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RESEARCH ARTICLE

TOXICITY AND ANTITRYPANOSOMAN ACTIVITY OF HEMI SYNTHESIS PRODUCTS OBTAINED FROM BIOACTIVE COMPOUNDS OF *MITRACARPUS SCABER* HARVESTED SOUTH OF BENIN

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ABSTRACT

Objective: Present work involve study of the toxicity and the antitrypanosomal activity of the hemi syntheses (total of thiosemicarbazones) extracted from the substrates (reagents of thiosemicarbazides) in 1N hydrochloric acid medium from *Mitracarpus Scaber* harvested in the region of Abomey-calavi.

Methods: The ethanolic, dichloromethane and hydroethanol extracts (50:50 v/v) yielded the alkaloid extracts with yields of 4.44%, 2.48% and 5.08% respectively leading to three products of hemi-synthesis P1, P2 and P3 whose larval toxicities have respective values LC_{50}: 78.1µg/mL, 95µg/mL, and 48.8 µg/mL whereas the toxicity tests of the alkaloid samples of ethanol, dichloromethane and hydroethanolic E1, E2 and E3 extracted from the evaluation of the same larval toxicity test gave values of 83.41µg/mL 102.51µg/mL and 52.91µg/mL respectively.

Results: Results were less toxic than those of semisynthetic products. Acute and sub-acute toxicity in non-pregnant NMRI female mice after oral gavage of P2 product has been shown to be non-toxic. The antitrypanosomal test was carried out according to the Alamar blue method, it revealed that P1 moderately inhibits trypanosome parasites (IC_{50}=18.06 µg/mL) as well as P2 and P3 with a respective IC_{50} of 17.24 µg/mL and 20, 68 µg/mL while the alkaloid totals had lower antitrypanosomal activity than the hemi-synthesis products.

Conclusion: Study concludes that, shrimp larvae were tolerant of ethanolic, dichloromethane and hydroethanolic extracts and therefore the leafy stems of the plant do not have a priori harmful effects on human cells 9 PS, 9 KB, A-549 and HT-29.

Keywords: Antitrypanosomal, hemi synthesis, *Mitracarpus scaber*, thiosemicarbazide, toxicities.

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INTRODUCTION

Toxicological studies conducted on thiosemicarbazones do not mention their aggressivity in the stomach and intestinal walls. The pharmacokinetics and clinical phase I trials of a compound of thiosemicarbazones (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) 3-AP by intravenous injection were studied. It appeared that the molecule induced no toxic effect even after injection of a high dose. Herbal medicine referred to a practice of medicine that used the medicinal properties of plants[1]. However, in the industrialized countries, this practice was rarely encountered in favor of the industrial synthesis of molecules. We can see several reasons: The quantities of active substances were in very small quantities in the plants. At the scale of a population, this would require huge amounts of plants; the synthesis was cheaper[2]. The active molecules were often mixed in the plants with other undesired substances, and the separation was not easy and some natural molecules have side effects. A chemical synthesis consists of making a new molecule from one

or more chemical reactions. The challenge of a synthesis was to start with molecules that were easy to obtain (inexpensive), with the highest possible yield, which includes the energy, invested (heating). The possible harmfulness/toxicity of certain reagents, intermediate products or reaction by-products must also be taken into account. Finally, it was necessary to provide for the extraction and purification of the molecule to be synthesized. A hemi synthesis was a special case of chemical synthesis. The starting molecule was directly derived from natural substances and corresponds almost in its structure to the molecule that one wishes to produce. The challenge of a hemi-synthesis was a slight modification in order to acquire the molecule or get properties it did not have, or make it more assimilable for a consumer. The interest compared to a synthesis was to save steps in the manufacture.

Biologically active molecules (drugs) derive their properties from their structure, but also from the organic functions they possess[2,3,4]. For example, the hemi synthesis of aspirin leading to salicylic acid which is a molecule naturally present in the willow (a tree) or in the queen of the near (a plant). It has analgesic (pain suppressing), antipyretic (fever fighting) and antiseptic properties (kills bacteria and viruses). However, by semi synthesis from salicylic acid, acetylsalicylic acid, better known as aspirin, was produced. Indeed, it was more effective as a drug, has anti-inflammatory properties, and was less harmful to the stomach. It retains the properties of salicylic acid, except the antiseptic effect. Aspirin can be produced by reaction of salicylic acid with ethanolic acid, also called acetic acid. However, the yield of this reaction was limited to 8%. In order to increase it, it was possible to replace the ethanolic acid with more reactive ethanolic anhydride. In modern medicine most of the drug alternatives come from complex molecules found in the nature of known properties and these molecules are often the source of other improved molecules whose spectra of action have been enlarged compared to those of the beginning and presenting diminished side effects. This work was part of the improvement of the biological properties of the bioactive products of the plant *Mitracarpus Scaber* from the hybrids obtained by hemi synthesis, from which the knowledge of the toxic properties of the extracts prepared from the plant proved indispensable[5].

MATERIALS AND METHODS

Plant material

Fresh samples of *Mitracarpus scaber* were harvested and identified as No AA. 6252/HLB at the National Herbarium of the University of Abomey-Calavi[6]. The aerial part of the plant was cut out and dried for 10 to 14 days in the dark, then reduced to powder using an electric grinder (MILLS of Nigeria Flour, El. MOTOR No. 1827) then the ground material was preserved until use. The brine shrimp larvae (*Artemia salina*, LEACH) marketed by the German firm JBL GmbH and Co.KG were used for the preliminary toxicity test.

Biological material

The brine shrimp larvae (*Artemia salina*, LEACH) marketed by the German firm JBL GmbH and Co.KG were used for the preliminary toxicity test Non-pregnant NMRI female mice at 8 and 12 weeks of age weighing 30.8±3.8g were used for the acute and sub acute toxicity tests. They are maintained under standard conditions in the animal facility: temperature 21±2°C, circadian rhythm day-night 12h-12h with free access to water and food. The relative humidity was maintained between 50 and 70% except during the cleaning of the room. The animals are kept in 110 cm x 50 cmx20 cm wire mesh cages equipped with small feeders and drinking troughs. The bottom of the cages was constituted by a system of removable drawer garnished with chips of wood gathering the feces and the urine. Mice have free access to drinking water and food. They were supplied with drinking tap water and their diet was made of a mixture of nutrients in the form of croquettes.

Preparation of raw extracts

The maceration method in accordance with the traditional method of preparation was used[7]. In this study, three types of dichloromethanolic, hydroethanolic and ethanolic extracts were prepared for each of the 03 products; it was a total of[9] extracts preserving heat-sensitive compounds and representing a common route of extraction in medicine traditional. One hundred grams (100g) of powder were mixed with five hundred milliliters (500mL) of solvent (dichloromethane, hydroethanol (50:50 v/v) or ethanol 95°C) depending on the type of extract. The mixture is stirred continuously for 72 hours and filtered; this filtrate was then evaporated with a rotavapor (Heidolph Laborota 4000 efficient) coupled to cooler (Julabo FL 300) water at 40°C and then oven dried at 40^0C. The dry residue obtained was weighed in order to determine the yield according to the relation:

$$\text{Yield}(\%) = \frac{\text{Mass of dry extract obtained (g)}}{\text{Initial mass of powder (g)}} \text{x100}$$

Preparation of crude Hemi extracts in situ synthesis of thiosemicarbazones

0.175 g of each crude and alkaloid extract was dissolved in 15 ml of 95° ethanol (SIGMA-ALDRCH) in an Erlenmeyer flask. In another Erlenmeyer flask containing 5 ml of the 1N hydrochloric acid solution (R.P. PROLABO), 1.82g of thiosemicarbazide (SIGMA-ALDRCH) were dissolved and stirred gently until complete dissolution The contents of the Erlenmeyer flasks were then mixed in a 100 ml flask kept on a non-heating magnetic stirrer (BIOBLOCK AM 3000 D). The reaction was allowed to continue for 45 to 60 minutes (or even 2 hours). After the filtration of the heterogeneous mixture, the crystals obtained were dried and recrystallized in ethanol at 95°C.

Larval toxicity test

The test was carried out against *Artemia salina Leach* by the method as followed by[8] and has proposed in the literature as a simple biological assay method for the evaluation of the preliminary toxicity of active natural products. The eggs of *Artenia salina* were incubated in seawater until hatching of the young larvae (48 hours). Then, a series of solutions of each crude extract tested at different concentrations and gradual were prepared.

A defined number of larvae (16) were introduced into each solution. All solutions and the control solution containing no active substance were left stirring for 24 hours. Microscopic counting of the number of dead larvae in each solution was used to evaluate the toxicity of the solution. In the case, where there were dead larvae in the control medium, the data were corrected by the Abbott formula:

$$\% \text{ Death} = \frac{\text{Test} - \text{Control}}{\text{Control}} x100$$

The data are transformed by logarithm and the LC_{50} was determined by linear regression. Trials were performed in triplicate.

Acute or sub-acute toxicity test

The method used here was based on OECD recommendations[8]. We carried out a limit test consisting of a sequential test using 3 female mice per step. They were fasted for 12 hours before a single dose by oral gavage of 2000 mg/kg. The animals were observed closely for the first 4 hours in search of toxicity symptoms and the survival rate was evaluated after 24 hours. The animals will remain under observation for the next 14 days. If no mortality was observed at the end of this first step, a second confirmation test was performed.

This procedure, adapted to traditional preparations with proven ethno evidence, allows the determination of an approximate LD_{50} using a minimum of animals. It was carried out in an approach that combines the work of [9] with those of[10] Two groups of 06 mice were constituted. The group 1 (control group) receives physiological saline for 14 days and group 2 (treated group) receives the test extract in per os daily also for 14 days at a dose of 1000 mg/kg body weight. The mice weighed daily will all be sacrificed on the fifteenth day and their main organs (heart, lung, kidney, liver, stomach and spleen) will be removed after dissection and weighed.

Statistical treatment of data

The analysis of the data collected during the survey was carried out on the basis of the descriptive statistics using "MICROSOFT EXCEL 2013" highlighting the percentages of the respondents in relation to the total number of respondents. This analysis was followed by a taxonomic identification of plant species identified at two levels. For the first level of identification, local names of plant species were compared to scientific names reported in the literature. A second level of identification was carried out at the laboratory of the National Herbarium of Benin where the scientific names of the plant species identified in the literature were confirmed.

***In vitro* antitrypanosomal test**

This test was carried out according to the method described by Ganfon *et al.*[11] and Räz *et al.*[12] and recently used by Brun *et al.*[13]. Trypanosomes were diluted to adequate cell density. The cell density was adapted so that after 72 hours of incubation in the control wells, the cells were at the end of the logarithmic growth phase (*Trypanosoma brucei*: 5x104 tryps/ml). The density of trypanosomes was adjusted by a counting device: the hemocytometer. Per plate, 2ml of trypanosome stock will be used. 50 µl of the medium at room temperature were added to each well except for the extreme wells and column 11 (columns 2 to 10 in row B to G). 75 µL of medium containing twice the highest concentration of compounds (stock solution II) were placed in the wells of column 11 to be tested (compound No. 1 in rows B, C, D and compound No. 2 in rows E, F, G). Three dilution series were prepared with a multi pipette (6 channels are needed). Volume of 25 µl of the 6 wells of column 11 are taken and then transferred to column 10. The mixture was subsequently stirred.

The dilution from right to left was continued until addition of 25 µl of column 5 to column 4. After mixing, the remaining 25 µL are removed (total volume in each well equal to 50 µL at this stage). The wells in columns 2 and 3 serve as control wells (without addition of compound). 50 µl of trypanosome suspension were added to the wells of rows C, D, E, F of columns 2 to 11; then 50 µL of medium without trypanosome in rows B and G of the same columns. These wells serve as blank control. After incubation for 72 hours at 37°C, with 5% CO_2, the plates were observed under an inverted microscope to determine MIC (minimum inhibitory concentration): the lowest concentration of compounds to which no trypanosomes with normal morphology and motility compared to control well cannot be observed. Additional information may be recorded such as insolubility of the compound, microbial contamination, etc. 10 µl of Alamar Blue Fluorescent Dye, which can be diluted 1: 1 in phosphate buffered saline and incubated for a further 4 hours at 37°C, were added to each well. The plate was read with a fluorescence plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The data can be transferred into a graphics program (Excel) and the IC_{50} values can be evaluated by linear interpolation (Excel sheet template).

RESULTS AND DISCUSSION

Yield of extractions

The yield of the extracts obtained is expressed as a percentage of dry extract contained in 100 g of powder of the plant. The values obtained are summarized in Table 1.

Table 1: Returns of the different extracts

Type of extract		Yield (%)
Ethanolic	Gross	19.23
	alkaloid	4.44
Dichloromethane	Gross	16.13
	alkaloid	2.48
Hydro-ethanolic	Gross	21.09
(50:50, V/V)	alkaloid	5.08

It can be seen from this table that the best yield was obtained with the crude hydroethanolic extract; which means that most of the chemical principles of the plant are polar. The same observation was made at the level of the corresponding total alkaloid extracts. It can therefore be deduced that the yield of the total alkaloids was closely related to that of the parent crude extracts. On the other hand, yields were more or less improved compared to previous work[14,15]. This allows us to say

that the yields of extractions often depend on the type of extraction performed. Depending on whether fresh plant material was passed to dry plant material, yield can vary significantly[16]. This yield therefore depends enormously on the state of the plant material.

Table 2: Pharmacological Properties of P1, P2, P3.

Products	CL_{50} (µg/mL)	CI_{50} (µg/mL)	IS
P_1	78.1	18.08	4.39
P_2	97.5	17.24	5.65
P_3	48.8	20.68	2.35

Products P1, P2 and P3 indicated the respective LC_{50} values of 78.1 µg/mL, 97.5 µg/mL and 48.8 µg/mL during these toxicity tests, while alkaloid extracts E1, E2 and E3 were 83.41µg/mL, 102.51µg/mL, and 52.91µg/mL sources of semisynthetic products P1, P2 and P3. All the matrices tested each have an LC_{50} value of greater than 30 µg/ml. According to the literature, they were not very toxic[17] with a tendency for alkaloid extracts to be less toxic than those of semi synthetic products. In order to assess the degree of toxicity of the extracts from the LC_{50} values, we conform to the correspondence (Table 3) established by[18,19]. When the CL_{50} was greater than or equal to 100 µg/mL, the extract was nontoxic. An LC_{50} between 50 µg/mL and 100 µg/mL indicates low toxicity while an LC_{50} between 10 µg/mL and 50 µg/mL gives a moderate toxicity. An CL_{50} less than 10 µg/mL gives the extract a high toxicity.

Table 3: Correspondence between LC_{50} and toxicity

CL_{50}	Toxicity
$CL_{50} \geq 100$ µg/mL	-
100 µg/mL > $CL_{50} \geq$ 50 µg/mL	+
50 µg/mL > $CL_{50} \geq 10$ µg/mL	+ +
$CL_{50} < 10$ µg/mL	+ + +

- : non toxic; +: low toxicity; ++: moderate toxicity; +++: high toxicity

Analysis of the CL_{50} values of the extracts tested with respect to the correspondence table above, allows us to say that the products of hemi ethanolic synthesis more active and less toxic in the range of concentrations analyzed, because the CL_{50} obtained were within the fixed limits (100 µg/mL> $CL_{50} \geq$50 µg/mL). The values of the IS (Table 2), taking into account the values of the CL_{50} and CI_{50}, show that all products used in current study were selective and that the semisynthetic product P2 was more selective (IS=5.65) than P1 and P3[20,21]. The P2 thiosemicarbazone extract was first subjected to the cytotoxicity test on WI-38 cells (human embryonic fibroblasts). For this test, the result was expressed by the half-inhibitory concentration (CI_{50}) which was greater than 100 µg/mL for the ethanol extract against 0.4 ± 0.2 µg/mL for the control (tetracycline). The CI_{50} was equal to 50 µg/ml for the half-synthetic product P2. Since the toxicity limit was set at 20 µg/mL[10,22] it can be concluded that P2 was not harmful to WI-38 cells. This result reinforces that of the larval toxicity which has previously made it possible to demonstrate the non-toxic nature of the P2 extract on shrimp larvae and consequently on the human cells of carcinoma and colon in reference to the previous work[22,23].

Acute toxicity

At the end of the acute toxicity study, mice given the P2 extract administered at a dose of 2000 mg / kg body weight showed no toxic symptoms and no deaths were observed. These results make it possible to locate the LD_{50} for the extract above 2 g/kg of body weight. According to the OECD[8] a substance with an $LD_{50} \geq 2$ g/kg was classified in category 5 grouping very low-toxicity substances.

Sub-acute toxicity

In the sub-acute toxicity study, daily administration of a 1000 mg/kg body weight dose of P2 resulted in no deaths at the end of the treatment period. Changes in body mass showed no significant weight gain in both the control and treated groups. It can therefore be concluded that the extracts have no significant effect on weight gain[25] (Figure 1).

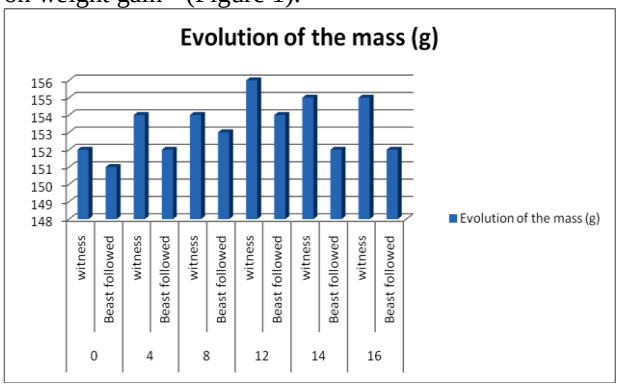

Figure 1: Evolution of body mass in % at the end of the treatment period

Comparison of organ masses (heart, lung, liver, kidneys, and spleen) and body mass between treated and control groups showed no significant difference. Macroscopic observation of these organs revealed no increase in volume, nor congestion, lesion or necrosis. These results reinforce the need for the use of certain plants in Traditional Medicine without risk of intoxication in order to enrich the therapeutic arsenal of modern medicine[26].

Antitrypanosomal activity of the extracts

The antitrypanosomal test performed according to the Alamar blue protocol, revealed that P1 moderately inhibits trypanosome parasites (CI_{50}=18.06 µg/mL) as well as P2 and P3 with CI_{50} of 17.24µg/mL and 20.68 µg/mL, whereas alkaloid totals showed lower antitrypanosomal activity than semi synthetic products (Table 2). It was important to remember that pest control tests were never performed from samples of present study; what makes this work, its originality in the search for trypanocidal molecules[27].

E1, E2 and E3: Total alkaloids from the hydroethanol, ethanol and dichloromethane extracts. P1, P2 and P3 respectively denote the semi synthesis products from these total alkaloids. The analysis of Table 4 shows that the hemi-synthesis was beneficial to the biological activity of the alkaloidal totals which have seen their antiparasitic activity which was initially weak increased one can conclude that this work brings a more in the fight against this formidable disease what

was trypanosomosis. However, data concerning the toxicity of P1, P2 and P3 products were fundamental for a better appreciation of this activity.

CONCLUSION

The shrimp larvae were tolerant of ethanolic, dichloromethane and hydroethanolic extracts and therefore the leafy stems of the plant do not have a priori harmful effects on human cells 9 PS, 9 KB, A-549 and HT-29. This preliminary study contributes to the valorization of the Beninese flora and deserves to be deepened by evaluating the anti-trypanocidal power of extracts and by quantifying the chemical groups proven to be responsible for this activity. In addition, the *in vivo* toxicity tests have shown that the extract was not harmful inside the body. The non-toxic nature of this extract, supported by the demonstrated antitrypanosomal activities, was a reason for further investigation of the P2 extract in order to purify and then isolate active molecules. In view of the importance of thiosemicarbazones in the field of health on the one hand and of these biological potentialities, it would be beneficial to continue investigations on the plant by exploring other lines of research. and it is in this context that the present work is inscribed.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

All the authors' participle in writing, giving feedback on this manuscript, have read and approved the final manuscript.

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