Preclinical Toxicity Evaluation of *Quassia amara* Extract with Potential Antimycobacterial Activity

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Abstract

This study aimed to evaluate the oral acute and sub chronic toxicity of *Quassia amara* (*Q. amara*) extract. For the acute toxicity study, six male and six female rats received 2000 and 5000 mg/kg *Q. amara* extract. Rats were observed for 14 days; after sacrifice, liver, kidneys, lung, spleen and heart were submitted to macroscopic analysis. No death occurred and just the liver presented significant difference compared to the control group. For subchronic toxicity, eight groups of 6 male and 6 female rats received 200, 400 and 800 μg/mL of extract of *Q. amara*. Prothrombin time, bilirubin, urea, creatinine and gamma glutamyl transpeptidase *(γ*-GT*), aspartate transaminase *(AST)*), alanine transaminase *(ALT)* and alkaline phosphatase *(ALP)* were used as markers of renal and hepatobiliary function, respectively. The male showed a slight rise in serum bilirubin and creatinine and the female slight rise of AST, ALT and ALP activities. The urinalyses *(pH, density, glucose and leukocytes)* showed no change relative to the control. The NOAEL of 35 mg/kg and RID 0.35 mg/kg/day encourage us to isolate the active principles in order to discover which molecules of this extract showed better antimycobacterial activity.

Keywords: Extract of *Quassia amara*; Assessment of toxicity; *Mycobacterium tuberculosis*; *Mycobacterium avium*; NOAEL; Reference dose

Abbreviations: LOAEL: Lowest Observed Adverse Effect Level; NOAEL: No Observed Adverse Effect Level; RID: Reference Dose; MABA: Microplate Alamar Blue Assay; OECD: Organization for Economic Cooperation and Development; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: gamma Glutamyl Transpeptidase; ALP: Alkaline Phosphatase; MIC: Minimal Inhibitory Concentration; PT: Prothrombin Time; BRB: Bilirubin; BUN: Blood Urea Nitrogen; RBC: Red Blood Cell; Plt: Platelet; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; Hb: Hemoglobin; Ht: Hematocrit; *Q. amara*: *Quassia amara*

Introduction

*Mycobacterium tuberculosis* (MTB), the pathogenic agent of tuberculosis (TB), is responsible for the death of 2–3 million people annually and for a global economic toll of US$12 billion each year [1]. Additionally, *Mycobacterium avium* is the most common cause of human infection due to non *Tuberculosis mycobacteria*. Initially *M. avium* was regarded as only an opportunistic pathogen, primarily in immunocompetent humans [2].

For the treatment of tuberculosis, the United States Food and Drug Administration (FDA), currently cites 10 medicines, divided into two lines of treatment. The treatment of first line consists of: isoniazid, rifampicin and pyrazinamide. The treatment of second line consists of: ethambutol and rifabutin. According to Zhang et al. [3] isoniazid and all other drugs in the first line show significant toxicity with adverse effects such as psychosis, convulsion and increase in hepatic enzymatic activity [Aspartate transaminase *(AST)*], Alanine transaminase *(ALT)* and alkaline phosphatase *(ALP)*]. Rifampicin is also reported to cause hepatitis, leucopenia and induces the activity of hepatic microsomic enzymes. The frequent use of this drug can cause acute interstitial nephritis, glomerulonephritis and proteinuria [4,5]. The fact that the main drugs listed for the treatment of tuberculosis can have strong adverse effects, justifies the search for new medicines that offer at least the same effectiveness, but with less adverse effect.

An approach to the search for new drugs is to look in nature, mainly for the extremely rich and varied flora of the tropical areas. In this search, information obtained from folk knowledge and traditional medicine of different cultures can be valuable. This trend in associating popular or alternative medicine with pharmaceutical research has been growing for some years [6]. However, even though the activity of a popular plant medicine may be known, as well as the form in which it should be used, it is still necessary to prove its harmlessness, in order to diminish the risks to health. Moreover, it must be considered that one medicinal plant may not only show an immediate effect, easily correlated with its ingestion, but also sub chronic and chronic effects [7,8].

The Simaroubaceae family has approximately 30 genus and 200 species. These plants are distributed across all the American continents, with greater abundance in tropical regions. The best known representative of this family is the *Q. amara* a large shrub between 3 and 8 meters in height, with palmate leaves of five leaflets, abrupt acuminate at apex and attenuated at base. *Q. amara* is popularly known as Murubá, Marupa, Quina da caína in Portuguese [9,10] and stave wood in English. Amongst the active principles of *Q. amara*, quassinoids

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constitute a class of chemicals found almost exclusively in plants of the Simaroubaceae family. These chemicals are oxygenated biodegradable triterpenes and they have a wide range of biological activity. Among the biological activities cited for quassinoids are anticancer, antimalarial, herbicidal, insecticidal, nematocidal, antituberular and others. Thus, in popular medicine, the best known uses for Q. amara bark and wood are against flatulence, diarrhea, anemia, dyspepsia, malaria, and stomach [11]. In a previous work was presented an in vitro screening against Mycobacterium genera of a series of plants, demonstrated that Q. amara inhibited the growth of bacilli with a minimal inhibitory concentration (MIC) of 62.5 μg/mL [12].

As the standard drugs for the treatment of tuberculosis have strong adverse effects on the liver and kidney, the objectives of this study was to determine the toxicity (in rats) of a daily preclinical dose of aqueous extract of Q. amara from Brazilian shrubby savannah giving special attention to the hepatobiliary and renal systems.

Material and Methods

Plant material

The Bark of Q. amara was collected in the savannah region of the State of South Mato Grosso, at Bataguassu 21° 42’ 51” S by 52° 25’ 20’’ W, and the State of Goiás, at Rio Verde 17° 47’ 53” S by 51° 55’ 53” W from March to June of 2006. The material was placed in plastic bags, conditioned in Styrofoam boxes with ice and the bags stored in a freezer at -20°C until the moment of use. The coordinates of this plant is also recorded in Toma et al. [13].

Extraction and isolation

The bark was washed in a current of water and then transferred to a drying greenhouse at a temperature of 40°C, with constant movement. The bark was then triturated and transferred to an Erlenmeyer flask containing dichloromethane at final concentration of 500 g/L. Flasks were covered and wrapped in aluminum paper to protect them from light and were rocked for 72 hours. After this period, liquid fraction was filtered in a Buchner funnel and transferred to a rotating evaporator under reduced pressure and protected from light. The solid part was returned to the Erlenmeyer where dichloromethane was added again to carry out a new extraction (this operation was repeated four times). The concentrated extract was transferred to a heating plate at 40°C ± 1 for four hours to evaporate the remaining solvent. The dried extract was mixed with 5% Tween-80 (v/v), enabling the dissolution of 250 mg of extract per ml in distilled water. This stock solution was stored in a freezer (-20°C) until used. From the stock solution, dilutions of 1200, 800, 400 and 200 μg/mL were prepared for oral administration to the test animals.

Toxicity

The experiments were carried out according to the protocols of the Organization for Economic Cooperation and Development (OECD 423 and 408) [14,15]. Wistar rats (60-day-old) were housed in plastic cages 30 (width)×40 (length)×20 (height) cm with shaving for acute toxicity (six per cage) and in metabolic cages for sub chronic toxicity (one per cage) in a climate controlled room at a temperature of 24°C ± 2, and relative humidity 55% ± 10, with light-dark cycle of 12 hours (light on at seven a.m.), with free access to tap water (for acute toxicity tests) and commercial food. All experimental procedures were conducted with the approval of the Research Ethics Committee of the School of Pharmaceutical Sciences of Araraquara, SP, Brazil in accordance with their guidelines for the care and use of laboratory animals (Resolution 44/2006).

Acute toxicity: Applying the oral limit test (as described by Protocol OECD 423) [14], all the doses of Q. amara were administered by gavage after overnight fasting in the morning, between 7 and 8 a.m. Two groups of six male rats and two groups of six female received a dose of 2000 and a dose of 5000 mg/kg/body weight of the Q. amara extract and two control groups of six male and six female rats received a solution of 5% Tween-80 (v/v) in water. The animals were observed after the gavage at half an hour, 1 h, 2 h, 4 h, 8 h, 24 h and then daily for 14 days, when they were sacrificed. Liver, kidneys, lungs, spleen and heart were weighed, dissected and examined macroscopically.

To calculate the relative weight we used the follow equation: [(organ weight×100)/(body weight)].

Sub chronic toxicity: After one week of acclimatization in individually metabolic cages (for individually assessment of urine), 20 male and 20 female rats, weighing on average 200 grams (initially) were divided into eight equal groups, six experimental and two controls. The animals received concentrations of 200, 400 and 800 μg/mL (highest concentration of Q. amara in the palatability test at which rats did not diminish the ingestion in relation to the control) of Q. amara extract in their drinking water. To assess the volume of intake, the extracts of the plant was maintained for 7 days in place of drinking water, in increasing concentrations. No differences were observed between the intake of control (water) and ingestion of the extract analyzed, until the concentration of 800 μg/mL. At higher concentration (1200 μg/mL), intake of the extract was reduced when compared with drinking water, characterizing the unacceptability of palatability. The control groups received only drinking water and the extracts were changed every three days (because the stability of the extract). The animals were observed during 90 days. After this period, the animals were sacrificed and blood was collected (in tubes with EDTA) by cardiac puncture. The following parameters were evaluated: full blood count; assay of the enzymes ALP, AST, ALT, gamma glutamyl transpeptidase (γ-GT); analysis of the plasma levels of bilirubin, urea, creatinine, estimation of prothrombin time (using a clinical chemistry kit Liquiform Labtest®); and assessment of the hepatic and renal profiles. Spleen, heart and lung were removed for microscopic inspection while liver and kidneys were analyzed microscopically. For histopathological assessment, the liver and kidneys portions was gently dissected and immersion-fixed in 10% neutral buffered formalin for 48 hours. The tissues were then processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Urinalyses of the animals were carried out for pH, density, glucose and leukocytes in the first and last weeks of the experiment.

Statistical analysis: Differences in biochemical parameters between groups were examined for statistical significance using ANOVA (Analysis of Variance), followed by Tukey’s test for multiple comparisons. These tests were run in Microsoft Office Excel 2007 for Windows. The criterion of significance was p<0.05 for all statistical analyses. All biochemical data are expressed as mean ± standard error mean (SEM).

Results

Acute toxicity

The animals that received 2000 mg/kg/body weigh of Q. amara extract showed irritability in two male and three female rats; for the
5000 mg/kg/body weight dose, irritability was also observed in three male and two female until 2 days after ingestion of the extract. No deaths occurred during the 14 days of the trial. The dissected organs (heart, lung, spleen and kidney) exhibited relative weights and macroscopic appearance perfectly within the standards of normality. Only the liver decreased statistically different values of relative weight (dose of 5000 mg/kg/body weight) from control animals (Figures 1a and 1b).

**Subchronic toxicity**

For animals that received different concentrations of the *Q. amara* extract no significant changes were found in hematological profiles compared with control group, as demonstrated by tables 2 and 4. In relation to the biochemical results it was verified an increase in serum bilirubin levels in male that received the dose of 800 µg/ml as shown in tables 1 and 3. Histopathological findings showed no significant changes compared with the control group (Figures 2a and 2b). No significant changes were found in the urinalyses profiles of any animals that received different concentration of *Q. amara* extract, comparing the first week with the last week of experiment, not even with the 800 µg/ml which was the highest concentration given to animals (Table 5).

**Discussion**

The dichloromethane *Q. amara* extract tested before against *M. avium* and *M. tuberculosis* showed a MIC value of 62.5 µg/ml. Tosun et al. [16] considered inactive the plant extracts that could not prevent growth of *M. tuberculosis* up to a concentration of 200 µg/ml and
Concentrations of crude extract of *Quassia amara* through drinking water after 90 days of exposure. Table 3: Serum biochemical profile of Wistar female rats treated with different concentrations of crude extract of *Quassia amara* through drinking water after 90 days of exposure.

Table 4: Serum hematological profile of Wistar female rats treated with different concentrations of crude extract of *Quassia amara* through drinking water after 90 days of exposure.
rats, neither cells under microscopy was observed nor alterations in physico-chemical parameters as pH, density and glucose were detected. These findings suggest that this extract of Q. amara did not interfere with the normal functioning of the kidneys.

Many authors use the serum activity of enzymes such as γ-GT, AST, and ALT to assess hepatocellular injury [23,24]. More specifically, Vetelainen et al. [25] used the plasma bilirubin level to assess indirectly the hepatic metabolic and excretory function, and the prothrombin time (PT) to evaluate the capacity for hepatoocyte protein synthesis. In the present study, levels of activity of ALT, AST, γ-GT and PT were determined to assess the hepatobiliary toxicity. In male rats, only bilirubin showed a significant increase in relation to the control at the concentration of 800 μg/ml. As no other parameter to use to test the tissue or hepatic function was modified, this suggests that the Q. amara extract did not cause hepatotoxicity in male rats. With regard to the female rats, a statistically significant rise of AST and ALT activity was observed only at the 800 μg/ml concentration. As no other parameter to use to test the liver function was modified, this suggests that the Q. amara extract did not cause hepatotoxicity in female rats.

Table 5: Urinalysis profile of Wistar male and female rats treated with 800 μg/ml of crude Quassia amara extract through their drinking water after 90 days of exposure.

### References


