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Analyses of *Ampelozizyphus amazonicus*, a Plant Used in Folk Medicine of the Amazon Region

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ABSTRACT
In Brazil, a popular preparation of *Ampelozizyphus amazonicus* Ducke is widely used to prevent malaria. Physical chemistry analyses such as atomic absorption spectrophotometer, HPLC and antioxidant activity by spectrophotometer were used to evaluate the raw botanic material (stem barks, leaves, twigs and root barks) and extracts of root barks. The chromatographic profile of the 1% root barks preparation of *A. amazonicus* showed 48.4% of saponins that are important point for the knowledge of this popular formulation. The concentration levels of macronutrients and micronutrients along with phosphorus were obtained from this species. The iron level of the root barks was higher than that observed in the other vegetal material. This result was considered relevant based on the fact that certain malaria-related processes involve the presence of iron and the root barks are the part of the plant used by the population. The DDPH assays showed that the one-percent extracts have no relevant ability as free-radical scavengers or hydrogen donors but these results may be associated to the parasite evolutive cycle and the popular use of the plant for the prophylaxis of malaria. The root barks preparation like the folk use did not present significant toxicity to brine shrimp.
KEYWORDS: *Ampelozizyphus amazonicus*; Folk medicine; Malaria; HPLC; Brine shrimp; Metals.

INTRODUCTION

Ampelozizyphus amazonicus belongs to the Rhamnaceae family. The plant is a liana that reaches 25 cm diameter as an adult tree. It can be found on the banks of *igarapés* and in the forest of the Amazon region, particularly in Manaus, Maués and the Trombetas River. It is popularly denominated "cervejeira, cerveja de índio, saracura, curupira-mirá and saracura-mirá" and is used as stimulant, energizer (1), antiseptic on wounds (2) and antimalarial in endemic areas (3). According to the population information, the popular recipe to prevent malaria considers a spoonful (soupspoon) of the grated root barks, prepared with water at room temperature, which must be drunk after withdrawing the intense foam produced by shaking the roots in water, on alternate days. Although the prophylactic activity of the crude extract has been proved (3), the mechanism of action and the active substances of *A. amazonicus* are not yet identified. Additionally, there are few investigations on its chemical composition and little knowledge about the potential influence of metals on the pharmacological effects of natural drugs obtained from medicinal plants. Such influence is possible either by direct metal binding to pharmacologically active substances or, indirectly, by influence of bioavailability, pharmacokinetics, or side effects of the drug (4).

Several nutrients may potentialize the morbidity of malaria through immune modulation and alteration of oxidative stress (5) and may implicate in resistance to many infectious diseases. These nutrients together with other essential elements are necessary for

growth, normal physiological function and survival (6). In view of the important utilization of *A. amazonicus* in the folk medicine, this work is aimed to determine the physical chemistry analyses of this species. The HPLC profile of the root barks aqueous preparation was investigated. The stem barks, leaves, twigs, root barks and aqueous extracts of the root barks were analyzed as to the macro and micronutrients. The antioxidant activity and toxicity to the brine shrimp were evaluated with a formulation prepared in accordance with the folk use.

MATERIALS AND METHODS

Plant material

The botanic materials were collected in Manaus (Km 38, Amazonas State). A voucher specimen (number 191532) has been deposited in the INPA Herbarium, Manaus, Brazil.

Mineral analysis of Ampelozizyphus amazonicus extracts

Preparation of the samples

Plant material

The parts of the plant (stem barks, twigs, leaves and root barks) were washed with deionised water to remove dirt and were dried to constant weight in an oven at 60°C. The dried samples were grounded and then stored in airtight plastic containers. For each nutrient, a weighed amount of each sample was analyzed, in triplicate. The samples were weighed separately (1g) in tube digester and prepared in triplicate, then 10 ml of nitric acid (HNO_3) were added and kept at rest for 24 h. After that, the samples were placed in block digester at about 120°C temperature, to ensure the complete digestion of the samples. After cooling, 2 mL of hydrogen peroxide (H_2O_2) were added to each sample and then placed into the block digester for over 24 hours to finish the acid digestion process. After cooling, the samples were put in a 100 mL flask and the volume completed with Milli-q water.

Preparations with the root barks

A spoonful (soupspoon) of the fresh root barks powder (contain 1g of vegetal material) was added to 100 mL of deionized water, the mixture was left for 2 min under agitation and the formed foam was removed before the lyophylization. This procedure was based on the popular use of *A. amazonicus* and repeated more twice. The suspension was filtered and then 10 mL of concentrated nitric acid were added to digest the sample. The resulting solution was reduced to 30 mL. The preparation was transferred into a 50 mL-volumetric flask and then completed with deionized water (7). For the purpose of comparative studies, the procedure was also made with 10% and 20% (w/v) of the fresh plant. The 1%-root barks preparation of *A. amazonicus*, after the lyophylization, produced 3.8 mg of material to be ingested.

Atomic absorption spectrophotometer

The digested and diluted samples were submitted to reading through analysis for atomic absorption spectrometry (AAS) in air-acetylene flame by model AA3300 (Perkin Elmer), hollow cathode lamps for Zn, Cu, Fe, Mn, Na, K, Mg, Ca, Cr, Ni and Co. The elements were measured under optimum operating conditions with an air-acetylene flame. Phosphorus levels of the plant samples were determined by colorimeter at 630 nm.

Reagents, glassware and stock solutions

All reagents used were of analytical grade purity and only ultrapure water (ELIX –Millipore, model – JBRLX03Y6) was used. For sample digestion, concentrated HNO_3 (Merck) was applied. La_2O_3 (Merck) was used for the analysis of Ca, Mg, Na and K with the purpose to mask the P, which might interfere in the reading of these macronutrients. Stock standard solutions of Zn, Cu, Fe, Mn, Na, K, Mg, Ca, Cr, Ni, Co and P containing 1000 ppm of each metal were used. Calibration standards of each element were obtained by appropriate dilution of the stock solutions. Glassware bottles were cleaned with 20% nitric acid and rinsed several times with deionised water.

Analytical procedure

All measurements were run in triplicate for the sample and standard solutions, after these procedures, the calibration curves were prepared for the reading of macro and micronutrients.

HPLC analysis of the 1% root barks preparations

Analytical HPLC analysis was performed on a Shimadzu LC-8Avp gradient chromatograph equipped with two LC-8Avp pumps, controlled by an interface module, an automatic injector and an SPD-M10Avp photodiode array detector (PAD) was used for peak purity test and analysis of compounds. Solvents were filtered using a Millipore system and chromatographic separation was carried out on a C18 column (150 mm x 4.6 mm, 5µm; Shimpack). The mobile phase consisted of 0.05% aqueous trifluoracetic acid (A) and acetonitrile (B) and the composition gradient was: (0-5 min) 3% - 10% (B), (5-60 min) 10%-30% (B), (60-65 min) 30-45% (B). 40 µL of the three 1% root barks preparations were injected (triplicate) and monitored at 210 nm. A constant flow-rate of 0.8 $mL.min^{-1}$ was used during analysis and a PAD range 200 – 600 nm. HPLC grade solvents and Milli-q water were used in the chromatographic study. All

chromatographic experiment was performed at room temperature.

DPPH radical-scavenging assay

The evaluation of the antioxidant activity was made by the radical-scavenging method of Chell et al. (2005) (8). The absorbance was measured by spectrophotometer at 517 nm, using a UV/VIS (Thermo Electron Corporation, model: BioMate^{TM} 3). Inhibition free radical DPPH in percent was calculated as follows: % scavenging effect = [(control$_{517nm}$ - sample$_{517nm}$)/control$_{517nm}$] x 100. The scavenging concentration of each sample at 50% (SC_{50}) was used to compare the levels of free radical-scavenging activity among the samples.

Toxicity testing against the brine shrimp (Artemia salina)

The bioactivity of the extracts was monitored by the brine shrimp lethality test, applying the method described by Pimenta et al. (2003) (9). The samples to be assayed were dissolved in dimethylsulfoxide (DMSO) (4 mg were dissolved in 800 µL of DMSO) and diluted serially (10, 20, 30 and 50 µL/5 mL) in seawater. In each case three replicates of each concentration were assayed. After 24 h the numbers of survivors were counted and percentage of death calculated. Criterion of activity: LC_{50} values of <1000 µg/mL for extracts (10, 11). Lapachol dissolved in DMSO was used as a positive control. The Probit analysis was used for attainment of the LC_{50} and respective intervals of confidence (12).

RESULTS AND DISCUSSION

Chromatographic Profile

The chromatographic profiles of the plant extract have been largely accepted and applied for the quality evaluation processes of medicinal plants. In this sense, HPLC coupled with the diode array detector (DAD) is an analytical tool of resolution used to obtain these chromatographic profiles (13). According to the literature (14), the characteristic UV spectrum of saponins was used to identify the peaks with similar profile in the chromatograms obtained by HPLC/DAD at 210 nm (Fig. 1). The three analyses of the 1% root barks preparations, prepared at the same time, showed similar profiles with small variation. The best chromatogram is presented at the figure 1. These peaks were numbered in the chromatogram and the analyses of them related to the UV spectra revealed the presence of 48.4% of saponins. Despite the withdrawal of foam, they remain as major constituents in the preparation. Jujubogenin and dammarane-type saponins were previously reported to this species (15, 16). This chemical class is related to various biological properties, for example, the dammarane-type saponins associated to the hepatoprotective effect (17). These results are important for the knowledge of this preparation, which is widely used as a folk medicine in Brazil.

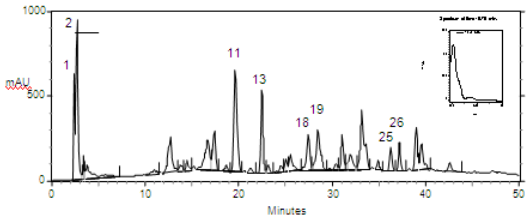

Figure 1. Chromatogram of Ampelozizyphus amazonicus 1% root barks preparation.

DPPH free radical-scavenging effect

The free radical scavenging activity of the two extracts prepared with 1% of the root barks and different solvents (ethanol and water) were determined by the DPPH method. The DPPH radical was used to test the ability of these samples as free-radical scavengers or hydrogen donors and antioxidant as it was previously described (18). The ethanol extract was used in the *in vivo* assays with the plant and the aqueous extract is used in the folk medicine. The highest SC_{50} was shown by ethanol extract from 1% of the root barks (119.06 µg/mL), while the lowest activity was observed for the aqueous preparation of 1% of the root barks [preparation as antimalarial use (SC_{50} = 590 µg/mL)]. The different values may be related to the nature of the solvent used in the preparation of extracts (19). These results showed no relevant ability of these one-percent extracts as free-radical scavengers or hydrogen donors. Additionally, they may be associated to the parasite evolutive cycle (20) and the action of *A. amazonicus* in the pre-erythrocitic stage (3), which corresponds to the liver stage in human malaria (21), considering that the events related to the oxidative stress due to the pathology have been established mainly in the blood stage of malaria (22 - 24).

Brine shrimp test

The brine shrimp lethality assay is considered a useful tool that is rapid, simple, reproducible and economical for preliminary assessment of toxicity (11). The tests were performed with the aqueous preparation of 1% of the root barks and the saponinic extract obtained according to Maciuk et al. (2004) (25). The results did not show any significant toxicity against *Artemia salina* with LC_{50} = 250 and 176 µg/mL, respectively. It is important to emphasize that the tests with *Artemia salina* present a good correlation with the toxicity

assays made *in vivo* as demonstrated by Parra et al. (2001) (26), which suggests that the low toxicity presented by the preparation represents a preliminary relevant result in the study of the plant, with positive reflex in the popular use.

Macro and Micronutrients

The aqueous preparations and vegetable organs of the *A. amazonicus* were analyzed as to the presence of 12 elements (Zn, Cu, Fe, Mn, Na, K, Mg, Ca, Cr, Ni, Co and P) by atomic absorption spectrometry (AAS). The table 1 shows the results of the macronutrients present in the samples of *A. amazonicus*. A minimum variation was observed when comparing all analyses of the vegetable organs to Na. On the other hand, a major concentration of K was observed on the leaves. Calcium and phosphorus concentrations were lower in the root, when compared with the other samples values. Magnesium presented a discrete difference of concentration among all the analyses. According to Ražić et al. (2003) (27), elemental profiles in herbal drugs are attributed to the differences in their botanical structures, their element mobility within the plant and absorption from soil.

The abundance of K, Mg and Ca in the results of these analyses was in agreement with previous findings, showing that these three metals are the most abundant in many plants (28, 29). The macronutrients and micronutrients concentrations found in the root barks of *A. amazonicus* compared with the daily acceptable values for consumption are not harmful (6).Table 2 presents the results of the micronutrients of the samples.

The iron concentrations varied from 1.80 to 12.57 mg Kg^{-1} to the samples of the aqueous preparations, and from 60.8 to 2540 mg Kg^{-1} to the raw materials of the different parts of the plant. The variation of this metal in the aqueous extracts was proportional to the amount of vegetal material used in the preparation of the sample. The levels of iron in the vegetable organs of *A. amazonicus* were higher than in other botanical species evaluated up to now (27, 30, 31). As to the root barks sample, the result (2540 mg Kg^{-1}) was even more interesting, since the iron makes part of some processes related to malaria and the root barks are the part of the plant used by the population. Nussenblat and Semba (2002) (5) observed convergence about malarial anemia and nutrients deficiency, especially to iron in morbidity and mortality caused by malaria. An interesting fact related with animals infected by malaria shows that the ones treated with *A. amazonicus* developed an even higher parasitaemia, a more severe anemia and died earlier than the ones not controlled against malaria. These results were attributed to the amount of saponins present in this species, which lysed the red blood cells, more fragile in the case of malaria infections (3), but they can also be associated to the amount of iron found in the plant, which contributed to the parasites multiplication. However, in spite of the importance of iron for the metabolism and growth of the parasite, there is no conclusive answer on how it is acquired by the host (32 - 34).

As to zinc, its importance was demonstrated in the regular immune function, being that its deficiency provokes adverse reactions in the development of malarial anemia, mainly due to the impaired immune function, increased parasitemia and oxidant damage (5). Aside from iron and zinc, little is known about the role of other trace elements, such as chromium and copper, during malaria in humans (5). The Cr verified in the root was 24 mg Kg^{-1}, which is a value allowed for daily consumption. Leaves present a higher amount of this element. The same conformity can be observed for manganese and nickel (6).

Table 1. Results of the macronutrients present in the samples of A. amazonicus.

Samples	Concentration (mg Kg^{-1})				
	Na	K	Ca	Mg	P
1% aqueous extract of root barks	1.64 ± 0.21	1.73 ± 0.11	6.47 ± 0.50	0.49 ± 0.08	0.37 ± 0.02
10% aqueous extract of root barks	2.62 ± 0.22	2.25 ± 0.15	8.97 ± 0.71	0.53 ± 0.06	0.48 ± 0.05
20% aqueous extract of root barks	3.18 ± 0.19	3.15 ± 0.17	12.20 ± 0.63	0.76 ± 0.01	0.60 ± 0.03
Stem Barks	613 ± 11.5	660 ± 25.3	2766 ± 31.4	904 ± 12.1	2026 ± 32.6
Leaves	651 ± 13.7	1083 ± 21.7	3335 ± 41.8	921 ± 25.3	2265 ± 12.8
Twigs	686 ± 10.5	820 ± 12.4	5183 ± 27.3	911 ± 18.3	2628 ± 23.6
Root Barks	623 ± 15.9	623 ± 13.7	1366 ± 22.2	922 ± 16.4	580 ± 9.8

Table 2: Results of the micronutrients present in the samples of A. amazonicus.

Sample	Concentration mg Kg^{-1}						
	Fe	Cu	Zn	Cr	Co	Mn	Ni
1% aqueous extract of root barks	1.88 ± 2.5	0.04 ± 1.8	0.26 ± 3.1	ND	ND	1.10 ± 7.2	0.33 ± 0.7
10% aqueous extract of root barks	7.74 ± 5.8	0.21 ± 0.7	0.50 ± 2.9	ND	ND	1.66 ± 0.71	0.36 ± 1.5
20% aqueous extract of root barks	12.57 ± 9.2	0.25 ± 1.1	0.64 ± 3.2	ND	ND	2.50 ± 10.5	0.43 ± 2.5
Stem Barks	100.2 ± 1.0	1.5 ± 0.6	2.6 ± 2.4	ND	5.3 ± 0.2	10.6 ± 1.5	5.3 ± 0.5
Leaves	150.5 ± 1.2	1 ± 0.1	6.66 ± 1.5	57.6 ± 4.4	ND	56.6 ± 4.0	23.0 ± 1.7
Twigs	60.8 ± 2.5	ND	8.00 ± 2.52	11.0 ± 4.6	5.6 ± 0.2	23.0 ± 1.0	2.3 ± 0.5
Root Barks	2540 ±15.6	ND	ND	24.0 ± 2.0	ND	12.3 ± 2.0	5.0 ± 0.1

CONCLUSIONS

The chromatographic profile of the 1% root barks preparation of the *A. amazonicus* showed 48.4% of saponins. Despite the withdrawal of foam, they remain as major constituents in the preparation. The analyses of the elements indicated that the levels of iron in the botanic material of *A. amazonicus* were high and that the root barks showed a major amount of this metal when compared to others. This last result was even more interesting, since iron makes parts of some processes related to malaria and the root barks are the part of the plant used by the population. Yet, the traditional use of 1g of the root barks of *A. amazonicus* to prepare the antimalarial formulation does not exceed the established daily dose, even when taken on alternate days, according to popular use. The 1% root barks preparation showed no relevant ability as free radical scavengers or hydrogen donors, but this result may be associated to the action described for *A. amazonicus*. Additionally, the test with *Artemia salina* demonstrated that this preparation did not present toxicity.

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