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Evaluation of *In vivo* Toxicity and *In vitro* Antimicrobial Activities of Crude Ethanolic Stem Bark Extract of *Anonidium mannii*

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Authors' contributions

This work was carried out in collaboration among all authors. Author JETM designed the study, supervised the work and corrected the first draft of the manuscript while author EEK wrote the protocol and carried out the antimicrobial activities, supervised by author JCAN. Author ELN carried out the study of toxicity, supervised by author EPP. Author XSN managed the literature searches and the analyses of the study while author BN wrote the first draft. Author BT corrected the last written version of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aims to evaluate the *in vivo* acute and sub-acute toxicity; and the antimicrobial activity of *Anonidium mannii* stem bark ethanolic extract.
Study Design: This is an experimental study.
Location and Duration of the Study: The work was conducted from 15 November 2020 to 31 May 2021 at several laboratories (the Faculty of Medicine and Pharmaceutical Sciences Laboratory of the University of Douala, the Animal Physiology Laboratory of the Faculty of Medicine and Biomedical Sciences at the University of Yaoundé 1 and in the Hematology and Bacteriology Laboratories of the District Hospital of the Cité des Palmiers, Douala, Cameroon).
Methodology: The *in vivo* acute and subacute toxicities studies were conducted following the guidelines of the OECD on Wistar rats, while the *in vitro* antimicrobial activity was evaluated using the microdilution method on bacterial (*Neisseria gonorrheae, Escherichia coli, Salmonela spp*) and fungal (*Candida albicans*) strains.
Results: Phytochemical screening revealed the presence of alkaloids, anthraquinones, coumarins, flavonoids, phenols, reducing sugars, tannins, triterpenes. An oral administration of the extract did not induce an abnormal variation of the physiological parameters in rats, at doses of 2000 and 5000 mg/kg body weight for 14 days. After twenty-eight days of observation during the study of subacute toxicity, the extract showed good safety in terms of subacute toxicity. No deaths were recorded at the different doses; and clinical and physiological parameters analyses revealed a non-significant statistical difference. All bacterial strains in the presence of the extract were inhibited, as well as *C. albicans*. The extract exhibited strong antibacterial activity against *N. gonorheae* (MIC 15.6 µg/mL), *E. coli* (MIC 62.5 µg/mL) and *Salmonella spp* (MIC 15.6 µg/mL).
Conclusion: These results could justify the use of *A. mannii* stem bark in the traditional pharmacopoeia for the treatment of gonococcal disease, diarrhea, abscesses and food poisoning.

Keywords: Phytochemical screening; acute toxicity; subacute toxicity; antimicrobial activities; Anonidium mannii.

1. INTRODUCTION

Medicinal plants used for thousands of years, constitute a vast biodiversity of vegetation throughout the world, including Africa and particularly in Cameroon. Indispensable for the balance of the ecosystem and the feeding of animals, plants have many therapeutic properties which today are not all studied. Approximately 60% of medicines come from plants and those produced synthetically come partly from natural molecules taken as the mainstay [1,2]. According to the World Health Organisation (WHO), 80% of Africans use traditional medicine to treat themselves [2,3].

“Traditional medicine, sometimes referred to as alternative medicine, is the sum total of knowledge, skills and practices based on culture-specific theories, beliefs and experiences that are used to maintain human health and to prevent, diagnose, treat and cure physical and mental illness” [1,4]. It is derived from the ancestral knowledge possessed by descendants of certain families, initiates in certain villages, herbalists and drug sellers. Faced with the limitations of modern medicine, socio-cultural habits and poverty, African populations have recourse to traditional medicine, which has proved to be effective for human health [3,5].

In view of the expected results, traditional medicine must be promoted in order to protect and perpetuate phytotherapeutic resources. Under the aegis of the WHO, many researchers, particularly in Cameroon, are working to evaluate the activities of plants throughout the country. In order to contribute to these studies, we chose *Anonidium mannii* (Oliv) Engl. and Diels.

The choice of this plant was motivated on the one hand by its traditional use in the treatment of dysentery, diarrhoea, gonorrhoea, infertility, abscesses and candidiasis [6-8]. On the other

hand, a review of the literature revealed that few studies have been carried out on *A. mannii* in terms of toxicity (acute and sub-acute) and antimicrobial activity, none of them on stem bark [9]. However, the phytochemical study, the antiprotozoal and cytotoxic activities of the stem barks [10,11], the antimycobacterial activities of the leaves and twigs [12,13], the antiplasmodial activities of the leaves and stem bark [14], the antioxidant, cytotoxic and antibacterial activities of the leaves [6,12] have already been studied. Therefore, we propose to study the toxicity (acute and subacute) and evaluate the antibacterial and antifungal activities of the ethanolic extract of the stem bark of *A. mannii*.

2. MATERIALS AND METHODS

2.1 Materials

Materials and instruments used throughout the study include: Plastic cages, drinkers, sanitizer, cotton wool, animal feeds, paper tape, universal bottles, 5 ml and 2 ml syringe, stainless plates, surgical glove, sensitive weighing scale, accu check strip, distilled water, heparinized capillary tube, phosphate buffer solution, petri dish, scalpel, centrifuge, measuring cylinder.

2.2 Experimental Animals

Healthy female and male Wistar rats, nulliparous, non-pregnant, aged from 06 to 09 weeks, which have not been subjected to previous experimental activities, were used. Their weights were determined prior to feeding. The rats were acclimatized for 2 weeks. The experimental animals were housed in standard plastic cages and provided access to food and water *ad-libitum*.

For antimicrobial assays, three bacterial strains were used: *Neisseria gonorrheae, Escherichia coli, Salmonela spp* and one fungal strain: *Candida albicans*.

2.3 Collection, Authentification, Preparation and Extraction of the Plant Material

The stem bark of *Anonidium mannii* was collected in the South Cameroon region, Department of Dja and Lobo, Sangmelima District, more precisely in a forest in the village of Nkolotou'outou and identified by a taxonomist at the National Herbarium of Cameroon under No. 50327/HNC by Mr Victor Nana.

The fresh stem bark of *Anonidium mannii* were air dried for four weeks. It was pounded and later blended to powdered form. The preparation of the ethanolic extract was carried out according to the protocol described by Mugiraneza et al in 2009 [15].

2.4 Animal Grouping

2.4.1 Acute toxicity

A total number of 12 female Wistar rats were randomly selected and divided into four (4) different treatments batches, each batch comprises of 3 healthy animals.

Batch 1: the neutral control group, treated with 1 ml / 100 g of distilled water body weight, Batch 2: the negative control group, treated with olive oil at 0.75 ml / 100 mg of body weight, Batch 3: treated with *Anonidium mannii* extract at 2000 mg / kg of body weight, and Batch 4: treated with *Anonidium mannii* extract at 5000 mg / kg of body weight.

2.4.2 Subacute toxicity

A total number of 30 Wistar rats were randomly selected and divided into five (5) different treatments batches, each batch comprises of 6 healthy animals (3 males and 3 females).

Batch 1: the neutral control group, treated with 1 ml / 100 g of distilled water body weight, Batch 2: the negative control group, treated with olive oil at 0.75 ml / 100 mg of body weight, Batch 3: treated with *Anonidium mannii* extract at 200 mg / kg of body weight. Batch 4: treated with *Anonidium mannii* extract at 400 mg / kg of body weight, Batch 5: treated with *Anonidium mannii* extract at 800 mg / kg of body weight.

2.5 Phytochemical Screening

Detailed phytochemical screening was performed on the ethanolic extract of *A. mannii* stem bark using standard methods, as reported in the literature [16-19]. Other specific phytochemical tests were also realized, all based on a precipitation reaction via the generation of insoluble complexes called precipitates, and on colorimetry reaction through the formation of colored soluble chemical species. The color reactions were carried out in test tubes in the presence of the reference positive controls. The following tests were used: Drangendorff test (alkaloids), Tannins (gallic tannins), Liebermann-

Burchard test (steroids and triterpenes), Shinoda (flavonoids), Cardiotonic glycosides test (cardiotonic glycosides), Borntrager (anthraquinones), Foam Index test (saponins), $FeCl_3$ test (polyphenols), Potash test (coumarin) and Reducing Sugars test. All observations were recorded.

2.6 Acute Toxicity

Acute toxicity experiment was conducted according to guideline 423 of the OECD protocol [20] at the Pharmacology and Toxicology Laboratory of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala. Nine-week-old female Wistar rats were fasted over the night preceding the experiment from 8 p.m. to 8 a.m. Two (2) batches of three (3) randomized rats received the ethanolic extracts of the stem bark of *A. mannii*, at doses of 2000 and 5000 mg/kg body weight, respectively. The control batch received distilled water. Once treated, the animals were observed for 2 hours (H) after the administration of the extract. They were then fed and observed after 4, 8 H and then 14 days during which the symptoms of intoxication (Stool appearance, Noise sensitivity, groupement, Locomotion, Motility, Coat modification, Reaction to noise, Grooming, Trembling, as well as deaths) were noted. The dead rats in each batch were counted for the determination of the median Lethal Dose (LD_{50}). The extract was administered to animals orally.

2.7 Subacute Toxicity

Subacute toxicity has been studied as per OECD Guideline 407 with slight modifications [21] at the Pharmacology and Toxicology Laboratory of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala. Nine-week-old adult, Wistar albino, male and female rats were divided into three experimental batches of six (6) animals each, three males and three females. They were fasted the night before the experiment from 8 p.m. to 8 a.m. The subacute toxicity tests were carried out on the three (3) batches of six (6) randomized rats which received the ethanolic extracts of the stem bark of *A. mannii*, at doses of 200, 400 and 800 mg/kg body weight, respectively. The two (2) control batches received distilled water. The administrations continued for 28 days with 6 days of administration out of seven per week. After 28 days, the organs that were removed including: liver, kidneys, heart, lung, spleen were rinsed with 0.9% saline solution (physiological solution), then observed *in situ* and weighed.

The biochemical parameters measured were serum urea, creatinine, aspartate aminotransferase (AST) analine aminotransferase (ALT). In hematology, the blood count and organ sections in histology.

2.8 Biochemical Analysis

The following serum parameters: Aspartate transaminase (AST), alanine aminotransferase (ALT), Creatinine (Creatine), Uric acid (UA), were measured by enzymatic methods. These assays were carried out at the Animal Physiology Laboratory Laboratories of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala.

2.9 Hematological Examinations

The hematological parameters for the blood count formula (SNF) were : white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) count, hematocrit (Hct), mean globular volume (MGV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) count and lymphocytes (Lym) count. These parameters were carried out by Medonic (Beckman Coulter, USA) at the Haematology Laboratory of the District Hospital of the Cité des Palmiers.

2.10 Histology Examination

The histological procedure was carried out by the method described by Biswas et al in 2010 with some modifications [22]. The liver, kidneys, heart, lung from both the treated and control groups was processed with automatic tissue processor (STP 120) by tissue processing method as described by Galen and Gambino, 1975 [23]. Histology preparation was done in 4 µm tissue sections with a Microtome (Leica, RM 2145). These sections were then deparaffinated in xylene, dehydrated through a graded ethanol series, and stained with haematoxylin-eosine and cleared in xylene I and xylene II and these organs were preserved for microscopic examination. The slides prepared by this process were observed under microscope (Model Nikon Labophot. 223425 Japan) and photographed through Nikon labophot Advanced Research Microscope, Model 223425 Japan, with Sony Digital 12.1 MEGA PIXELS. This assay was carried out at the Animal Physiology Laboratory of the Faculty of Medicine and Biomedical Sciences of the University of Yaoundé 1.

2.11 Antimicrobial Activity

The evaluation of the antimicrobial activity was carried out after the sterility control of the ethanolic extract of *A. mannii* stem bark. In order to determine the parameters of inhibition: "The Minimum Inhibitory Concentration (MIC) and the Minimum Microbicidal Concentration (MMC)". We followed the principle based on the microdilution method, adopted by the Société Française de Microbiologie (SFM) and updated in their report with the EUCAST in 2019 (European Committee on Antimicrobial Susceptibility Testing). This method consists of observing the color change of the colored indicator (phenol red) which passes either from red to yellow due to the release in the medium of acid metabolites, or from red to pink when the products resulting from the metabolism of the bacteria during their growth are basic. In the absence of growth, there is no change in coloration [24]. This assay was carried out at the Bacteriology Laboratory of the District Hospital of the Cité des Palmiers.

2.12 Data Analysis and Statistical Parameters

Excel software was used to record the data of rat weight tracking, organ masses and biochemical marker concentration. Both disk GraphPad Prism 8.0.1. software allowed us to plot the curves and perform statistical analysis by one-way ANOVA and two-way ANOVA methods through the parametric test of DUNNETT with a significance level of 0.5%.

3. RESULTS AND DISCUSSION

3.1 Extraction

The extract was obtained by double maceration of 5 kg of stem bark powder with 25 l of ethanol, which is a practical polar solvent chosen in our study for it's low toxicity, for 3 days at room temperature. The collected solution was filtered through Whatman No. 1 paper and concentrated using a rotary evaporator at 40°C, making 120 rpm. A black viscous substance was collected and left for 3 weeks in a flask covered with aluminum foil. The masses were measured after every two days until a constant mass of 123 g was obtained corresponding to an extraction rate of 2.46%.

3.2 Phytochemical Screening

Phytochemical screening (Table 1) had shown that ethanolic extract of *A. mannii* stem bark contain alkaloids, anthraquinones, coumarins, flavonoids, phenols, reducing sugars, tannins, triterpenes while saponins and steroids were not detected.

Table 1. Phytochemical screening of ethanolic extract of *Anonidium mannii* stem bark

Chemical compound family	Ethanolic extract
Alcaloids	+
Anthraquinones	+
Coumarins	+
Flavonoids	+
Phenols	+
Saponins	-
Steroids	-
Reducing sugars	+
Tannins	+
Triterpenes	+

+ = *presence;* **-** = *absence*

3.3 Acute toxicity

3.3.1 Physiological parameters of rats

The LD_{50} is the concentration of substance in mg/kg causing the death of 50% of a given animal population under precise experimental conditions. After oral administration of a single dose of the ethanolic extract of *A. mannii* stem bark, abnormal variation of the physiological parameters was not observed during 14 days of the assay for batches 3 and 4 of rats compared to the rat control group apart from the aspect of the stools which was pasty, for the batches having received olive oil (batches 2 to 4) for 14 days compared to those in batch 1 who received distilled water (Table 2). Therefore, ethanolic extract of *A. mannii* stem bark was considered as non-toxic at doses of 2000 and 5000 mg/kg. Consequently, the LD_{50} can be considered as greater than 5000 mg/kg.

After administration of the ethanolic extract of the stem bark of *A. mannii*, the animals were individually weighed at least once every 2 days: an evolution of the weight mass of the rats during the study was therefore recorded. The result of the study as illustrated in Table 2 showed, in the same way as for the determination of the LD_{50}, that the ethanolic extract of *A. mannii* stem bark has no impact on the weight mass of the rats (Fig. 1).

Table 2. Observation of physiological parameters of rats of acute toxicity

Parameters observed	Batch 1 (distilled water 10 ml/kg)	Batch 2 : olive oil	Batch 3 (extract at 2000 mg/kg)	Batch 4 (extract at 5000 mg/kg)
Number of rats	3	3	**3**	**3**
Stool appearance	N	P	P	P
Noise sensitivity	N	N	N	N
Groupement	N	N	N	N
Locomotion	N	N	N	D
Motility	A	A	A	A
Coat modification	N	N	N	N
Reaction to noise	N	N	N	N
Grooming	N	N	N	N
Trembling	A	A	A	A
Death	0	0	0	0

A = Absent, N = Normal, P = Pasty, D = Decreases, 0 = Zero.

3.3.2 Evolution of the weight growth of rats

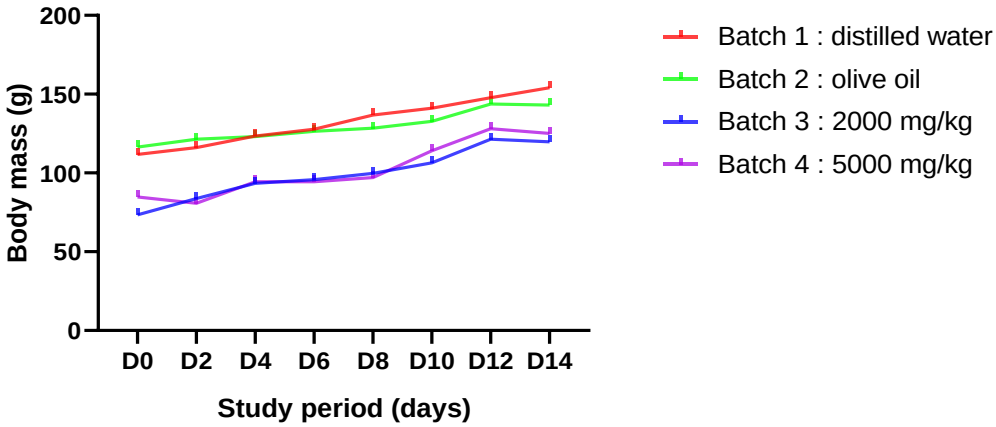


Fig. 1. Evolution of the weight growth of rats during acute toxicity

Acute doses were well tolerated. No substance-related deaths were observed. According to the Hodge and Sterner scale at the oral LD_{50} above 5000 mg/kg (5000 < LD_{50} < 15000 mg/kg) the substance is almost non-toxic [25].

3.4 Subacute Toxicity

3.4.1 Physiological parameters of rats

Ethanolic extract of the stem bark of *A. mannii* was administered to the rats and the observation was made during the first 4 h after administration, and then for 28 days. All the parameters observed showed no abnormality during the four weeks of observation apart from the aspect of the stools which was pasty, slowed growth and reduced nutrition for the batches having received olive oil (batches 2 to 5) for 28 days compared to those in batch 1 who received distilled water (Table 3).

3.4.2 Variations in the average mass of batches of rats

Fig. 2 shows the effect of the ethanolic extract of the stem bark of *A. mannii* on the evolution of the weight of rats during subacute toxicity. These results let us affirm that a slow general growth of the rats was observed in comparison to the neutral control. Moreover, the weight analysis of the rats reveals a slowed increase compared to the neutral control with an extremely significant statistical difference (p-value < 0.0001).

Table 3. Observation of physiological parameters of rats of subacute toxicity

Parameters observed	Batch 1 (distilled water 10 ml/kg)	Batch 2 : Olive oil	Batch 3 (extract at 200 mg/kg)	Batch 3 (extract at 400 mg/kg)	Batch 3 (extract at 800 mg/kg)
Number of rats	**6**	**6**	**6**	**6**	**6**
Stool appearance	N	P	P	P	P
Noise sensitivity	N	N	N	N	N
Groupement	N	N	N	N	N
Locomotion	N	N	N	N	R
Nutrition	N	D	D	D	D
Coat modification	N	N	N	N	N
Reaction to noise	N	N	N	N	N
Grooming	N	N	N	N	N
Trembling	A	A	A	A	A
Death	0	0	0	0	0

A = Absent, N = Normal, P = Pasty ,R = Slow down, D = Decreases, 0 = Zero.

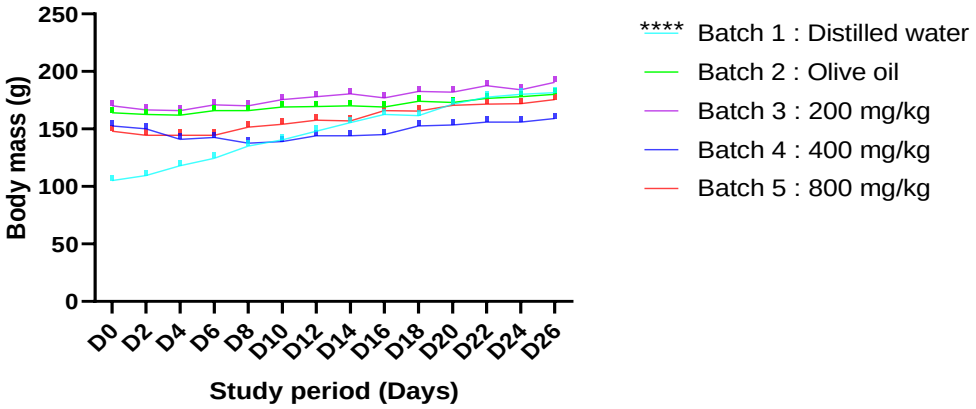


Fig. 2. of the average of body **masses of the batches of the subacute toxicity**

p > 0.05 = not significant; p < 0.05 (*) = insignificant; The p < 0.01 (**)= significant; The p < 0.001 (***) = very significant; The p < 0.0001 (****) = extremely significant.

The observation of the behavioral parameters of the subacute toxicity allowed us to observe during the 28 days that the rats that received the olive oil showed the appearance of pasty stools and a slow growth. This was most likely due to the extract being dissolved in olive oil and we found that the animals given the olive oil had reduced appetites and consequently slow growth. Mean body weights of rats from all batches increased over the observation period. Statistical studies revealed non-significant differences in batch-to-batch comparison with p-values > 0.05. These results corroborate those of Benhaddad et al in 2019, in a study of the effects of olive oil on the parameters of oxidative stress in wistar rats made diabetic with streptozotocin, which is manifested by the decrease in volume or weight of the rats [26]. These results are contradictory to those of Etame et al in 2017 and Etame-Loe et al in 2018 for which the weight gain of rats generally increases regardless of the batch chosen [27,2].

3.4.3 Average masses of the internal organs of the batches of rats during the experiment

The weighing of the masses of the internal organs for male rats yielded the histogram in Fig. 3 that showed little comparative variations in organ weights with slight decreases in livers and lungs. In kidneys and heart, there is an increase at the dose of 200 mg/ml. The differences observed compared to the neutral control having

received distilled water could be due to the use of olive oil which proved to be the best dissolution solvent in comparison to DMSO (poorly soluble) and distilled water. The analysis of the weight mass of the organs of the different batches in male rats, in accordance with the multi-comparative test 2 WAY ANOVA of the Graphpad Prism software version 8.0.1, showed that the average values of the mass of the organs in comparison batch by batch for each organ according to the doses have non-significant statistical differences according to the column factors with p-value > 0.05.

The weighing of the masses of the internal organs for female rats yielded the histogram in Fig. 4 that showed the decrease in weight of the livers, an increase in the weight of the kidneys of the batch at the dose of 400 mg/kg, an increase in the weight of the hearts of the batches at the doses of 200 and 400 mg/kg, of the weight of the lungs of the batches at the doses of 400 and 800 mg/kg and an increase in the weight of the spleens of the group at the dose of 200 mg/kg. The analysis of the weight mass of the organs of the different batches in males, in accordance with the multi-comparative test 2 WAY ANOVA of the Graphpad Prism software version 8.0.1, showed that the average values of the mass of the organs in comparison batch by batch for each organ according to the doses had non-significant statistical differences according to the column factors with p-value > 0.05.

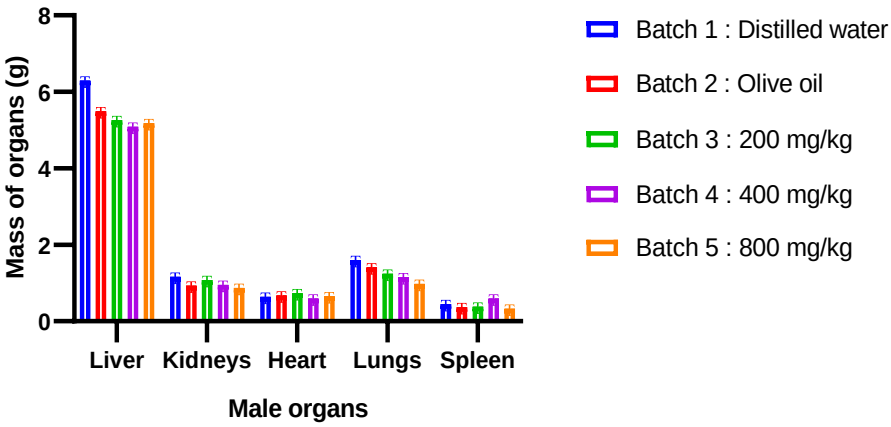


Fig. 3. Average masses of the internal organs of male rats groups

p > 0.05 = not significant; p < 0.05 (*) = insignificant; The p < 0.01 (**)= significant; The p < 0.001 (***) = very significant; The p < 0.0001 (****) = extremely significant

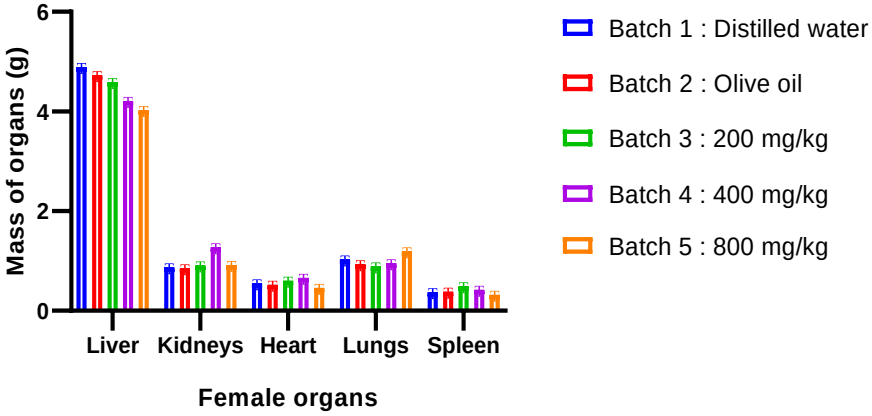


Fig. 4. Average masses of the internal organs of female rats groups

p > 0.05 = not significant; p < 0.05 (*) = insignificant; The p < 0.01 (**)= significant; The p < 0.001 (***) = very significant; The p < 0.0001 (****) = extremely significant

Table 4. Calculated means for biochemical parameters in all batches of rats

	Urea (mg/dL)		Creatinine (mg/dL)		AST (IU/L)		ALT (IU/L)	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Batch 1 (neutral control)	48.28	40	0.98	0.97	90.21	94.87	44.81	59.36
Batch 2 (negative control)	30.34 ± 0	35.4 ± 2.981	1.04 ± 0.074	0.9 ± 0.05	91.18 ± 14.702	88.46 ± 4.542	37.24 ± 4.742	33.76 ± 0
Batch 3 (200 mg/kg)	38.62 ± 7.397	31.26 ± 5.137	0.87 ± 0.077	1.09 ± 0.094	111.55 ± 4.038	118.14 ± 67.683	43.46 ± 3.352	52.92 ± 11.174
Batch 4 (400 mg/kg)	28.96 ± 0.975	31.03 ± 1.463	1.19 ± 0.046	0.88 ± 0.028	135.89 ± 9.671	128.62 ± 9.465	47.72 ± 0.411	14.55 ± 1.646
Batch 5 (800 mg/kg)	41.38 ± 3.518	30.34 ± 0.976	0.774 ± 0.131	1.13 ± 0.167	123.96 ± 23.169	121.83 ± 23.827	45.69 ± 4.733	54.66 ± 18.329

Data are presented as mean plus or minus mean standard error (MSE) in mg/dl for urea and creatinine concentrations, also in IU/L for those of AST and ALT of neutral, negative and test batches. Multi-comparative analyses 2 WAY ANOVA and Duncan's post-hoc test performed by Graphpad Prism 8.0.1 software, were used to compare the concentrations of the batches on each marker

Table 5. Calculated means for hematological parameters in all batches of rats

Parameters studies	**Batch 1 (distilled water 10 ml/kg)**		**Batch 2 : olive oil**		**Batch 3 (extract at 200 mg/kg)**		**Batch 4 (extract at 400 mg/kg)**		**Batch 5 (extract at 800 mg/kg)**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
	Complete Blood Count									
RBC (10^6/mm^3)	5	4.6	5,5	4.19	5.5	5.5	5.8	6.1	5.4	5.5
WBC (10^6/mm^3)	8.3	4.4	4,5	3.5	5.8	4.03	6.1	5.8	5.5	5.4
Hemoglobin (g/dl)	14	8.8	11	11.05	11	11	11.6	12.2	10.8	11
Hematocrit (%)	44	54	53	53	55	54	58	61	54	55
MGV (µm^3)	86	41.5	55	67	55	55	58	61	54	55
MCH (pg)	28	44	45	32	58	39	61	58	51	59
MCHC (g/dl)	41	44	55	29.9	55	55	58	61	54	55
PLT (10^6/mm^3)	143	144	155	153	155	155	158	161	154	155
Lym # (10^3/µl)	0.7	0.4	0.45	0.35	0.58	0.6	0.61	0.58	0.55	0.54

Each value represents the average of 3 measurements ; RBC = red blood cell ; WBC = White Blood Cell ; Hb = Hemoglobin ; Hct = Hematocrit ; MGV = mean globular volume ; MCH = Mean Corpuscular Hemoglobin ; MCHC = Mean Corpuscular Hemoglobin Concentration, ; PLT = Platelet ; LYM = Lymphocytes.

These results corroborate those of Benhaddad et al in 2019, in a study of the effects of olive oil on oxidative stress parameters of Wistar rats rendered diabetic with streptozotocin, which showed macroscopic changes and atrophy in organ weights [26].

3.4.4 Biochemical parameters of rats

The determination of biochemical markers urea and creatinine of renal function as well as AST and ALT of hepatic function was carried out on Wistar rats for the subacute toxicity of the sample submitted for study. The wavelengths measured by the spectrophotometer were used to draw the Table 4 here represented.

Administration of the ethanol extract during the 28 days of treatment did not cause any significant change in serum urea, creatinine and ALT levels in the negative control and 200, 400 and 800 mg/kg body weight dose batches. However, the slightly elevated AST values could indicate the onset of toxicity. The urea analysis, in accordance with the 2 WAY ANOVA multi-comparative test of the Graphpad Prism software version 8.0.1, showed that the mean values of the concentrations of the test batches in comparison with the control batches were statistically insignificant with p-value > 0.05. In the males, the creatinine of the batch of rats at the dose of extract of 400 mg/kg was higher than those of the other batches while in the female rats, at the same dose of 400 mg/kg, the creatinine level was lower than that of the other batches. The creatinine analysis, in accordance with the 2 WAY ANOVA multi-comparative test of the Graphpad Prism version 8.0.1 software, showed that the mean values of the concentrations in comparison batch by batch had a non-significant statistical difference with p-values > 0.05. In both sexes, we observed the growth of AST in the test batches compared to the controls with a higher peak on the batch having received the extract at a dose of 400 mg/kg. The analysis of the AST, in accordance with the 2 WAY ANOVA multi-comparative test of the Graphpad Prism version 8.0.1 software, showed that the average values of the concentrations in comparison batch by batch had a statistically significant difference with p-values < 0.01. Non-considerable variations in ALT were observed in the males and cis-tooth regressions in the females, particularly more marked on the negative control and the batch having received the dose of 400 mg/kg. The ALT analysis, in accordance with the 2 WAY ANOVA multi-comparative test of the Graphpad Prism version 8.0.1 software, showed that the mean values of the concentrations in comparison batch by batch had a non-significant statistical difference in the two sexes of p-value > 0.05. With the exception of AST, the results for urea, creatinine and ALT are similar to those of Etame et al in 2017 [27].

3.4.5 Hematological parameters of rats

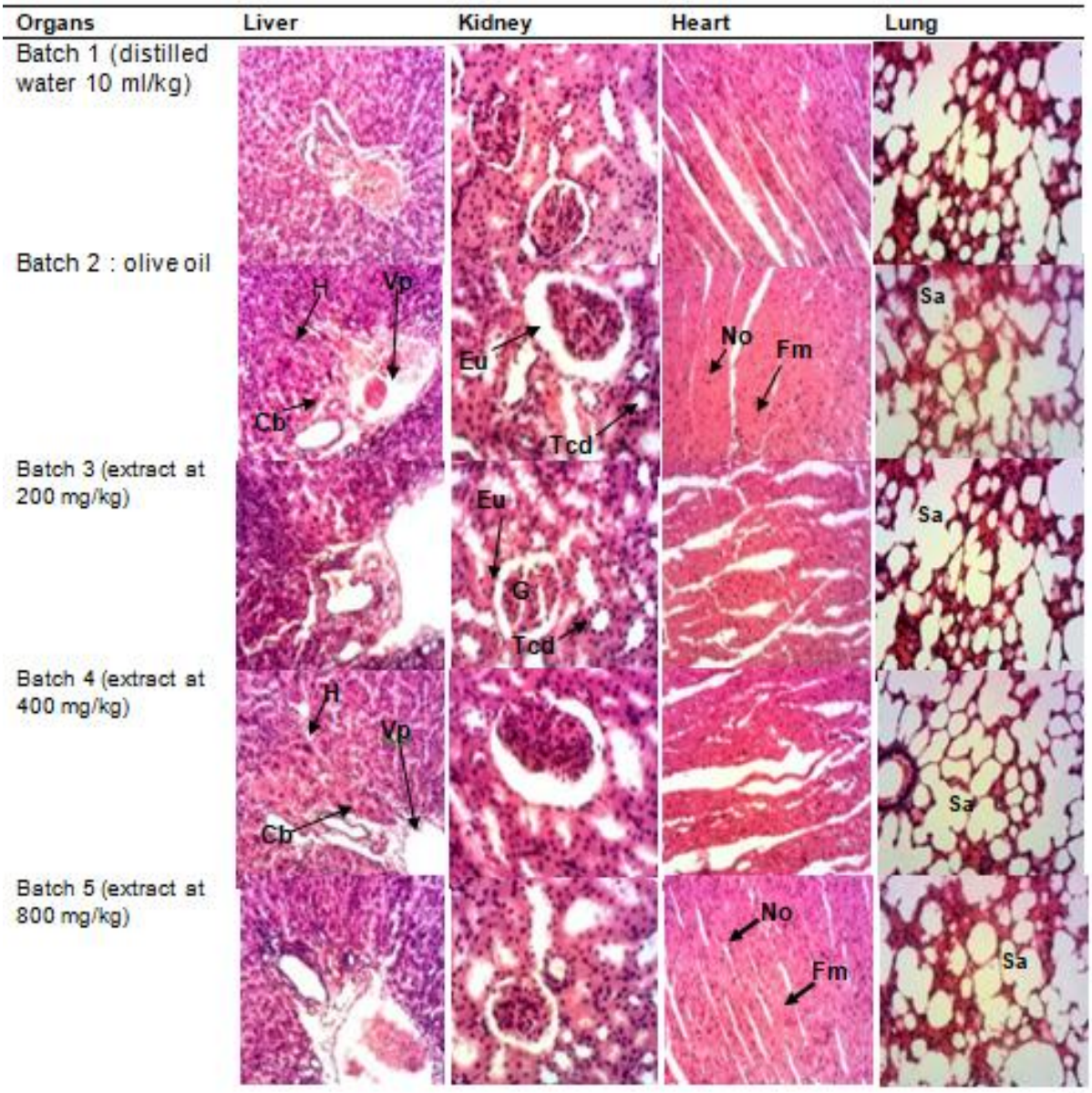
Analysis of the hematological parameters of the rats' (Table 5) showed that there was no change in the level of red blood cells between the male animals treated with the plant extract and the controls serving as controls. In females, the values were higher by 1 to 2 x 106/mm^3 compared to controls. Also, no significant difference was observed in mean corpuscular volume (MCV) and hematocrit (Hct) between these different groups. Hemoglobin (Hb) concentration (g/dl), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin concentration (MCHC) did not vary significantly between the extract-treated groups and the group control. Similarly, the white blood cell (WBC) per liter of blood, the lymphocyte rate (LYM) and the number of platelets (PLT) did not undergo a significant variation between the tested animals, compared to the control animals.

3.4.6 Histological parameters of rats

Analysis of the histological parameters of the females rats' (Table 6) showed a normal overall architecture of the liver (hepatic parenchyma with a centrilobular vein and clearly distinct hepatocytes), kidney (normal parenchyma with a clearly distinct glomerulus and urinary space), heart (muscle fibers and clearly distinct nuclei) and lung (pulmonary epithelium, alveolar sac). No signs of significant alterations in its organs were observed.

Similarly for the male organs (Table 7), we noted the same observations, i.e. a normal overall architecture of the liver (hepatic parenchyma with a central-lobular vein and very distinct hepatocytes), of the kidney (normal parenchyma with a glomerulus and a distinct urinary space), the heart (muscle fibers and distinct nuclei) and the lung (pulmonary epithelium, alveolar sac). No signs of significant alterations in his organs were observed.

Table 6. Micrographs of liver (X100), kidney (X200), heart (X100), and lung (X100) of female rats; Hematoxylin-eosin staining



Liver: Vp = hepatic portal vein; He = Hepatocyte; Cs = Capillary sinusoid; Cb = Bile canaliculus; Il = Leukocyte infiltration; Heart: No = Nucleus of cardiac muscle fibers; Fm = cardiac muscle fibre; Kidneys: G = Glomerulus; Eu = Urinary space; Tcd = distal convoluted tubule; Lung: Sa = alveolar sac

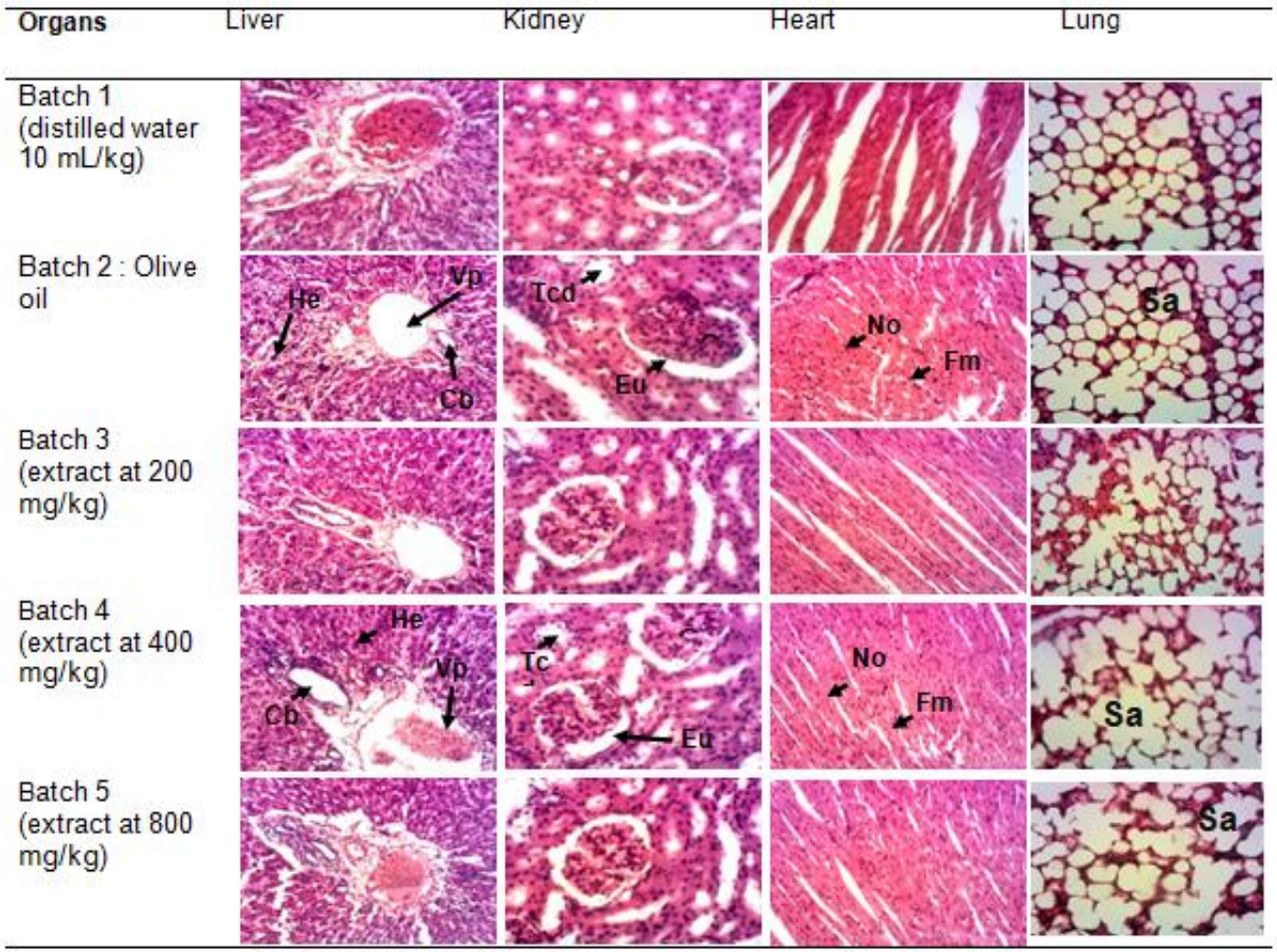
No sign of significant alterations in tests rats organs was observed, which is close to the results of Etame-Loe et al [2].

3.5 Antimicrobial Activity

The activity of the extract on bacteria and fungi related to the infections traditionally treated by the stem bark of *A. mannii* allowed us to make the microbiological control of the samples and to determine the minimum inhibitory and microbicidal concentrations.

The microbiological control allowed us to establishing the sterility of the extract after inoculation of the sample in EMB, Sabouraud, Muller hinton, Chapman, Hektoen and blood agar media. No growth was observed after 48 H of incubation. Determination of the minimum inhibitory and microbicidal concentrations resulted in Table 8 shown here.

Table 7. Micrographs of liver (X100), kidney (X200), heart (X100), and lung (X100) of male rats; Hematoxylin-eosin staining



Liver: Vp = hepatic portal vein; He = Hepatocyte; Cs = Capillary sinusoid; Cb = Bile canaliculus; Il = Leukocyte infiltration; Heart: No = Nucleus of cardiac muscle fibers; Fm = cardiac muscle fibre; Kidneys: G = Glomerulus; Eu = Urinary space; Tcd = distal convoluted tubule; Lung: Sa = alveolar sac

Table 8. Antimicrobial activity of ethanolic crude extract of the stem bark of *Anonidium mannii*

Sample	*N. gonorheae*			*E. coli*			*Salmonella spp*			*C. albicans*		
	MIC	**MMC**	**MMC/MIC**	**MIC**	**MMC**	**MMC/MIC**	**MIC**	**MMC**	**MMC/MIC**	**MIC**	**MMC**	**MMC/MIC**
Extract	15.6	62.5	4.006	62.5	> 62.5	/	15.6	**>** 15.6	/	250	> 250	/
References[a]												
Ceftriaxone	0.002	0.008	4	4	4	1	4	8	2			
Fluconazole										> 1000	/	/

/. Not determined because the sample was not soluble or not active by the disc diffusion test. MIC: minimum inhibitory concentration (µg/ml); MMC: minimum microbicidal concentration (µg/ml). [a]Reference antibiotics (ceftriaxone for bacteria and fluconazole for fungi)

The antibacterial and antifungal activities of the extract were performed in liquid medium by the microdilution method on three bacterial strains (*N. gonorheae, E. coli* and *Salmonella spp*) and one fungal strain (*C. albicans*), and we obtained MICs of 15.6; 62.5 and 15.6 µg/mL respectively for the bacteria and 250 µg/ml for the fungus.

According to the study conducted by Kuete in 2010 [28], "the activity of an extract was considered significant if the MIC values were below 100 µg/ml, moderate if between 100 and 625 µg/ml and low for values higher than 625 µg/ml". Accordingly, the extract had strong antibacterial activity on *N. gonorheae, E. coli* and *Salmonella spp* and moderate on *C. albicans*. According to the scale of Tabouguia et al. in 2017, the MMC/MIC ratio of extract tested against *N. gonorheae* was greater than 4, so the extract can be considered bactericidal [29]. In our study, this ratio was not determined due to the MMC which was not obtained.

These results corroborate those of Traoré et al in 2012 [30] and Adesokan et al in 2008 [31] who showed that the aqueous extract of the leaves of *Annona senegalensis* and the ethanolic extract of the stem bark of *Annickia chlorantha*, which bollonguing to the same family as *Anonidium mannii* (Annonaceae), possessed strong antimicrobial activities on *Salmonella spp, E. coli* and *C. albicans*

4. CONCLUSION

At the end of our study, the phytochemical screening revealed the presence of alkaloids, anthraquinones, coumarins, flavonoids, phenols, reducing sugars, tannins, triterpenes and the absence of saponins and steroids. The acute toxicity test of the extract administered at single doses to animal populations allowed us to determine the LD_{50} which was between 5000 and 15000 mg/kg body weight, indicating that the extract was almost non-toxic. The extract showed good safety with respect to subacute toxicity. No deaths were recorded at the different doses. However, we noted that the olive oil that was used as a solvent for dissolving the extract contributed to slowing down of the growth of the rats. All bacterial strains in the presence of the extract were inhibited, as well as *C. albicans*. All the observations and results obtained, allowed us to think that the ethanolic extract of the stem bark of *Anonidium mannii* has a very interesting antibacterial and antifungal activity on *N. gonorrheae, E. coli, Salmonella spp* and *C. albicans* and presents quasi-harmlessness for its medium term use by oral route. It is therefore of good quality for the formulation of improved traditional drugs with antimicrobial properties. This is the first report on acute, sub-acute toxicities and antimicrobial activities of the stem bark of *A. mannii.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors declare that 'ethical clearance was obtained from the Institutional Ethics Committee of the University of Douala for the conduct of this study and for the publication of this article'. All experiments were reviewed and approved.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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