

Acute toxicity of *Brosimum gaudichaudii* Trécul. root extract in mice: determination of both approximate and median lethal doses

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RESUMO: “Toxicidade aguda de extrato de raízes de *Brosimum gaudichaudii* Trécul. em camundongos: determinação da dose letal aproximada e da dose letal mediana”. O *Brosimum gaudichaudii* Trécul. (BG) família Moraceae, é largamente distribuído nas regiões de cerrado do Brasil e é popularmente conhecido como “mamacadela” e possui, na sua composição química, furocumarinas fotossensibilizantes. O principal uso terapêutico do BG é no tratamento do vitiligo, uma doença despigmentante da pele com prevalência de aproximadamente 1-2% da população mundial. Há um produto no mercado (Viticromin®), que é comercializado com tal finalidade, havendo poucos dados disponíveis acerca da toxicidade experimental, ao lado de eficácia clínica comprovada. Portanto, o desenvolvimento de estudos de toxicidade pré-clínica foi proposto (dose letal aproximada, DLA e dose letal mediana, DL50) para o exsudato da raiz de BG em animais de laboratório. Foram utilizados camundongos machos, albinos, pesando entre 25 e 30 g, em boas condições sanitárias, recebendo água e ração *ad libitum*. O pó de BG foi suspenso em solução salina 0,9% com 0,5% de cremofor e administrado p.o. (gavage) e intraperitonealmente. A DL50 foi obtida através do método de regressão linear de Lichtfield & Wilcoxon (1949), utilizando um *software* de domínio público. Foram formados 6 grupos de 10 animais para cada via de administração, incluindo grupo controle, e o número de mortes foi observado durante 14 dias; o grupo controle recebeu uma emulsão de solução salina 0,9% com 0,5% de cremofor. Antes de estabelecer a DL50 e visando reduzir o número de animais utilizados, determinou-se a DLA, utilizando 1 animal por dose (a próxima dose 50% maior do que a dose anterior), até identificar a dose letal para 1 animal. Assim, a DLA p.o. foi de 3750 mg/kg e a DLA i.p., 2920 mg/kg. A DL50 p.o. foi de 3517,54 mg/kg e a DL50 i.p. foi 2871,76 mg/kg. O produto foi considerado possuir baixa toxicidade, considerando apenas uma dose neste ensaio de toxicidade aguda.

Unitermos: *Brosimum gaudichaudii*, Moraceae, toxicidade aguda.

ABSTRACT: The *Brosimum gaudichaudii* Trécul. (BG) Moraceae, with a high frequency in regions of the Brazilian *cerrado* (shrubland) and popularly known as “mamacadela”, holds photosensitizing furocoumarins. The BG’s main therapeutic use is the treatment of vitiligo, a skin depigmentation disease that takes hold of approximately 1-2 % of the world population. There is one product on the marketplace (Viticromin®), for which there is no scientific research available about its experimental toxicity, in spite of its proven clinical efficacy. Therefore, the development of acute pre-clinical toxicity trials has been proposed (approximate lethal dose, ALD, and median lethal dose, LD50) of the BG root exsudate in laboratory animals. Male, albino mice weighing between 25 and 30 g, in good sanitation conditions have been used. The mice received water and food *ad libitum*. The BG powder was mixed in 0.9% saline solution and 0.5% cremophor and was administered *po* and *ip*. The LD50 was obtained through the Lichtfield and Wilcoxon (1949) method. A public domain software was used for the calculations. Ten mice/dose were used - 6 groups for each administration via, including the control group - and the number of deaths within up to 14 days was considered; the control group received an emulsion made up by 0.9 saline solution and 0.5% cremophor. Before establishing the LD50 and aiming at reducing the number of animals used, the DLA was determined when 1 animal per dose was used (the next dose always 50% higher than the previous dose), until identifying the dose that led to the death of 1 animal. Thus, the DLA *po* was 3750 mg/kg and the DLA *ip*, 2920 mg/kg. The LD50 *po* was 3517.54 mg/kg and the LD50 *ip* was 2871.76 mg/kg. The product was considered as of being of low toxicity, taking into account only the single dose acute toxicity.

Keywords: *Brosimum gaudichaudii*, Moraceae, acute toxicity.

INTRODUCTION

Since ancestral times, medicinal plants have been largely used by mankind for preventing and curing illnesses. Along thousands of years of successful and unsuccessful experiences, knowledge has been accumulated about both their benign and malign effects. Today, many illnesses can be treated by means of this natural resource. Beginning with the true laboratory provided by nature, it has been possible to obtain a good degree of pharmaceuticals that are presently used, such as: morphine (poppies), salicylic acid (willow), cocaine (coca leaf), atropine (belladonna), digoxine (foxglove), quinine (cinchona or quinine-tree), caffeine (coffee), neuromuscular blocking agents (curare), among others.

The employment of pharmaceutical technology has provided for the diffusion of industrialized drugs as a predominant therapeutic resource, but very costly for patients. Therefore, the social and economic conditions in force, especially in third world countries, have led the lower classes to search for much cheaper and relatively safe therapeutic alternatives such as, for example, medicinal herbs.

At the same time, in recent years a "return to the origins" has been observed, provided by the need for new drugs, with lower toxicity levels and lower costs. The pharmaceutical industry has encouraged this trend, as it recognizes an opportunity to provide the marketplace with a pharmacotherapeutic arsenal with scientifically proven alternatives.

A detailed study of natural products becomes necessary in order to discover its potential collateral effects and main recommendations. Thus, the population will be offered additional and accessible options in its fight against the so many illnesses. Additionally, medicinal herbs constitute the main hope in the treatment of illnesses whose causes are unknown and that very often remain without treatment. Estimates are that our planet contains about 250,000 botanic species. Of these, only an expressionless percentage has been investigated as to its pharmacology and toxicology.

Encouraging research about natural products would not only benefit the poor population, but also the Brazilian health system as a whole, as it would reduce the demand for public services, since it encourages oriented self-medication, of a much lower cost and usually quite low risk (Ribeiro et al., 2005; Silva et al., 2006; Veiga-Junior, 2008; Carvalho et al., 2008)

The roots and the lower part of the stem of the *Brosimum gaudichaudii* Trécul. (BG), a plant of the Moreaceae family, frequent in the *cerrado* region and Northeast of Brazil, which is popularly known as "mamica de cadela", "mamacadela" and "algodão" (cotton), are employed by popular medicine in the fight against vitiligo and skin diseases (Macedo & Ferreira, 2004; Agra et al., 2007 and 2008). Its fruit, of a yellow-orange color, is chewed as chewing gum, due to the

latex it contains and due to its flavor, while the powder obtained by scrapping the roots is used as a fragrance (Hoehne, 1946).

Usually plants used for fighting vitiligo (leucomelanoderma), contain furocoumarins. Furocoumarins are of a broad spectrum of application in the treatment of leucomelanodermias, with photosensitizing activity upon the skin (Dean, 1963; Alchorne, 2001). Many studies have been carried out about this type of activity of the furocoumarins (Pozetti, 1966; Azambuja, 1981; Parsad et al 1998; Jimbow, 1998). It is known that "all fluorescent substances found in nature, whatever the chemical function they belong to, are active therapeutically and pharmacologically without exception" (Pozetti, 1969).

We wish to highlight that vitiligo is made up by achromic spots, of irregular shape, clear contours, sometimes of symmetric disposition, other times distributed by chance, that occur in any part of the tegmen, followed or not by depigmentation of hair, with a 2 to 3 times larger incidence on the female gender than on the male gender; it affects 1 to 2% of the population in general, 30% of all cases being of hereditary origin (Auad, 1973; Lerner & Nordlund, 1977; Azambuja, 1981; Alchorne, 2001). The treatment of vitiligo has been made and/or attempted through the use of several substances and/or mixtures of substances, such as bergamot essence at 20-25%, oral and local corticosteroids, adrenocorticotrophic hormone (ACTH) and the placenta extract (Alchorne, 2001). In 1947, El-Mofty launched a treatment based on the use of photosensitizing substances obtained from *Ammi majus* Linn, which contains amoidine-8-methoxypsoralen, amoidine-8-isoamylinsoralen and bergapten as active principles, that depended on exposure to sun rays in order to promote re-pigmentation (Azambuja, 1981; Sampaio et al. 1983).

The employment of 8-methoxypsoralen and trimethoxypsoralen, followed by exposition to ultraviolet light (photochemotherapy with UV-A, PUVA) of benzoquinone monobenzylic ether are being studied nowadays and since the 1980's for repigmentation (Martins et al., 1974; Azambuja, 1981; Parsad et al. 1998; Jimbow, 1998).

As has already been mentioned, in our region the roots and the lower part of the stem of *Brosimum gaudichaudii* Trécul. have been used as a source of photosensitizing furocoumarins. In this plant, a few substances - furocoumarinic derivates, such as: xanthyletine, bergapten, psoralen, luvangentine and the gaudichaudine (Figure 1) - have been isolated and identified, all of which have photosensitizing capability (Pozzetti, 1969; Anderson & Voorhees, 1980; Vieira et al., 1997). Synthetic similar products have already been found in the pharmaceutical market for use in the formulation of world-class drugs for the treatment of vitiligo and other depigmentating skin diseases.

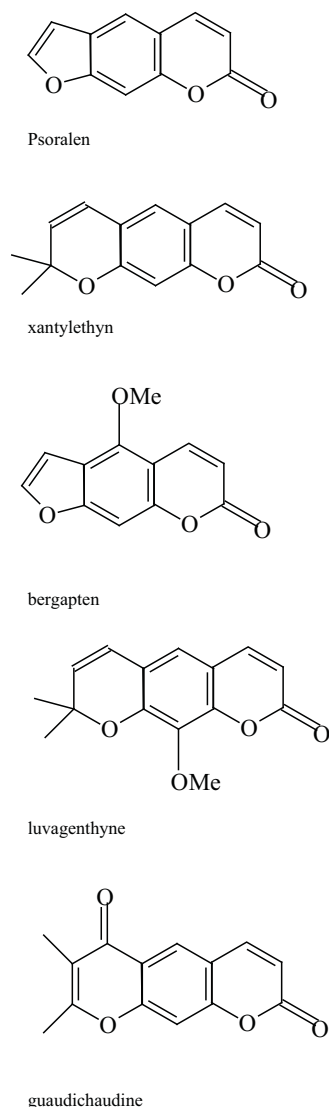


Figure 1. Phytochemical profile of the *Brosimum gaudichaudii* Trécul. roots, highlighting a few furocoumarinic active principles.

There is a commercial product on the marketplace for the treatment of vitiligo (Viticromin[®], AUAD Química), the main active component of which is made up by the lower part of the stem and of the roots of *mamacadela* (*Brosimum gaudichaudii*), that are harvested, dried, peeled, ground and prepared in pharmaceutical forms for internal (as tablets) and external (as creams and solutions) use (DEF, 2000).

The recommended dose of Viticromin[®] to be employed is 2 to 4 tablets for at least 6 months to 1 year and gradual exposure to sunlight (5 - 60 minutes). After a couple of weeks of oral ministration and exposure to the morning sun, the topical treatment with hydro-alcoholic solution or cream and exposure to the afternoon sun is started. Inadvertent exposure to the sun

should be avoided, due to the risk of burning (Sampaio et al., 1983; Azambuja, 1981).

The tablets, prepared with the vegetal raw material (root bark), hold 80% (p/p) of root powder of *Brosimum gaudichaudii* (400 mg in 500 mg *qsp.* excipient), while the cream contains 20% (p/p) of powder (6 g in 30 g *qsp.* excipient) and the solution contains a 20% concentration (p/v) (12 g powder in 60 ml *qsp.* excipient). The differences in concentration between the product for internal use and the products for external use must reflect eventual losses during the pharmacokinetic route of the active principles, in the stages of absorption, distribution, biotransformation and excretion, until they reach the action site, which corresponds to the area of epidermis suffering the depigmentation characteristic of the several forms of manifestation of vitiligo.

Although Viticromin[®] is registered since 1968 at the Brazilian Ministry of Health and in spite of the existence of some clinical studies that demonstrate an up to 100% efficacy in terms of repigmentation in some cases (Azambuja, 1981; Anderson et al. 1980; Auad, 1973), for the long run treatment of vitiligo (5 to 18 months) there is no study about the pre-clinical and clinical toxicity (acute, sub-acute and chronic) and pharmacology. First of all, it is necessary to assess the acute toxicity of *Brosimum gaudichaudii*. in mice, by determining the approximate lethal dose (ALD) and the median lethal dose (LD50), as a part of regulatory requirements (Brasil, 1994; Brasil, 2000; Brasil, 2004).

MATERIAL AND METHODS

All botanical material for the toxicity study was provided by AUAD Química Ltda., with a certificate of origin and quality (Batch n^o. 515/00), part of this material being deposited at our laboratory.

Of the *Brosimum gaudichaudii* - BG (Figure 2.1), we have used the root bark (Figure 2.2) still fresh and duly clean, which was crushed and pressed in order to obtain the exsudate that, later on, was dried in a stove with hot air ventilation.

In macroscopic terms, the bark presents itself in either curved or plane fragments, not rolled in, of varied length and width, 5 to 10 mm in thickness. The external surface is made up by a brown rugose suber. The stem bark surface presents itself even more rugose and dark. Once the suber is removed, the light brown cortical parenchyma, somewhat striped in the longitudinal direction, appears. The internal surface is light brown and fibrose. It has characteristic odor and flavor. When the root bark is ground, sifted and taken into the oven for drying, initially at temperatures between 60 °C to 80 °C, and later at temperatures between 40 °C and 60 °C until reaching constant weight, a powder is obtained. This powder, in microscopic terms, presents itself as pieces in varied shapes and sizes, in the amber color, semi-transparent when observed under the microscope

under direct light. When observed under a diffuse light or with the naked eye, it presents itself as a very fine powder, of characteristic odor and acrid flavor. This raw material, rich in bergapten and psoralen, is used in the manufacturing of the Viticromin® drug. The dosage methodology of the bergapten and psoralen biomarkers in the used exsudate is in a development stage.

For determining the acute toxicity of BG (approximate lethal dose, ALD, and average lethal dose, LD50), 130 albino male mice were used, weighing between 35 and 45 g (39.9 ± 4.8 ; Median: 40.6), and in good sanitation conditions, receiving autoclaved water and food *ad libitum*, confined in polypropylene cages in an environment with a 12-hour light-dark cycle and temperatures oscillating between 22-26 °C. Twelve hours before the experiment started, animals were submitted to food fasting, and the food returned two hours after the drug was ministered to each animal.

Emulsions in ultrasound bath (saline + cremophor at 0.5% v/v) were prepared with the BG powder, in concentrations sufficient for ministering the *po* dose (gavage feeding) and *ip* dose in constant volume of 0.1 ml/10 g of the animals living weight; initially, doses of 500 mg/kg were used for the ALD study, with 50% increases, up to the maximum value of the lethal dose for 1 animal (ALD) through the per oral (*po*) and intraperitoneal (*ip*) vias - in spite of the fact that this last via is not used for therapeutic purposes and is not the most appropriate for the best dissolution of the BG extract. 1 ml polypropylene syringes subdivided at every 0.1 ml and with a needle adapted for gavage feeding were used.

For determining LD50, the mice were divided into 2 large groups, according to the administration via: per oral and intraperitoneal. Each one of the groups was subdivided into 6 sub-groups of 10 animals each:

1 control sub-group (salina + cremophor at 0.5%, *po*) and 5 test sub-groups, which have received estimated increasing doses, starting from the *po* (1400, 2000, 3000, 4000 and 5000 mg/Kg) and *ip* (1000, 1500, 2250, 3500 and 5000 mg/Kg) ALD of BG.

Signs of general toxic effects, such as effects upon locomotion, behavior (agitation, drowsiness, reduced activity), respiration, salivation, watering of eyes, hemorrhage, consistency of feces, hair standing on end, cyanosis of the tail and the mortality of the animals were monitored at regular intervals during the first 24 hours and after that, on a daily basis, for a 14-day period (Malone & Robichaud, 1962).

The animals that died during the experiment were examined macroscopically, aiming at identifying eventual morphological alterations. Some organs (liver, heart, small intestine, stomach, lung, kidney, spleen, brain) were forwarded for histopathological study. At the end of the experiment, all surviving animals were sacrificed by means of inhalation of chloroform and were macroscopically examined.

The LD50 and its confidence interval were calculated according to probit methods, with the support of a public domain statistical program based on the traditional Lichtfield & Wilcoxon (1949) method by means of linear regression techniques.

RESULTS

Oral administration of BG root exsudate induced animals to death, proportionally to the administered doses (Table 1). The approximate lethal *po* dose was 3750 mg/kg, and through the *ip* via, 2900 mg/kg. Logarithms and probits were determined for each dose, aiming at calculating the median lethal dose (DL50), which has resulted in 3517.54 mg/kg (*po*;



Figure 2.1. Macroscopic aspect of the fruit and leaves of *Brosimum gaudichaudii* Trécul. (mamacadela).



Figure 2.2. Macroscopic aspect of the roots of *Brosimum gaudichaudii* Trécul. (mamacadela).

IC95% 3094.25 - 3998.74 mg/kg). The *ip* DL50 was calculated by the same procedure, producing a value of 2871.76 mg/kg (IC95% 2389.69 - 3451.07 mg/kg) (Table 2).

The 2000 mg/kg dose is established as a dose ceiling of the plant extract for the determination of DL50 because, above this value, the result obtained may be considered out of the product's standards for therapeutic use and, therefore, unrealistic, without significance under the conditions foreseen for the its use (Brasil, 1996; WHO, 1993; Lapa et al., 1983).

Some signs related to adverse and/or dose-dependant toxic effects of the BG exsudate have been noticed, that is: in doses up to 2000 mg/kg, about 40% of all animals presented diarrhea and abundant defecation. In higher doses (up to 5000 mg/kg), in addition to

diarrhea in all animals, some of them have shown signs of drying of the ocular mucosa (dry eye) and eyelid occlusion, ocular hemorrhage, epistaxis, weight loss and tachypnea. The seriousness of the intoxication signs was dose-dependant (Table 3). Deaths occurred from the 24 hour period until 5 days after the administration. The higher doses have led to lethality in a much shorter period of time.

A deviation of the hepatic sinusoids in the 3, 4 and 5 test groups were noticed in 75% of all animals and in 60% of the animals of the control group (*po* group), what could also be explained as a consequence of the acute effects of the inhalation of chloroform, the same fact having occurred, in similar proportions, to the *ip* group.

A difference in the amount of hemosiderin

Table 1. Mortality of male mice after intra-gastric administration of *Brosimum gaudichaudii* root exsudate (Control group: 10 male animals; no death).

Group	Dose (mg/kg)	Log dose	Number of animals	Response	Expected Frequency	Probit
1	1400	3.146128	10	0	0.001435	1.373116
2	2000	3.301030	10	0	0.131166	2.777255
3	3000	3.477121	10	3	2.654829	4.373467
4	4000	3.602060	10	7	6.935715	5.505999
5	5000	3.698970	10	9	9.168910	6.384459

Results: DL50 = 3517.54 mg/Kg; Log (DL50) = 3.546239; Weighed linear regression: probit = 9.065 x Log (Dose) -27.146; Standard error of Log (DL50) = 2.841083*10⁻²; Inclination = 2.424905; IC95% of DL50 = 3094.25 – 3998.74 mg/Kg; Degree of Freedom = 0; QUI-SQUARE test not performed.

Table 2. Mortality of male mice after *ip* administration of the *Brosimum gaudichaudii* root exsudate (Control group: 10 male animals; no death).

Group	Dose (mg/kg)	Log dose	Number of animals	Response	Expected Frequency	Probit
1	1000	3.000000	10	0	0.026565	2.212597
2	1500	3.176091	10	1	0.430763	3.283948
3	2250	3.352182	10	2	2.595607	4.355299
4	3500	3.544068	10	6	6.994242	5.522745
5	5000	3.698970	10	10	9.285638	6.465177

Results: DL50 = 2871.76 mg/Kg; Log (DL50) = 3.458148; Weighed linear regression: probit = 6.084062 x Log (Dosis) -16.039583; Standard error of Log (DL50) = 4.071824*10⁻²; Inclination = 1.442219; IC95% de DL50 = 2389.69 - 3451,07 mg/Kg; Degree of Freedom = 0; QUI-SQUARE test not performed.

Table 3. Clinical signs observed in mice after the *po* administration of *Brosimum gaudichaudii* extract.

Group	Dose (mg/kg)	Number of animals	Clinical signs observed (no. of cases)
1	1400	10	Diarrhea (4)
2	2000	10	Diarrhea (4)
3	3000	10	Diarrhea (10), dilated pupil, ocular lesion with hemorrhage, half shut and dry eyes (4)
4	4000	10	Diarrhea (10), semi-shut and dry eyes (4)
5	5000	10	Diarrhea (10), dyspnea (2) and weight loss (2).

was noticed in the spleen of the test group animals, in comparison to the control group. In test groups 3, 4 and 5, 70% of all animals demonstrated an increase in hemosiderin in the spleen, while only 10% of the control group showed the same effect, which also happened in the *ip* group. Some kidney tissue cuts demonstrated the occurrence of vessel dilation and hemorrhage. The influence of such alterations upon the death cause must be taken into account in sub-acute and chronic toxicity tests.

The increase of hemosiderin was the only statistically meaningful difference between the test and control group animals in the histopathological study. The presence of a high amount of hemosiderin in the spleen indicates it suffered previous hemorrhage, attributed to the high dose of the applied product. The observed hemosiderin is the result of the destruction of red blood cells in this organ, liberating its content. Thus, when the hematoxylin-eosin technique is employed the coloring becomes yellowish.

The other analyzed innards presented a normal appearance, demonstrating no alteration caused by the product applied to the animals.

DISCUSSION

As the use of phytotherapeutical products is a common practice in our country and considering that several botanical species present toxic effects, establishing the experimental toxicity can contribute to avoiding or preventing organic damage (Lapa et al., 1983), in addition to contribute to the determination of doses for the elaboration of the dose-response profile in pharmacological studies (Brito, 1994; OECD, 1999).

In the case of the botanical species under study, there are no citations in literature about the toxicity of BG root bark powder. It was suspected to be of low acute toxicity, due to its extensive and intensive traditional use by the populations of *cerrado* regions (Ortêncio, 1997). Such hypothesis - of low oral acute toxicity - was partially confirmed by the present study. When Tables 1 and 2 and the DL50, as well as the high ALD value, are observed (>2000 mg/kg), and applying the rules established by the Brazilian Ministry of Health (Brasil, 1996; Brasil, 2000; Brasil, 2004) and by the Organisation for Economic Co-operation and Development (OECD, 1999), DL50, whenever above 2000 mg/kg classifies the product as being of low acute toxicity and would hardly be therapeutically useful above such dose.

Additionally, among the several botanical phytotherapeutical species revised, it was observed that DL50 values are very variable, from the very toxic - such as garlic oil and extract (*Allium sativum*; DL50, 100 mg/kg in mice *po*) up to 731 mg/kg for pomegranate (*Punica granatum*) (Joseph et al., 1989; Vidal et al., 2003), demonstrating a high level of safety for the BG exsudate, when the fixed dose acute toxicity study is

considered.

The daily therapeutic dose of BG Trécul. root exsudate for human adults is 400 mg twice a day (800 mg). Considering a 80-kg individual, the daily dose per body weight would be of 10 mg/kg, completely dissonant from the value obtained for ALD *po* (375 times higher than the standardized therapeutic dose) and *ip* (290 times the therapeutic dose) and for DL50 *po* (351 times higher than the therapeutic dose) and *ip* (287 times the therapeutical dose) of the aforementioned product, demonstrating the low acute toxicity of the *Brosimum gaudichaudii* root bark extract.

Nevertheless, toxicity studies of repeated dose (sub-acute and chronic) are required in order to assess how far organic systems may be affected when animals are treated in the medium and long run, since the product is recommended for lengthy treatments.

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