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In vivo evaluation of the genotoxicity of the total aqueous extract of *Sacoglottis gabonensis* (Baille) Urban (Humiriaceae) stem barks in albino Wistar rat

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Abstract
According to the WHO, 80% of people in developing countries rely on medicinal plants for their health care. Some of these plants are reputed to be toxic and can present a real danger. The objective of this study was to assess the genotoxic potential of the total aqueous extract of *Sacoglottis gabonensis* stem bark (ETASg) in *Wistar* rat.
130 rats with body weights ranging from 73 to 103 g were divided into eight groups, i.e. groups 1, 2, 3, 4 and 5 of 20 rats and groups 6, 7 and 8 of 10 rats for reversibility. Groups 1 and 6 received distilled water; groups 2, 3 received ETASg at doses of 3.5 and 35 mg/kg bw respectively; groups 5 and 7 received ETASg at a dose of 350 mg/kg bw for 90 days orally; groups 5 and 8 received urethane at a dose of 400 mg/kg bw intraperitoneally 24 h prior to general euthanasia. After euthanasia, bone marrow was harvested from the femur for smears to test for micronuclei or micronucleated polychromatophylytic erythrocytes.
This study showed greater micronucleus formation in urethane-treated groups than in the control group. Rats treated orally with ETASg at doses of 3.5, 35 and 350 mg/kg bw showed a rate of micronucleus induction relatively close to that of control rats.
In short, ETASg is not genotoxic. It would be interesting to evaluate the effect of this extract on stem cell proliferation in rats.

Keywords: Medicinal plant, *Sacoglottis gabonensis*, genotoxicity, rat

Introduction
The environment contains many chemical compounds, some of which are responsible for mutagenic or carcinogenic effects in humans [1]. The induction of these effects is considered more or less predictive of mutagenicity and carcinogenicity linked to the misuse of chemical substances [2]. Some natural substances, such as medicinal plant extracts, can induce genotoxic effects [3]. Genotoxicity is assessed using *in vivo* tests, the most reliable of which is the micronucleus formation test [4]. This test detects the cytogenetic toxicity of certain substances. With this in mind, we initiated research on *Sacoglottis gabonensis*, a medicinal plant used in the treatment of Buruli ulcer in Côte d'Ivoire. *Sacoglottis gabonensis* has been the subject of several research projects. In ethnobotanical terms, the treatment of Buruli ulcer with *Sacoglottis gabonensis* consists of using the decoction of the plant's stem bark as a drink and as a local application for a period of six months, depending on the patient's condition [5]. In addition, the aqueous extract of this plant's stem bark had an inhibitory effect on various strains of *Mycobacterium ulcerans* [6]. Acute and sub-acute toxicity tests in mice and rats, respectively, showed that total aqueous extract from *Sacoglottis gabonensis* (ETASg) stem bark has an LD_{50} greater than 5000 mg/kg bw.
In the present work, the objective is to assess the effects of ETASg on genotoxicity through micronucleus formation in rat bone marrow after 90 days of oral administration.

Materials
Plant Material
Sacoglottis gabonensis stem bark was collected at Ingrakon, in the Alépé department, 45 km from the city of Abidjan. A sample is identified and kept in the herbarium of the Centre National de Floristique d'Abidjan (Côte d'Ivoire), under number 131.

Animal material

The experiments were conducted on albino rats of the *Wistar* strain *Rattus norvegicus*. The rats were six to eight weeks old and weighed between 73 and 103 g. They were bred in the animal house of the Laboratory of Physiology, Pharmacology and Pharmacopoeia at Nangui Agrogoua University, in accordance with good laboratory practice [7]. Rats were acclimatized to a temperature of 22 ± 2°C and to 12 hours of alternating light and 12 hours of darkness. They were fed daily with IVOGRAIN® (Côte d'Ivoire) pellets and had unlimited access to tap water in their feeding bottles.

Technical equipment

All the equipment consisted of a grinder (Retsch SM 100) to grind the *Sacoglottis gabonensis* stem bark into a fine powder; a Denver S-234 precision balance (Belgium) for weighing; a Selecta® drying oven (Spain) to dry the extracts; an Ovan MCG05E magnetic stirrer (Europe) to homogenize extracts; a Heraeus SEPATECH centrifuge (Germany) to centrifuge bone marrow contents; an Optika optical microscope (Italy) to observe smears; a Samsung ST72 digital camera (China) to capture microscope images. The small equipment consisted of a funnel, absorbent cotton and wattman N°1 filter paper for filtering the decoctate; a 500 mL graduated cylinder for measuring the quantities of water consumed by the animals; 500 and 1000 mL beakers for preparing NaCl 9 ‰; 4% formalin and a gastric tube for force-feeding the animals. Also, a dissection kit was used for organ and femur removal; a 5 cc syringe for bone marrow content removal; hemolysis tubes for bone marrow content recovery; a 50 µL micropipette; tips for pellet removal and object holder slides for smears.

Chemicals and reagents

Methanol was used for fixation; urethane **CAS No. 51-79-6** for micronucleus induction; 4% formalin for bone marrow cell fixation; ethyl ether for euthanasia; May Grünwald-Giemsa for smear fixation and staining.

Methods

Preparation of total aqueous extract from *Sacoglottis gabonensis* stem bark

Total aqueous extract from *Sacoglottis gabonensis* stem bark (ETASg) is obtained using the method described by [8]. Freshly harvested *Sacoglottis gabonensis* stem bark is carefully cleaned, then dried in the laboratory for three weeks at room temperature (25 ± 2°C). After drying, the bark was ground to a fine powder using an electric grinder (Retsch SM 100). Four hundred grams (400 g) of stem bark powder were placed in 2 L of distilled water and boiled at 100°C for 30 minutes. The resulting decoctate undergoes double filtration on absorbent cotton and Whatman n°1 filter paper. The filtrates are evaporated and oven-dried at 50°C for 48 hours. A black-brown powder weighing 31 g, with a yield of 7.75%, was obtained and stored in a refrigerator at 7°C until use.

Treatment of animals and administration of substances

The experiment was conducted in accordance with OCDE guidelines 408 and 474 [9, 10]. One hundred (100) rats were evenly divided into five groups of 20 rats each, with ten males and ten females defined as follows:

- Group 1, the negative control, received distilled water ;
- Groups 2, 3 and 4 received 3.5, 35 and 350 mg/kg b.w. of ETASg respectively, 3.5 mg/kg b.w. being the tradipratician's therapeutic dose ;
- Group 5, the positive control, received a single intraperitoneal urethane dose of 400 mg/kg bw 24 hours before the end of the 90-day period.

Three additional so-called satellite groups of ten rats each, five males and five females, were included to observe reversibility, persistence or late appearance of micronuclei 30 days after cessation of administration, i.e. an experimental duration of 120 days :

- Group 6, satellite negative control, received distilled water.
- Group 7, satellite test, received 350 mg/kg bw ETASg ;
- Group 8, positive control satellite, received distilled water daily for 90 days and a single intraperitoneal dose of urethane 400 mg/kg bw at 2 mL/100 g bw, 24 hours before the end of the 90 days.

Satellite group receive no further treatment after cessation of treatment, but are fed daily during the 30-day observation period.

Observation of the general condition of the animals

General clinical observations of the animals are made twice a day, in the morning and in the evening throughout the study. These clinical observations covered behavior, gait, coat and respiration.

Slide preparation, staining and reading

Slide preparation

At the end of the 90-day experiment, all rats used in the study are euthanized in a metabolic bell containing ether-soaked cotton for two to three minutes, in accordance with good laboratory practice [7]. Immediately after euthanasia, bone marrow was harvested from the femurs of rats from groups 1, 2, 3, 4 and 5. The animal's femur is delicately freed from its flesh, then using a pair of scissors, one end of the femur is opened using the method of [11]. Then, using the method of [12], 2 mL of 9 ‰ NaCl solution is injected into the femur using a syringe. The bone contents are recovered, placed in hemolysis tubes and centrifuged at 3,000 rpm for seven min. Finally, after separation of the supernatant, 1 mL of 4% formaldehyde is added to the pellet to keep the cell cytoplasm intact. A volume of 50 µL of the cell suspension is withdrawn with a micropipette and spread onto a slide. The slides are then dried for 10-15 min at room temperature and fixed in absolute methanol for ten minutes [12].

This operation is repeated at the end of 120 days to study the reversibility of micronucleus formation.

Slide staining

After fixation, slides are covered with a 1:1 solution of May-Grünwald/distilled water for five minutes. Slides are carefully rinsed with a gentle stream of water to remove excess dye. The slides are then immersed for ten minutes in Giemsa solution diluted one-tenth, before being rinsed again with a gentle stream of water and air-dried for five to ten minutes [13].

Slide reading

Slide reading is based on the search for micronucleated polychromatophylytic erythrocytes. An increase in their number indicates the presence of chromatic alteration [14]. Per slide, 1000 polychromatophilic erythrocytes (PCE) are examined and micronucleated polychromatophilic erythrocytes (MNPCE) are counted under the microscope at x

1000 magnification. Micronuclei are strongly stained, independent of the main nucleus and contained in the same cytoplasm with a diameter smaller than that of the nucleus [15, 16].

Calculation of micronucleus frequency

The percentage (%) micronucleus frequency is determined by the ratio of micronucleated polychromatophilic erythrocytes (MNPCE) to observed polychromatophilic erythrocytes (PCE).

$$f\,(\%) = \frac{\text{MNPCE}}{\text{PCE}} \times 100$$

Statistical analysis

GraphPad Prism software version 5.0.1 for Windows (GraphPad Software Inc, San Diego, MO, California/USA, 2007), was used for analysis. Values were expressed as means followed by the standard error on the means (M ± SEM). To assess genotoxicity, each group is compared with the positive control group. Comparisons of means were made using a one-factor analysis of variance (ANOVA 1) followed by Bonferroni's post-hoc test.

Results

Observation of the general condition of the animals

Before administration of the extracts, all rats were in good health. However, after exposure to the different doses, rats at 350 mg/kg bw were much calmer than control rats and those at the other ETASg doses. Among the signs of toxicity investigated in rats, respiratory distress, circling and death were observed in animals at doses 35 and 350 mg/kg bw (Table 1). Respiratory distress was observed in batch 3 in four rats, 60 days after administration of the extract, and in group 4 in three animals, 30 days after administration of ETASg. Round-walking was only observed in group 4 during the entire ETASg administration period, in a total of four rats. Death was observed from day 60 in group 3 and day 30 in group 4, with 25% and 65% mortality respectively. Furthermore, all the animals in groups 3 and 4 that experienced respiratory distress died. Similarly, animals that walked in circles did not survive to the end of the 90 days. However, the surplus of deaths (four deaths) on day 60 in group 4 is thought to be due to other *in vivo* toxic manifestations.

Effects of ETASg on micronucleus formation

Intraperitoneal administration of urethane to group 5 rats induced a very highly elevated ($p<0.001$) rate of micronucleus formation (Figure 1) compared with rats treated orally with distilled water (Table 2). Furthermore, rats treated orally with ETASg at doses of 3.5, 35 and 350 mg/kg bw showed a rate of micronucleus induction relatively close to that of rats from the control group treated with distilled water.

One month after cessation of ETASg administration, micronuclei formation in rats treated with 350 mg/kg bw was similar to that in negative control rats, whereas in urethane-treated rats the rate remained high ($p<0.001$) (Table 3).

Table 1: General condition of rats after administration of ETASg

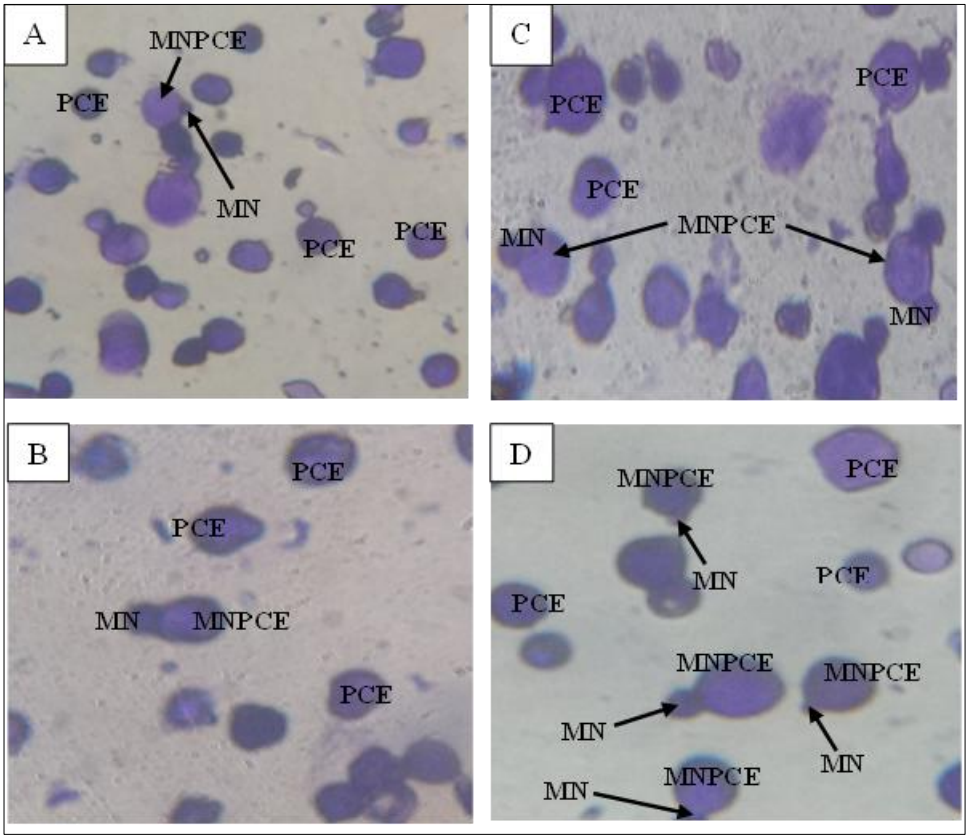
	Groups	**1**	**2**	**3**	**4**
	Doses (mg/kg bw)	**0**	**3,5**	**35**	**350**
Respiratory distress	D_{30}	0	0	0	3
	D_{60}	0	0	4	1
	D_{90}	0	0	1	1
	D_{120}	0			0
Walking in circles	D_{30}	0	0	0	1
	D_{60}	0	0	0	2
	D_{90}	0	0	0	1
	D_{120}	0			0
Death	D_{30}	0	0	0	4
	D_{60}	0	0	4	7
	D_{90}	0	0	1	2
	D_{120}	0			0
	Total number of deaths	0	0	5	13
	Percentage of mortality	0	0	25	65

n = 20, number of rats that received the substances for 90 days; n = 10, number of rats in which administration of the substances was stopped after 90 days; 0 = none; D_{30} = 30 days, D_{60} = 60 days, D_{90} = 90 days, D_{120} = 120 days.

Table 2: Effect of ETASg on micronucleus formation in rats.

Groups	**1**	**2**	**3**	**4**	**5**
Doses (mg/kg bw)	**0**	**3,5**	**35**	**350**	**Ur 400**
PCE observed	2567 ± 172,6	2639 ± 206,2	2389 ± 232,7	2147 ± 241,4	2962 ± 307,0
MNPCE observed	1,40 ± 0,18[a]	2,00 ± 0,23[a]	2,70 ± 0,36[a]	3,15 ± 0,25[a]	28,50 ± 0,96[C]
Frequency MNPCE (%)	0,06 ± 0,01[a]	0,09 ± 0,02[a]	0,12 ± 0,02[a]	0,16 ± 0,01[a]	1,35 ± 0,25[C]

PCE: polychromatophilic erythrocytes, MNPCE: micronucleated polychromatophilic erythrocytes. n=20 animals/group. Comparisons are made between the positive control and other groups, and between the negative control and groups treated with different doses. Values bearing the same letters are statistically equal; a = $p<0.05$, c = $p<0.001$. Ur 400: positive control group treated with urethane; 0: negative control group treated with distilled water; 3.5: group treated with extract at 3.5 mg/kg bw; 35: group treated with extract at 35 mg/kg bw; 350: group treated with extract at 350 mg/kg bw.



PCE: Polychromatophilic erythrocytes, MNPCE: micronucleated polychromatophilic erythrocytes, MN: micronuclei. A: group treated with distilled water; B: group treated with extract at 3.5 mg/kg bw; C: group treated with extract at 35 mg/kg bw; D: group treated with extract at 350 mg/kg bw.

Fig 1: Rat bone marrow smear (G x 1000).

Table 3: Effect on micronucleus formation after cessation of ETASg administration

	Groups	**6**	**7**	**8**
	Doses (mg/kg bw)	**0**	**350**	**Ur 400**
PCE observed	PCE observés	2452 ± 113,8	2123 ± 300,0	2330 ± 122,3
MNPCE observed	MNPCE observés	1,50 ± 0,22 [a]	1,90 ± 0,31[a]	23,30 ± 1,54[C]
Frequency MNPCE (%)	Fréquences MNPCE (%)	0,06 ± 0,01 [a]	0,11 ± 0,02 [a]	1,02 ± 0,09[C]

PCE: Polychromatophilic erythrocytes, MNPCE: micronucleated polychromatophilic erythrocytes. n=10 (M±ESM); D_{120} = 120 days. Ur 400: batch treated with urethane; 0: control group treated with distilled water; 350: group treated with 350 mg/kg b.w. ETASg; a = $p<0.05$; c = $p<0.001$.

Discussion

The study of the subchronic toxicity of ETASg by the oral route revealed signs of toxicity such as uncoordinated gait movements, respiratory distress and death at medium and high doses of 35 and 350 mg/kg bw. These various signs of toxicity observed in the course of this study would appear to be due to the chemical composition and excessively high dose of the extract, which seems to act not only on respiration, but also on the central and peripheral nervous systems.

Thus, the effects of a toxic substance on respiration manifest themselves as irritation, shortness of breath leading to breathing difficulties and even death. On the central nervous system, these effects translate into depression, loss of appetite and drowsiness, and on the peripheral nervous system, into motor impairment such as weakness, tremors, incoordination and convulsions [17]. Our results are contrary to those of [18], who, after subchronic administration of methanolic extract of Euphorbia hirta (Euphorbiaceae) in rats, did not observe any clinical signs of toxicity.

The micronucleus test is recognized as an easy and reliable way of studying genotoxicity. It is also considered one of the markers of early biological effects of genotoxic substances [19] and focuses on micronucleus formation [4]. Urethane administered at a dose of 400 mg/kg bw in positive control rats caused the formation of a significant number of micronuclei with a frequency of 1.35 ± 0.25%, confirming the genotoxic potency of urethane. The micronucleus frequency of 0.06 ± 0.01% in negative control rats has been reported by other authors to be 0.05 ± 0.01% in the bone marrow of mice treated with distilled water [20]. The observation of this frequency by authors in negative control animals, would assume the existence of endogenous factors that induce their formation. Indeed, in the evolutionary process of the red cell lineage, micronuclei can be observed at the reticulocyte stage [14, 21]. However, damage leading to micronuclei can be repaired by cellular machinery [22]. Thus, the slight variations in micronucleus frequency observed from these negative controls would be linked to the species and immune system of the experimental subject [3].

Furthermore, the administration of different doses of ETASg had no significant effect on micronucleus formation. For doses of 3.5, 35 and 350 mg/kg bw, respectively, ETASg induced micronucleus formation frequencies of 0.09 ± 0.02%, 0.12 ± 0.02% and 0.16 ± 0.01%. These frequencies are much

lower than those of urethane, and virtually identical to those obtained in rats treated with distilled water. The micronucleus-inducing effect of ETASg is virtually nil compared with that of urethane. These results are similar to those of authors who evaluated in vivo the micronucleus-inducing effect of hydo-ethanolic and dichloromethanic extracts of Piliostigma reticulatum stem bark in rats for 28 days [23]. At doses of 250, 500 and 1000 mg/kg bw for the hydroethanol extract of Piliostigma reticulatum stem bark, they observed micronucleus frequencies that were 0.04 ± 0.01%; 0.08±0.02%; 0.17 ± 0.04%, respectively. The micronucleus-inducing effect of ETASg is relatively similar to that of hydroethanol and dichloromethanol extracts of Piliostigma reticulatum stem bark. However, the dose variations between the hydroethanol extracts of Piliostigma reticulatum stem bark and ETASg are explained by a difference between the animals and the chemical constituents of the plants [3].

Conclusion

Testing the safety of ETASg has favored the evaluation of general condition and genotoxicity in rats. This study showed that ETASg induced toxic effects such as respiratory distress, circling and death in rats tested with high doses. As far as genotoxicity is concerned, the micronucleus-inducing capacity of ETASg is almost negligible.

It would be advisable to carry out a chronic study on the histology of certain organs such as the brain to elucidate the uncoordinated movements, but also on vital organs such as the heart, liver and kidneys to elucidate the death of the rats.

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