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Evaluation of Cytotoxic and Mutagenic Activities of *Tabebuia aurea* (Silva Manso) Benth. & Hook. f. ex S. Moore.

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Abstract : With antimicrobial and antiseptic properties, *Tabeuia aurea* is used in folk medicine to treat pain, inflammation, and several conditions such as cancer, tuberculosis, malaria, stomach problems, influenza, nasal congestion, snake bites, and skin lesions. This study assessed the toxicological and mutagenic effects of methanolic and aqueous extracts of the bark of *T. aurea*. The microcrustacean *Artemia salina* was used as bioindicator in the cytotoxic analysis. Also, cytotoxicity and mutagenicity were investigated using the *Allium cepa test*. The methanolic extract had LD_{50} of 4608 μg/mL, while for the aqueous extract the LD_{50} value was 104656 μg/mL, indicating that they are atoxic. No extract induced significant changes in mitotic index in roots of *Allium cepa*. However, the methanolic extract 100 μg/mL and 50 μg/mL was cytotoxic, reducing root growth. No extract induced the formation of micronuclei, indicating no mutagenic effect. The results show that the extracts tested are not mutagenic to *A. salina*, with no significant changes in mitotic index. In turn, the extracts were cytotoxic, but not mutagenic, in the *Allium cepa* test.

Keywords - Allium cepa, Artemia salina, Medicinal plants, Tabeuia aurea, Toxicity

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I. INTRODUCTION

As a country enjoying considerable biodiversity, Brazil has a large body of traditional practices and knowledge about the management and use of medicinal plants [1,2,3]. According to the World Health Organization (WHO), between 65% and 80% of the population in developing countries use medicinal plants [4]. More specifically in countries in tropical regions, such as Brazil, numerous plant species are used to treat diseases based on health practices, traditional knowledge, and religious beliefs. These species are widely used *in natura* in spiritual healing practices based on a variety of methods, such as infusion, maceration, ointment, cream, syrups, and capsules [5]. Such practices are common in inland areas, where public health services are hard to reach, such as the Amazon and Pantanal wetlands. Incidentally, these are the regions that also exhibit a considerable diversity of native species with medicinal potential [6].

Comprised by 120 genera and approximately 800 species, Bignoniaceae is one of the plant families with interesting medicinal potential. With 32 Bignoniaceae genera and 350 species, Brazil is considered the center of diversity of the family [7]. One of the genera considered to have medicinal applications is *Tabebuia*, whose species have been used to treat simple conditions such as pain and inflammation as well as more complex diseases like cancer, tuberculosis, malaria, stomach conditions, influenza, nasal congestion, skin lesions, and snake bites. Its species are also held to have antimicrobial and antiseptic activities [8,9,10,11,12].

Popularly known as silver trumpet tree and tree of gold, *Tabebuia aurea* is one of the species of the genus *Tabebuia* considered to have interesting medicinal potential [13]. The species blooms annually between August and November, when individuals are leafless [14].

Phytochemical studies have determined the presence of secondary metabolites such as alkaloids, pyrogallol tannins (i.e. hydrolysable tannins), phlobatannins (i.e., condensed tannins), xanthones, saponins, steroids, triterpenoids, and flavonoids. These classes of compounds are responsible for the biological activities exhibited by *T. aurea* [15].

Tabebuia aurea has been used empirically as source of anti-inflammatory, antitumor, and antimicrobial agents [16]. However, so far no study has addressed the toxicity and mutagenic potential of its chemical

constituents in function of the doses administered for a given purpose. In this scenario, the present study assessed the acute cytotoxicity and the mutagenic potential of the bark of *T. aurea*.

II. MATERIALS AND METHODS

2.1 Plant material

Healthy, fertile specimens of *T. aurea* bearing fruit and flowers were collected in the urban area of the municipality of Ji-Paraná (10º85'94.68"S, 64º96'45,02"W), state of Rondônia, Brazil. All individuals were identified as *T. aurea* in the Herbarium Antônio Dalla Martha, Lutheran University Center (ULBRA), and exsiccata were deposited in the same herbarium (JPCU 322).

2.2 Preparation of plant extracts

Parts of bark and stem were washed in running water and sprayed with ethanol 70% to remove contaminants [17]. Next, the material was dried in a fan-forced oven at 40ºC for 24 h. The material was then ground in a knife mill as described by Broglio-Micheletti [18]. The aqueous extract (AE) was prepared boiling 12 g of the bark of *T. aurea* in 240 mL of water. This ratio of bark-to-water is normally used in traditional folk medicine preparations [19].

The methanolic extract (ME) was prepared weighing 75 g of the material and transferring it to an amber glass vial containing 250 mL methanol. The extraction procedure lasted seven days and was carried out at 30 ± 5ºC with a daily shaking session [20]. After extraction the material was strained using filter paper, and the liquid phase was evaporated in a rotary evaporator (Q344B1, Quimis) at 400 psi and 40ºC. The bottom was used in the experiments.

2.3 Acute cytotoxicity in *Artemia salina*

The cytotoxicity test was conducted using nauplius larvae of the microcrustacean species *Artemia salina*. Briefly, *A. salina* eggs were transferred to a sea salt solution (35 g/L, pH 8.5). Larvae were allowed to hatch in an environment at 25 ± 2ºC with constant aeration and light (100 W) for 48 h, ensuring complete cyst hatching [22].

Ten *A. salina* larvae were used in each treatment. The concentrations of EM used were 100% (3.66 mg/mL), 50% (1.8 mg/mL), 25% (0.9%), 12.5% (0.45 mg/mL), and 6.25% (0.25% mg/mL). The EA solutions used were 100% (50 mg/mL), 50% (25 mg/mL), 25% (12.5 mg/mL), 12.5% (6.25 mg/mL), and 6.25% (3.125 mg/mL). The negative control used was a sea salt solution (pH 8.5), whereas the positive control was a potassium dichromate 0.1% solution [23]. Each treatment was carried out in triplicate. Twenty-four hours later the surviving and dead individuals were counted in each treatment, and the median lethal dose (LD_{50}) was calculated.

According to Meyer et al. [22], an aqueous extract is toxic (or active) when LD_{50} is below 1000 µg/mL and atoxic (or inactive) when LD_{50} is above 1000 µg/mL. In turn, for Dolabela et al. [24], a methanolic extract is toxic when LD_{50} is below 100 µg/mL, moderately toxic when LD_{50} is between 100 µg/mL and 500 µg/mL, and atoxic or mildly toxic when the value is above 500 µg/mL.

The percent mortality rate and log-transformed concentration data were used to construct charts in the software 2003.

2.4 Cytotoxic and mutagenic effect in *Allium cepa*

Six treatments were prepared, namely ME 100% (3,66 mg/mL), 50% (1.8 mg/mL), 25% (0.9 mg/mL), and 12.5% (0.45 mg/mL). Also, for AE, two treatments with *in natura* samples were used, which were 100% (50 mg/mL) and 50% (25 mg/mL). Distilled water and copper sulfate 0.0006 mg/L were used as negative and positive control, respectively.

The treatments with ME and AE were conducted using seven sterile plastic tubes containing 50 mL of the respective concentrations. Each tube was used to expose one bulb, whose basal plate was kept submerged. Next, 48 h into treatment, midsized roots were removed from bulbs and soaked in an acetic acid solution in methanol (3:1) for 12 h. Next, these roots were washed in distilled water and hydrolyzed in hydrochloric acid in a double bath at 60ºC for 6 min and newly washed in distilled water again [25].

Slides were prepared in duplicate and sequentially stained using the Quick Panoptic LB Kit. Slides were inspected in an optical microscope (1000x) to detect the formation of micronuclei in 1000 cells per slide and the formation of interphase, prophase, metaphase, anaphase, and telophase cells were determined for each treatment, which represents the number of mitotic cells divided by the total number of cells. The result was multiplied by 2000 [26,27].

Five days into the experiment, the longest root in each bulb was measured using a caliper to analyze the cytotoxic effect of the extracts of *T. aurea* [28].

2.5 Statistical analyses

The statistical data are expressed as means plus standard deviation, and were analyzed using a one-way ANOVA in the softwares Prisma 5 and OriginPro 2017. The results were compared with the means using the Tukey test at significance levels 1% and 5%.

III. RESULTS

The results show that *T. aurea* ME 100% and 50% induced 20% and 10% mortality rates in *A. salina*, respectively. Mortality decreased with concentration. Linear regression indicated LD_{50} of 4608 µg/mL for ME of *T. aurea* (Fig. 1A).

In turn, treatment with *T. aurea* AE 100% and 50% induced the death of 40% and 26.6% of the larvae, in that order. Similarly to ME, mortality decreased with concentration of AE, and linear regression produced LD_{50} value of 104656 µg L^{-1} (Fig. 1B).

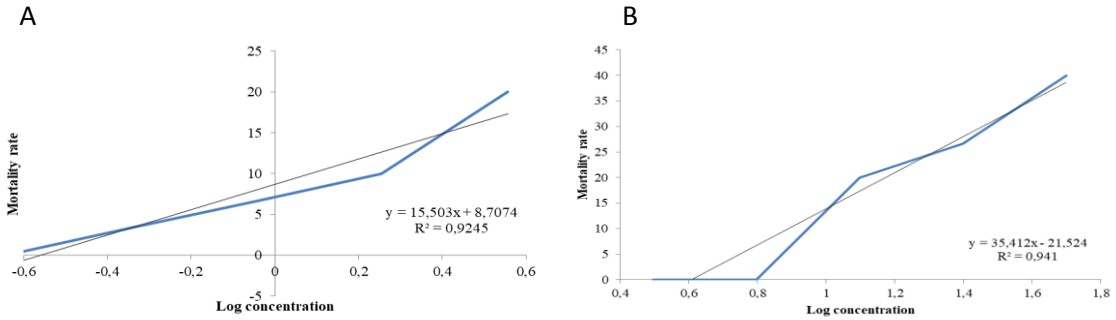

Fig. 1 Linear mortality rate and log-transformed concentration for the methanolic (A) and aqueous (B) extracts of the bark of *T. aurea* (Manso) Benth. & Hook. F. ex S. Moore.

The *A. cepa* test showed that no treatment affected the mitotic index significantly, compared with the controls (5% significance level, Table 1).

Table 1 Number of mitotic cells analyzed and mitotic index obtained for each treatment with the bark of *T. aurea* (Manso) Benth. & Hook. F. ex S. Moore.

Treatment	Interphase	Mitotic phase				MI
		Prophase	Metaphase	Anaphase	Telophase	
NC	13856	113	13	7	11	1.03
PC	11995	189	21	42	52	2.47
ME 100	13949	43	2	1	3	0.35
ME 50	13947	52	3	0	8	0.45
ME 25	7916	86	13	3	12	1.42
ME 12.5	11915	70	6	8	2	0.72
AE 100	13897	83	10	3	7	0.74
AE 50	9855	191	49	26	12	2.74

NC= negative control; PC = positive control; ME = methanolic extract (100%, 50%, 25%, and 12.5%); AE = aqueous extract (100%, 50%); IM= mitotic index.
ANOVA-Tukey test at 5% significance ($p < 0.05$), OriginPro 2017 software.

The results also showed that *T. aurea* ME 100% and 50% induced a significant cytotoxic effect in *A. cepa* roots, indicating a likely allelopathic activity. However, *T. aurea* AE 100% and 50% did not promote significant changes in root growth of *A. cepa* five days into treatment (Fig. 2).

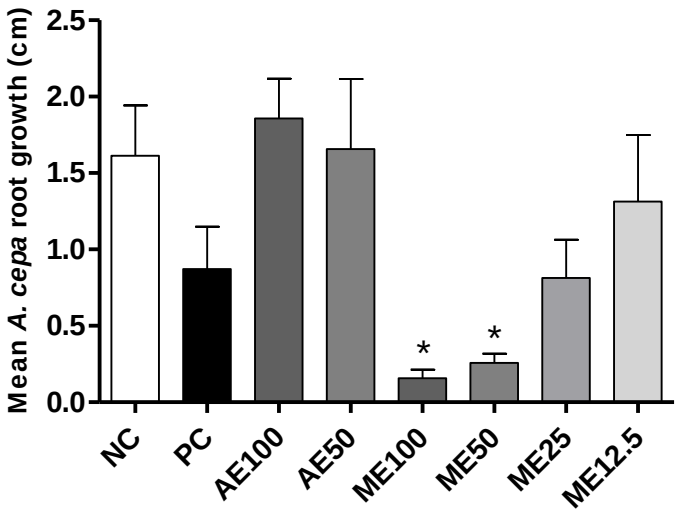

Fig. 2 Mean root growth of *A. cepa* roots after treatment with the methanolic extract of *T. aurea* bark NC = negative control; PC = positive control; ME = methanolic extract (100%, 50%, 25%, and 12.5%); AE = aqueous extract (100%, 50%); $p < 0.05$, ANOVA and Tukey test.

The micronucleus test in *A. cepa* roots showed that neither AE nor ME induced any mutagenic effect, regardless of concentration, compared with the positive control.

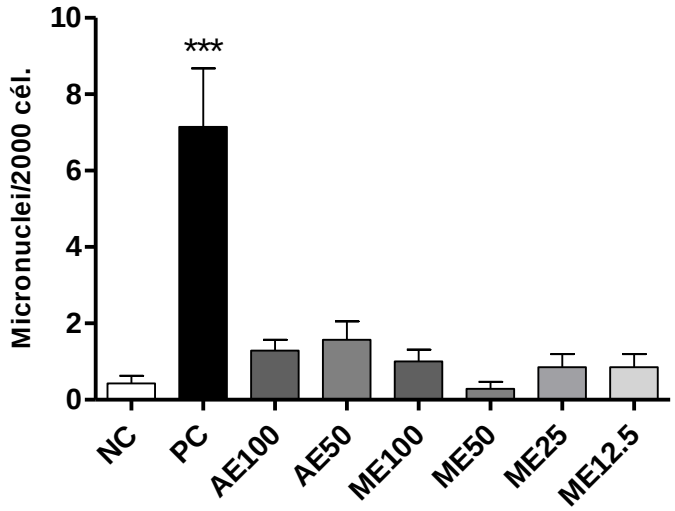

Fig. 3 Mean micronucleus counts after treatment of the aqueous and methanolic extracts of *T. aurea* bark. NC = negative control; PC = positive control; ME = methanolic extract (100%, 50%, 25%, and 12.5%); AE = aqueous extract (100%, 50%); $p < 0.05$, ANOVA and Tukey test.

IV. DISCUSSION

Medicinal plants are widely used in folk medicine. This popularity is explained in view of the misconception that botanical medicinal preparations are healthy simply because they are not industrially processed. But the various chemical constituents of plants may trigger a variety of physiological mechanisms in an organism, eventually manifesting as undesired reactions and side effects such as poisoning, for instance [29,30].

The unsupervised use of medicinal plants may become a hazard, since sometimes species are difficult to identify accurately, have variable chemical composition, and may be relatively toxic [31]. Most plants are toxic when consumed as high concentration extracts; therefore, it is important to investigate the toxicity of these preparations [32]. In addition to the variety of chemical constituents, the likely adverse reactions caused by

plants in humans may be ascribed to the presence of contaminants and the fact that homemade medicines are not always reliable [33].

The results obtained in the present study show that *T. aurea* did not induce any cytotoxic effect in *A. salina*, regardless of the concentration tested. But in a study that analyzed the cytotoxicity of flowers of *Tabebuia serratifolia* (Vahl) [34], the results indicated that the same concentrations as used in the present study induced LD_{50} of 679 μg/mL, which is indicates cytotoxicity according to the classification system devised by Meyer [22]. These differences in result may be imputed to the part of the plant used to prepare extracts, cultivation conditions, seasonality, water availability, temperature, altitude, ultraviolet radiation, mechanical factors, attacks by pathogens, and genetic differences.

Another study revealed that *Zeyheria tuberculosa*, which also belongs to the family Bignoiaceae, like the species analyzed in the present study, was atoxic in the *A. salina* test [35]. The authors used concentrations varying between 10 μg/mL and 100 μg/mL of an ethanolic extract prepared with the stems of the plant.

Similar findings to the present study were observed by Byeon et al. [36] in a study that analyzed the toxicity of the liber of *Tabebuia avellanedae* using aqueous extracts (50 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL) and the MTT assay in macrophages, with no toxicity detected [36].

Genotoxic substances pose a continuous hazard to organisms. They may trigger gene duplication, gene transcription, and chromosome changes, eventually causing death [37]. In this sense, the *A. cepa* test may be used as an indicator of cell proliferation, helping detect appropriate proliferation based on the mitotic index [38]. The test reveals the existence of disturbance in the mitotic process and the presence of toxic substances in cells. These disturbances may be determined based on the instability of the mitotic test [39].

The development of indicator effects as revealed by the mitotic index, when they are affected by toxic agents, may affect cells negatively and induce tumorigenesis [40].

In the present study, no significant difference was observed in the mitotic index for *Allium cepa* roots exposed to ME and AE of *T. aurea*. This may be the consequence of the short treatment time of these roots to test solutions. It is known that the time the test is conducted for may increase or decrease cell division rate. The literature cites no study addressing the analysis of the mitotic index for this species or its family as a whole.

Cytotoxicity is measured using the cell growth rate, and may be high or low [41]. For Silva [42], a significant decrease in mitotic index compared to a negative control indicates changes in growth rate that may be caused by secondary metabolites in the extract tested.

In the present study, the analysis of the effect of extracts on root growth in *A. cepa* shows that *T. aurea* EM 100% and 50% inhibited root growth, with significant cytotoxic effect compared with the negative control. Similar results were obtained for the same plant species by Póvoas et al. [43] in a study that analyzed the ME of leaves between 10 μg/mL and 100 μg/mL and the MTT test in macrophages. But the authors observed that treatment with the 500 μg/mL dose induced strong cytotoxicity. The toxicity recorded in the present study may be associated with the secondary metabolites such as anthraquinones and alkaloids. The toxicity of anthraquinones is a function of the affinity for plasma proteins [44]. Alkaloids have wide structural diversity, which is responsible for the biological activities exhibited by these compounds, especially cytotoxicity [45].

During the germination and development of model plant species, phenolic compounds as well as tannins and terpenes may work individually or concomitantly to affect physiological processes [46]. Also, flavonoids influence osmosis potential, inhibiting absorption and reducing root growth [47]. Previous research showed that the inhibitory action of flavonoids in the aqueous extracts of plants influence root growth [48]. Moreover, terpenes, anthraquinones, alkaloids, and flavonoids have been identified in *T. aurea*, and are the metabolites responsible for the toxicity of the species.

Although plants produce metabolites that are beneficial to health, they may also produce or have cytotoxic, mutagenic, and/or genotoxic compounds, which may interact with DNA and induce the emergence of mutations in cells. Mutations are observed based on micronuclei, which are small corpuscles containing DNA formed from chromosomal breaks and containing whole chromosome sequences that, at the moment of cell division, do not attach to the spindle. As a result, the chromosome fragment and the whole chromosome do not interact with the new nucleus, generating a small, individual nucleus, called micronucleus [25].

The plant species investigated in the present study did not induce mutagenic effects regardless of concentration of extracts. Similar findings were published by Moraes et al. [49] in a study that assessed the mutagenic and antimutagenic activities of the bark of *Tabebuia impetiginosa*, also from the Bignoniaceae family. The authors observed no micronuclei in the model organism, irrespective of concentration of extracts. A study carried out by Lourenço et al. [50] likewise did not report any mutagenic, antimutagenic, or cytotoxic action of the extract of *T. impetiginosa* flowers in the micronucleus test.

Using the *Salmonella*/microsome assay, Resende [51] analyzed the effect of the hydroalcoholic extract of the bark, leaves, and roots of *Arrabidaea brachipoda*, family Bignoneaceae. The authors observed that only the aqueous fraction of the leaves was mutagenic, inducing an increase in the number of revertant colonies. This was explained by Duarte e Silva et al. [52] in a study that found out that leaves exhibit high levels of flavonoids,

which are responsible for the high mutagenic action detected. For this reason, experiments carried out using leaves produce high mutagenicity findings, while barks and roots are not mutagenic because they have low levels of flavonoids.

Flavonoids comprise one of the largest groups of secondary plant metabolites. These compounds are abundant in fruit and leaf of plant species as well as in teas and vines. As the constituents responsible for the pigmentation of plants, flavonoids protect organisms against oxidizing agents in what has been described the most important of their numerous roles [53].

The present study was carried out using the bark of *T. aurea*. Since the bark of the species is not rich in flavonoids, no mutagenic effect was observed.

Therefore, it is important to assess the mutagenicity, cytotoxicity, and genotoxicity using several tests, which are usually accepted by regulation agencies. These tests indicate the hazards and the safety of use of a given substance [54,55,56]. However, the variety of results observed in research may be associated with the part of the plant used, amount of substrates, the concentrations tested, exposure times, and the secondary metabolites the plant produces.

V. CONCLUSION

The extracts of *T. aurea* bark were atoxic in the acute treatment of *A. salina*, as shown by the micronucleus counts. No significant differences were observed in mitotic index after treatments, although *T. aurea* EM induced no cytotoxic effects nor mutagenicity, regardless of the concentration used. Further studies have to be carried out to identify secondary metabolites using new techniques and other solvents, as a means to enlarge the body of knowledge obtained in the present study.

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