

Toxicity of brazilian medicinal plant extracts on *Macrobrachium amazonicum*

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Received: January 10, 2021 Accepted: March 24, 2021 Published: April 18, 2021

doi:10.5296/jas.v9i2.18162

URL: <https://doi.org/10.5296/jas.v9i2.18162>

Abstract

Medicinal plants from Amazon have been commercialized for decades, but few scientific studies prove their effectiveness and safety in use in aquaculture activities. The objective of the present study was to use the Amazon river prawn *Macrobrachium amazonicum* to predict the toxicity of the natural extracts of nine medicinal plants viz pariri *Arrabidaea chica*, muirapuama *Ptychopetalum olacoides*, anauerá *Licania macrophylla*, barbatimão *Ouratea hexasperma*, faveira *Vatairea guianensis*, sacaca *Croton cajucara*, jacareúba *Calophyllum brasiliense*, pau d'arco *Tabebuia* sp. and verônica *Dalbergia subcymosa*, in concentrations of 1, 10, 100, 500 and 1000 µg/mL. The media was prepared in 0.5% dimethyl sulfoxide (DMSO) diluted with water. Ten post-larvae (0.5 ± 0.1 g) were added to each triplicate and, after 24 h, the mortalities were evaluated, with the results of median lethal concentration expressed as LC_{50-24h} using the Probit statistical method. To obtain the concentrations of a common bioactive compound of plant extracts, the concentrations of flavonoids were analyzed using a methodology based on the formation of chromophores. The results of acute toxicity indicate variability in the toxic effects of medicinal plants, taking into account the concentration of total flavonoids, with the least toxic *Tabebuia* sp. (LC₅₀ = 758.31 µg/mL) and the most toxic *C. cajucara* and *V. guianensis* (LC₅₀ = 72.16 and 75.23 µg/mL), respectively. The extracts demonstrated lethality against *M. amazonicum*, which predicts toxicity and warns of its use them as herbal medicines. More studies must be carried out to determine other bioactive compounds in the plant extracts used since there is an unparalleled availability of chemical diversity.

Keywords: Amazon, aquaculture, lethality, medicinal plants, post-larvae

1. Introduction

The use of medicinal plants is a traditional form of treatment for human use and with great potential for use in aquaculture. In Brazil, because of its rich biodiversity, many researches have been focused on natural products, from new renewable sources of energy, from biomass (biofuels), or other industrial uses, to those directed to drugs. Currently, the pharmaceutical

industry handles high numbers worldwide, selling derivatives of medicinal plants (Albuquerque & Hanazaki, 2006). Among the medicinal plants studied for this purpose, the following stand out:

Arrabidaea chica (Pariri)

A scandent shrub traditionally indicated to treat symptoms of inflammation. Its ethanolic extracts are chemically investigated and tested against yeasts and dermatophyte fungi (Vicentino & Menezes, 2007).

Ptychopetalum olacoides (Muirapuama)

The indigenous tribes of Brazil use the bark and roots in infusion for many purposes as a treatment for neuromuscular disorders, cardiac and gastrointestinal asthenia acting as a muscle tonic (Velasco et al., 2008).

Licania macrophylla (Anauerá)

It is popularly known as “anauerá” or “anuera”, being found mainly on the lowland margins of the lower Amazon regions. Amazon communities use the stem bark of this plant as an antibiotic in the treatment of amoebic parasites and dysentery disorders. The *Licania* genus is rich in bioactive compounds such as flavonoids, terpenoids, steroids, etc (Lopes et al., 2012).

Ouratea hexasperma (Barbatimão)

The *Ouratea* genus comprises 300 tropical species that occur mainly in South America and has been reported to be used in folk medicine for the treatment of inflammation (Rocha & Galende, 2014).

Vatairea guianensis (Faveira)

Native to the Amazon, known as “fava bolacha” or “faveira”. Its fruits, stem bark, roots, leaves and juices are used for treatment as a dermatological antifungal (Araújo, 2010).

Croton cajucara (Sacaca)

Popularly known as “Sacaca”, it is a shrub with odorous leaves, common in the Amazon. In northern Brazil, the leaves and bark are used in teas, tinctures, or tablets as a hepatic-protective and antibiotic substance (Brasil, 2014).

Calophyllum brasiliense (Jacareúba)

It is of great interest in popular medicine and is used in the treatment of diabetes, and also as a healing and anti-viral substance (Carvalho et al., 2013).

Tabebuia sp. (Pau d’arco)

It is a species from the North and Northeast regions of Brazil, known as “ipê-roxo” and used in traditional medicine to contain inflammation (Carnevale et al., 2013).

Veronica officinalis (Verônica)

Used popularly to treat hemorrhage, and as antioxidant. It has properties such as healing, antifungal, antimicrobial, anthelmintic and anti-inflammatory (Saha et al., 2013).

Toxicological studies assess the harmful effects (acute and chronic) of chemical substances on living organisms. It operates on the principle that it is based on exploring the risk of animal and human exposure to various products to be able to establish safe conditions (Klüver et al., 2015).

Lethality is the inherent and potential ability of the toxic agent to cause harmful effects and death in living organisms. The toxic effect is generally proportional to the concentration of the toxic agent against the site of action (Klaassen & Watkins, 2012).

The use of plant extracts has increased in aquaculture as an alternative for prophylactic control. The extracts have some advantages over synthetic products for the cultivation of aquatic organisms, such as, less toxicity because they are less concentrated; they have multiple modes of action, resulting in less likelihood of causing microbiota resistance; in addition to reducing the environmental impact, as these are biodegradable (no environmental pollution), helping in the quality of cultivation, and reducing production costs (Coimbra et al., 2006).

There are several medicinal plants that have potential to help aquaculture in this regard. For use with prophylactic purposes, it is essential to know the concentrations that may cause toxicity (chronic and lethal) to the organisms. Toxicity tests with herbal products aim the safety use of plant extracts without causing harmful effects to organisms kept in captivity (Claudio et al., 2012; Aguinaga et al., 2014).

The bioactive compounds of plant extracts are substances that the plant synthesizes and stores during its growth. The active ingredients are not evenly distributed in the vegetable. They concentrate preferentially on flowers, leaves and roots, and sometimes on seeds, fruits and bark. These compounds are responsible for helping plants adapt to environments they are in, being sources of biologically active substances (Fumagali et al., 2008). In the plant extracts studied, flavonoids (phenolic compounds with activities such as antioxidant, anticancer, antibacterial, cardioprotective agents, anti-inflammation, immune system promoting, and so on) were analyzed, as they form a large group, due to the number of their natural constituents and wide distribution in the plant kingdom (Nacz & Shahidi, 2006; Khan et al., 2014; Panche et al., 2016; Metodiewa et al., 2017; Tungmunnithum et al., 2018). Flavonoids can be found in diverse structural forms, but their fundamental nucleus has 15 carbon atoms that form a tricyclic compound (Zuanazzi & Montanha, 2004). We believe in the relationship between the toxicity of plant extracts and their constitution in terms of flavonoids.

In order to test the different plant extracts from North of Brazil, we choose an important species of crustacean, the *Macrobrachium amazonicum*. This prawn is a Brazilian native species and can be found in the Amazon Basin, São Francisco Basin, Paraguay Basin, North and Northeast Coastal Basins, as well as countries like Guyana, French Guiana, Venezuela, Ecuador, Peru and Bolivia (Coelho & Ramos-Porto, 1985; Pereira et al., 2017). It is also a species with great potential for aquaculture (New & Valenti, 2000; Anjos et al., 2014).

Social aspects are also relevant when it comes to *M. amazonicum*, because there are riverside populations that use species fishing as a means of survival in certain locations, such as “Pará” state, Brazil (Freire & Bentes, 2008), where the activity generates jobs and income for families (Silva et al., 2017).

The objective of the present research was to use *M. amazonicum*, as a model to predict the toxicity of natural products (taking in consideration the flavonoids as bioactive compounds), for later use in aquaculture as aids in the treatment of animal diseases and organ disorders.

2. Method

The experiment was carried out with a completely randomized design with ten treatments (nine extracts and control) and three repetitions in each.

Herbal extracts containing tinctures from pariri, mairipuama, anauerá, barbatimão, faveira, sacaca, jacareúba, pau d’arco, and verônica were purchased from the Scientific and Technological Research Institute of Amapá (IEPA), in commercial packaging. The commercial samples obtained were sent to the mariculture sector of the Aquaculture Laboratory of the Federal University of Minas Gerais (UFMG) for the appropriate analysis and tests (ANVISA, 2010; Wannan et al., 2010).

Briefly, the content of total flavonoids was determined according to the methodology and determination equations described by Wannan et al. (2010), using aluminum chloride that reacts with the sample flavonoids generating a chromophore, which represents the characteristic molecule by identifying the characteristic yellow color of the flavonoids, which is evaluated at 420 nm in UV spectrophotometer. A calibration curve was plotted with rutin. The result was expressed in mg of rutin equivalents/g of extract. The validated parameters were linearity, specificity, precision, accuracy and robustness, as well as the determination of impurities and identification of the extract.

Ten post-larvae of *M. amazonicum* (Amazon river prawn) weighing 0.5 ± 0.1 g were used per treatment, maintained in 500mL-beaker with aeration, containing the extracts under analysis. The concentrations tested were 1, 10, 100, 500 and 1000 $\mu\text{g/mL}$ and were prepared in dimethyl sulfoxide (DMSO) and diluted in water, not exceeding 0.5% DMSO in the final solution (Oliveira et al., 2019a).

After 24 hours, the dead prawns were removed. The tests with the nine extracts were performed in triplicates. Safety levels were defined based on the most estimated values for pollutants in relation to the median lethal concentration for 50% of the population (LC_{50}), multiplying the LC_{50} by the application factor proposed by Sprague (1971).

For the statistical tests, the Probit program (Finney, 1952) was used to estimate the LC_{50} , and the Kruskal-Wallis test was used to determine the different toxicities between the extracts at the 5% level of significance using the Infostat version 2019 program (Casanoves et al., 2012).

Regarding the study of total flavonoids for the different plant extracts, the results obtained are shown in the table 1.

3. Results

The LC₅₀-24h of plant extracts for *M. amazonicum* post-larvae with the respective upper and lower limit values are shown in figure 1.

The extracts that had the greatest toxic effect were sacaca LC₅₀-24h of 75.96 µg/mL, faveira LC₅₀-24h of 79.19 µg/mL, and anauera LC₅₀-24h was 99.94 µg/mL.

Barbatimão and verônica showed the same toxicity to *M. amazonicum* post-larvae, the LC₅₀-24h were 102.5 µg/mL (Tab. 2). The jacareúba showed LC₅₀-24h of 322.2 µg/mL, followed by pariri and muirapuama plants with the respective values of 433.3 and 493.9 µg/mL.

Based on the results obtained, the least toxic medicinal plant for Amazon river prawn was pau d'arco, with the LC₅₀-24h of 722.2 µg/mL. Table 2. shows the safety levels of the tested extracts.

Table 1. Total concentration of flavonoids for the different plant extracts from Brazil

Extract	Total flavonoids (mg/g ± SD)
<i>Licania macrophylla</i>	9.65 ± 0.25
<i>Calophyllum brasiliense</i>	8.05 ± 0.15
<i>Veronica officinalis</i>	7.18 ± 0.13
<i>Vatairea guianensis</i>	6.33 ± 0.80
<i>Arrabidaea chica</i>	6.20 ± 0.07
<i>Croton cajucara</i>	6.00 ± 0.83
<i>Ouratea hexasperma</i>	2.59 ± 0.14
<i>Tabebuia</i> sp.	N.D
<i>Ptychopetalum olacoides</i>	N.D

N.D = no detected

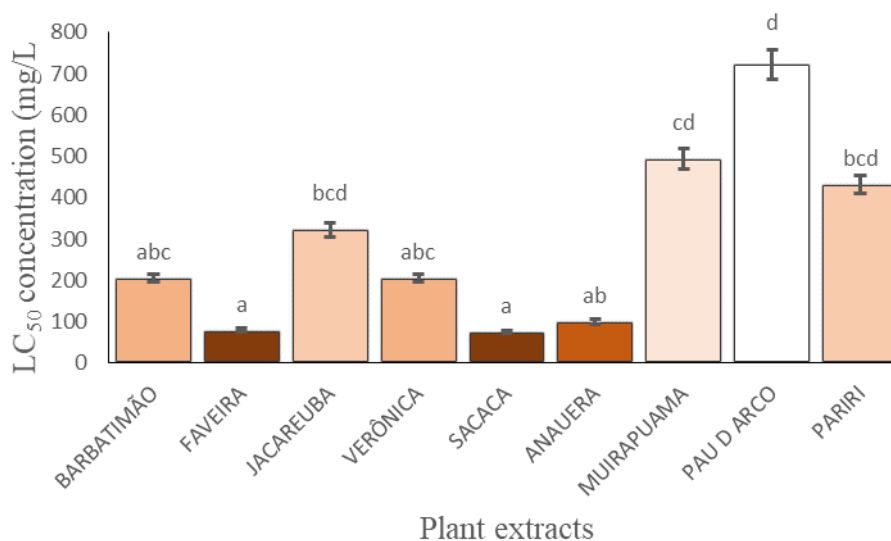


Figure 1. Median lethal concentration (LC₅₀) 24 h of plant extracts to Amazon freshwater prawn *Macrobrachium amazonicum*. Different letters show significant differences (P <0.05) by the Kruskal Wallis test. Toxicity a > b > c > d.

Table 2. Safety levels for the use of Amazon plant extracts for freshwater prawn *Macrobrachium amazonicum*.

Plant Extract	Safety Level* (µg/mL) ±SD
<i>Tabebuia</i> sp. (Pau d'arco)	72.22 ± 3.62
<i>Ptychopetalum olacoides</i> (Muirapuama)	49.39 ± 2.48
<i>Arrabidaea chica</i> (Pariri)	43.33 ± 2.18
<i>Calophyllum Brasiliense</i> (Jacareúba)	32.2 ± 1.60
<i>Veronica officinalis</i> (Verônica)	10.3 ± 0.52
<i>Ouratea hexasperma</i> (Barbatimão)	10.3 ± 0.52
<i>Licania macrophylla</i> (Anauerá)	10.0 ± 0.50
<i>Vatairea guianensis</i> (Faveira)	7.9 ± 0.40
<i>Croton cajucara</i> (Sacaca)	7.6 ± 0.38

*10% LC₅₀-24h.

4. Discussion

Regarding the study of flavonoids, some identifications for *Licania* were observed in a publication reporting the traditional, phytochemical, and pharmacological use of the genus (Feitosa et al., 2012). These authors described the chemical composition of several species of *Licania*, finding potential for flavonoids in some of them, such as *Licania pettiere* and *L. carii*. Additionally, Neto et al. (2013), conducted studies with *Licania* sp. and *Parinari* sp., showed more than six positive results for flavonoids. Medeiros & Medeiros (2012) also discovered a new flavanol, licanol, in *L. macrophylla*. In the present study, a very favorable potential for flavonoids was identified in the species *L. macrophylla*, 9.65 ± 0.25 mg/g.

In a phytochemical approach performed on leaves of *A. chica*, Alves et al. (2008) reported the presence of different chemical classes, among them flavonoids. Our quantification of total flavonoids performed in this work identified 6.20 ± 0.07 mg/g in *A. chica*.

Calophyllum brasiliense is a species of great medicinal interest in the treatment of diabetes, however in the literature there were no phytochemical studies related to flavonoids. In the present work, flavonoids were identified and quantified by spectrophotometry in the samples with a mean concentration of 8.05 ± 0.15 mg/g.

Ptychopetalum olacoides did not show potential for flavonoids in the analyzes performed in the present work. In the literature, no studies were found regarding the quantification of flavonoids performed with the species.

In a phytochemical study carried out with the bark of the *C. cajucara* tree, Tieppo et al. (2006) demonstrated the presence of several diterpene clerodanes such as trans-dehydrocrotonina, trans-crotonina, cis-cajucarina and sacacarina. Pharmacological analyses were performed with *C. cajucara*, and anti-inflammatory, antimicrobial, analgesic, antiulcerogenic or antilipemic properties were described. The bark essential oil has also been reported to have gastroprotective activity. According to Zou et al. (2013), a new flavonoid was found in *C. cajucara*, which represents a rare group of flavanols with specific cyclization. In the present study, the mean concentration of flavonoids for this species was 6.00 ± 0.83 mg/g.

There is no quantification of flavonoids in *O. hexasperma* in the literature. In the present study, the potential for flavonoids in that species showed a low result, with 2.59 ± 0.14 mg/g.

Veronica officinalis is a common species in the Amazon. (Nikolova, 2011), and studies were carried out regarding the identification of flavones in 29 species of *Veronica* and, in fact, found eight flavone aglycones. There are no comparative data regarding the quantification of flavonoids in *V. officinalis* in the literature. In the present study, one of the highest mean concentrations (7.18 ± 0.13 mg/g) was identified in this species.

Vatairea guianensis presented few scientific references that mention biological activities for the species, as well as no data regarding flavonoids. Although Souza (2013) reported for the first time four isoflavones and five triterpenes identified in a lupeol mixture. The quantification of total flavonoids was 6.33 ± 0.80 mg/g for *V. guianensis*.

Govindappa et al. (2013) in studies carried out regarding the antioxidant activity and

phytochemical screening endophytes, noted that *Tabebuia* sp. proved to be a rich source of tannins, flavonoids, steroids, alkaloids, etc, especially phenolic and polyphenol. Such substances have been classified as cytotoxic, antimicrobial, and antifungal due to the presence of anthraquinone compounds. In our study, we did not identify total flavonoids in *Tabebuia* sp.

The study of flavonoids contained in plant extracts aims to quantify one of the main bioactive compounds that can cause intoxication in animals when used incorrectly. Although flavonoids are commonly found in plants for medicinal purposes, other bioactive compounds must be quantified in the extracts of the nine plants studied in the present study, in view of the additive effect of them on the biota treated with plant extracts.

Some crustaceans are widely used in ecotoxicological tests. Once the safety levels of plant extracts are defined to different animals, they can be used to treat them in closed cultivation systems without compromising their normal development. For this, it is also necessary to know their effectiveness in deal with specific pathogens.

Several toxicological studies are carried out with in small crustaceans observing some aspects such as mobility in *Daphnia similis*, reproduction in *Ceriodaphnia dubia* or lethality in *M. amazonicum* post-larvae (Oliveira-Filho, 2013).

Plant extracts may have antibacterial activity against pathogens that affect humans (Ushimaru et al., 2007) and animals (Dal Pozzo et al., 2011), and the replacement of current antimicrobials with herbal products in aquaculture is not utopia. Several medicinal plants have shown activity to control and prevent pathogenic bacteria in fish, such as *Aeromonas hydrophila* (Harikrishnan et al., 2009; Harikrishnan et al., 2010), *Streptococcus iniae* (Abutbul et al., 2004; Zilberg et al., 2010), *Streptococcus agalactiae* (Rattanachaikunsopon & Phumkhachorn, 2010), *Flavobacterium columnare* (Tavechio et al., 2009), *Pseudomonas fluorescens* and *Edwardsiella tarda* (Harikrishnan et al., 2009).

These plant extracts can be of low cost to the producer and some are considered immunostimulants that increase the resistance of animals and stimulate non-specific responses of the immune system (Melo et al., 2008).

In an acute toxicological study carried out with *Artemia salina* exposed to three species of medicinal plants of the genus *Phyllanthus*, in Northeastern Brazil, the LC₅₀-24h were similar to those found in this work for the species muirapuama, pau d'arco and pariri. The authors estimated the LC₅₀-24h from 404.43 ± 49.64 to 770.84 ± 51.78 µg/mL for *Phyllanthus niruri*, 837.65 ± 61.45 to 1,075.89 ± 70.72 µg/mL for *Phyllanthus amarus* and 534.60 ± 46.83 µg/mL to 1,003.62 ± 65.15 µg/mL for *Phyllanthus tenellus* (Araujo et al., 2010).

The medicinal plant *A. chica* (pariri) is used as an anti-inflammatory, astringent, and against intestinal colic, diarrhea, anemia and skin diseases (Amaral et al., 2012). It presented low toxicity to *M. amazonicum* post-larvae, corroborating the study of Oliveira et al. (2009). These authors observed that this medicinal plant had practically no toxic effect on *M. amazonicum* post-larvae, demonstrating the low toxicity of the ethanolic extract present in the leaves.

In addition, pariri also presented low toxicity to some mammals, as demonstrated in a study carried out with rats, whose median lethal dose (LD_{50}) = 2g/kg (Oliveira et al., 2019b). Barbatimão, which is also used as an herbal medicine, should be administered with caution due to the phytotoxicity presented to animals such as Wistar rats (Rodrigues et al., 2013) and *A. salina* (Ramos et al., 2014).

Tests with *A. salina* exposed to ethanolic extract of Anauerá bark showed low toxicity to this small crustacean (Silva et al., 2011). In the same way, Faveira presented low toxicity for Wistar Rats, which can be used to treat integumentary tissue (Rodriguez et al., 2004). The use of trans-dehydrocrotonin from Sacaca in rats demonstrates a gastro-protective effect; however, at doses of 100 mg/kg, this plant caused liver damage (Oliveira et al., 2014). The use of Jacareúba extract in rats above 1,000 mg/kg demonstrated deleterious effects and intoxication signals such as agitation and depression (Peters & Guerra, 1995). Studies with verônica extract in pregnant rats did not caused embryo-toxicity and the authors indicate its use as an anti-inflammatory (Prósperi, 1993).

In general, *M. amazonicum* post-larvae are also important for monitoring the natural environments where the species occurs, as described in the literature (Silva, 2002; Costa et al., 2008; Pimentel et al., 2011; Hirota et al., 2012). Therefore, toxicity tests with prawn post-larvae are used in several research areas, such as aquaculture, medicinal chemistry, pharmacology, agriculture and ecotoxicity, the latter being of great importance in the evaluation of the toxic potential of extracts and isolated substances (Ikhwanuddin et al., 2014). Some authors have reported the effect of bioactive compounds from Brazilian plant extracts against bacteria (by antimicrobial activity) (Suffredini et al., 2004), favoring the raise of prawn *M. amazonicum* in captivity (Rocha et al., 2014; Brilhante et al., 2015).

In the present study, it was possible to verify the toxicity of plant extracts and their safety levels for use with Amazon prawn post-larvae. However, further work is needed to test its phytotherapeutic action (bioactive compounds) against pathogens, such as anti-inflammatories and other uses in the cultivation of *M. amazonicum*. The less toxic extracts (pau d'arco, pariri, muirapuama, and jacareúba) with anti-inflammatory and protective function of the organism can be used in the treatment of various disorders (Vicentino & Menezes, 2007; Velasco et al., 2008; Carnevale-Neto et al., 2013; Carvalho et al., 2013). The other extracts (verônica, sacaca, barbatimão, anauerá, and faveira) must be used with greater care due to their toxic potential and has application as fungicides and bactericides in the systems and in treatments against these pathogens (Araújo, 2010; Lopes et al., 2012; Saha, 2013; Brasil, 2014; Rocha & Galende, 2014).

5. Conclusions

The method carried out to flavonoid determination showed high specificity at 420 nm for the extracts of the species provided by the Institute of Scientific and Technological Research of Amapá (IEPA), giving reliability in the quantification of flavonoids.

Sacaca and faveira extracts were the most toxic to post-larvae of *M. amazonicum*, with the LC_{50-24h} = 72.16 and 75.23 $\mu\text{g/mL}$, respectively, and the least toxic medicinal plant was pau

d'arco, with the $LC_{50-24h} = 722.2 \mu\text{g/mL}$. The post-larvae of *M. amazonicum* showed sensitivity to the nine extracts evaluated, which predicts care in view of the toxic effects in the use of products derived from these plants if they are used in the cultivation of this crustacean.

The chemical diversity of the natural extracts encourages the study of other bioactive compounds (in addition to flavonoids) contained in the plant extracts tested. The research carried out with medicinal plants from the Amazon region was of paramount importance, in view of the plant richness of that region. Thus, the sequence of this research becomes important to assist the use of plant extracts in aquaculture.

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