential of the samples tested was: NA – no activity; LA – low activity (inhibition of cell growth ranging from 1 to 50%); MA – moderate activity (inhibition of cell growth ranging from 50.1 to 75%); HA – high activity (inhibition of cell growth higher than 75%), reported in the literature by Almeida et al. (2014) and the ISO / TC 194 Technical Committee (2009).

Phytochemical trial

The phytochemical trial consists of a chemical analysis to detect the presence or the absence of secondary metabolites using staining or precipitation reagents, according to the methodology adapted from Matos (2009), Falkenberg, Santos and Simões (2003).

Results and Discussion

The phytochemical trial tests indicated the presence of flavonoids, simple phenols, coumarins (only in the branches), tannins, triterpenes or steroids and deoxy sugars (Table 1). The results obtained for *T. papyrus* corroborate other results obtained in studies of the genus *Tibouchina*, where we may mention: *T. paratropica*, phenolic derivatives (Tracanna et al., 2015); *T. pulchra*, anthocyanins, phenolic compounds and flavonoids derived from kaempferol (Rezende et al., 2019); *T. pereirae*, flavonoids (Dias, 2013); *T. lepidota*, flavonoids derived from quercetin and kaempferol (Hendra & Keller, 2016).

The extracts were shown to be atoxic, once that no deaths above 50% were observed for the *T. papyrus* extracts (Table 2). For the TPBH (*T. papyrus* branch hexane), TPLH (*T. papyrus* leaf hexane), TPBA (*T. papyrus* branch ethyl acetate*)* and TPLA (*T. papyrus* leaf ethyl acetate) extracts, the the mean lethal concentration (LC_{50}) were >2,000, being thus considered as atoxic according to Nguta et al. (2012).

Table 1: Results obtained from the assays of preliminary phytochemical prospection, made with the powder from the leaves and branches of *T. papyrus*.

Metabolite class	Type of reaction	Result	
		leaves	branches
Flavonoids	Shinoda	-	+
	Oxalate-Boric	-	-
	Sulfuric acid	+	+
Simple phenols	Alkalin Hydroxides	+	+
	Aluminum chlorides	-	-
	Ferric chloride	+	+
Coumarins	-	-	+
Tannins	Reaction with jelly	+	+
	Reaction with alkaloids	+	+
	Reaction with metal salts	+	+
	Reaction with alkaline hydroxides	+	+
Alkaloids	Mayer	-	-
	Dragendorff	-	-
	Bouchardt	-	-
	Bertrand	-	-
	Hager	-	-
Steroid or Triterpenes	Tannic acid	-	-
	Liebermann-Buchard	+	+
	Pesez	+	+
Deoxy sugars	Keller-Killiani	+	+
Lactone ring	Kedde	-	-

Table 2: Evaluation of the toxicity against *A. salina* nauplii (LC_{50}) of the *Tibouchina papyrus* extracts and their confidence intervals.

Extract	LC_{50} (µg.mL^{-1})	95% IC		Classification Nguta et al. (2012)
		Lower limit	Upper limit	
TPBM	1,277.71	1,135.90	1,419.51	Atoxic
TPLM	1,596.47	1,402.65	1,790.29	Atoxic
$K_2Cr_2O_7$	34.56	23.94	45.18	Toxic

Legend: IC - Confidence Interval; TPBM - *T. papyrus* branch methanol; TPLM - *T. papyrus* leaf methanol; $K_2Cr_2O_7$ -potassium dichromate. Confidence interval in %.

The results achieved with the antibacterial activity assay performed against the *S. aeruginosa* pathogens are shown in Table 3. The TPBH, TPLA and TPBA extracts showed high activity of cell growth inhibition against the HL60 cell line, whose results are shown in Table 4. The TPLM and TPBM extracts, against the same cell line, presented moderate activity. The same extracts against non-tumor cell line (L929) showed low cytotoxicity, indicating a possible selectivity for the tumor line.

Viable cells, with active metabolism, convert MTT into formazan, with absorbance reading at 540 nm, whereas dead cells lose such capacity of conversion (RISS et al., 2016). The absorbance values were adjusted in their respective inhibition rates.

Table 3. Antibacterial activity assay of the *Tibouchina papyrus* extracts in µg.mL^{-1}.

Extract/ ntimicrobial	Gram positive			
	S a		*S e*	
	MIC	BMC	MIC	BMC
TPBH	>2,000	>2,000	>2,000	>2,000
TPBA	>2,000	>2,000	2,000	>2,000
TPBM	500	2,000	250	>2,000
TPLH	>2,000	>2,000	>2,000	>2,000
TPLA	>2,000	>2,000	>2,000	>2,000
TPLM	500	2,000	500	1,000
Chloramphenicol	4.0	-	4.0	-
Gentamicin	-	-	-	-
Extract/ ntimicrobial	Gram negative			
	E c		*P a*	
	MIC	BMC	MIC	BMC
TPBH	>2,000	>2,000	>2,000	>2,000
TPBA	>2,000	>2,000	>2,000	>2,000
TPBM	>2,000	>2,000	>2,000	>2,000
TPLH	>2,000	>2,000	>2,000	>2,000
TPLA	>2,000	>2,000	>2,000	>2,000
TPLM	>2,000	>2,000	250	>2,000
Chloramphenicol	4.0	-	4.0	-
Gentamicin	1.0	-	1.0	-

Legends: MIC: Minimal Inhibitory Concentration (µg.mL^{-1}); BMC: Bactericidal Minimal Concentration (µg.mL^{-1}); TPBH: *T. papyrus* branch hexane; TPBA: *T. papyrus* branch ethyl acetate; TPBM: *T. papyrus* branch methanol; TPLH: *T. papyrus* leaf hexane; TPLA: *T. papyrus* leaf ethyl acetate; TPLM: *T. papyrus* leaf methanol; Sa: *Staphylococcus aureus*; Se: *Staphylococcus epidermidis*; Ec: *Escherichia coli*; Pa: *Pseudomonas aeruginosas*.

Table 4: Cell Inhibition Rate ((% ± Confidence interval 95%) of the extracts of leaves, branches and stem bark of *Tibouchina papyrus* against cell lines in a sole concentration of 100 µg.µL^{-1} and their classifications as non-active (N), low (L), moderate (M) or high (H) antibacterial activity.

Extract	HL 60	HCT 116	MCF7
TPBH	93.31±1.85 (H)	50.27±3.77 (M)	38.02±1.39 (L)
TPBA	80.43±3.64 (H)	65.52±3.12 (M)	35.23±5.32 (L)
TPBM	66.39±6.97 (M)	52.59±18.77 (M)	38.16±1.76 (L)
TPLH	3.32±13.01(L)	-24.96±18.53 (N)	33.53±3.82 (L)
TPLA	83.42±2.07 (H)	42.47±9.02 (L)	8.92±3,00 (L)
TPLM	73.41±1.98 (M)	52.05±12.55 (M)	36.92±0.37 (L)
Extract	SNL-19	PC3	L929
TPBH	13.47±2.47 (L)	46.49±3.13 (L)	41.55±3.57 (L)
TPBA	45.9±2.39 (L)	57.53±0.68 (M)	30.27±5.4 (L)
TPBM	18.05±6.48 (L)	36.8±1.75 (L)	51.28±7.01 (M)
TPLH	7.12±7.19(L)	26.34±5.35 (L)	24.95±1.4 (L)
TPLA	24.34±1.21 (L)	28.56±4.28 (L)	41.55±3.57 (L)
TPLM	14.72±4.75 (L)	30.44±8.34 (L)	30.27±5.4 (L)

Legends: CI%: Cell inhibition rate ± SD: standard deviation of the means; SNB-19 (astrocytoma), HCT-116 (human colon carcinoma), PC3 (prostate carcinoma), HL60 (promyelocytic leukemia), MCF-7 (breast carcinoma) and L929 (murine fibroblasts); TPBH: *T. papyrus branch hexane; TPBA (T. papyrus branch ethyl acetate); TPBM (T. papyrus branch methanol); TPLH (T. papyrus leaf hexane); TPLA (T. papyrus leaf ethyl acetate); TPLM (T. papyrus leaf methanol)*.

The samples with good inhibitory activity show inhibition rate above 75%. Nonetheless, the IC_{50} (Mean Inhibitory Concentration) was not determined because they did not reveal an inhibitory profile in at least two lines of different cells. The other compounds showed either moderate or low toxicity against the other lines tested. The data on the selectivity indexes (SI) were supplied based on IC_{50} values and as these values were not calculated, the SI values were not supplied either. Studies performed with the extracts and fractions of *Miconia burchellii*, a species also belonging to the Melastomataceae family, indicate that there is a strong inhibitory activity against the HL60 cell line ranging from 75.29 to 91.45%. The IC50 was determined to be 37.66 µg.mL-1 with a selectivity index of 2.0. These results corroborate for the expansion of studies in species of this family (Cunha, 2021).

Conclusion

We here in report the first study on the biological potential of the species *Tibouchina papyrus*. The phytochemical trial suggests the presence of secondary metabolite groups in the genus *Tibouchina*, which have already been identified in other studies. Although atoxic against *A. salina*, the extracts were shown to be cytotoxic against the HL-60 cell line and with moderate antibacterial activity against the pathogens tested. This study paves the way for a phytochemical investigation of the extracts.

Acknowledgments

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