Chemical identification, antimicrobial activity, acute and subacute toxicity of the leaves of *Combretum grandiflorum* antidiarrheal plant

Abstract

*Combretum grandiflorum*G. Don is traditionally used in ethnic medicines for the treatment of different diseases in Benin. Despite its widelyuse, no studies have been undertaken on its potential toxicity.The study was designed to assessthe antimicrobial activity and potential toxicity of aqueous and hydroethanolic extracts of *C. grandiflorum*.
Qualitative phytochemistry was carried out by a differential staining or precipitation method. The antibacterial and antifungal effects were evaluated by the solid medium diffusion method. Cytotoxicity was evaluated on *Artemia salina* larvae while acute toxicity was evaluated with Wistar strain rats via the oral route.The qualitative screening revealed the presence in the plants of Polyphenols, Flavonoids, Tannins, catechicand gallic tannins. Both types of extracts inhibited microbial growth. The hydroethanolic extract was active on all the bacterial strains used with a high inhibition diameter compared to the aqueous extract. On strains of *Shigella Sonnei* and *Salmonella spp*, the antibacterial effect of the hydroethanolic extract is significantly ($p<0.05$) better compared to that of the aqueous extract. Cytotoxicity tests showed no toxic effects at the doses evaluated. At a dose of 2000 mg/kg, the aqueous and hydroethanolic extracts of *C. grandiflorum* induced neither mortality nor alteration of the physiological parameters of Wistar rats (renal, hepatic parameters, hematological constants, body weight). These extracts therefore do not present acute oral toxicity for Wistar rats at the limit dose of 2000 mg/Kg/bw in Wistar rats.These results justify sometherapeutic indications for these plants. They could therefore be used for the development of improved phytomedicines.

Key words: *Combretum grandiflorum;*Benin; antimicrobial activity;Cytotoxicity;acute toxicity

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Introduction

Early civilizations relied on herbal and traditional medicinal practices to treat their health problems [1]. The plants are used in developing African countries, people from disadvantaged and resource-poor communities as traditional medicine [2,3]. In cause, some modern synthetic drugs cause unwanted side effects [4, 5], while others have low potency against certain pathogens [6, 7]. Additionally, indiscriminate use of antibiotics has led many bacterial strains to develop resistance to synthetic antimicrobial agents [6, 7, 8]. Therefore, alternative sources of pharmacologically active compounds are required to effectively treat diseases, leading to intensified research on medicinal plant species [9]. Plants contain a wide range of bioactive phytocompounds [10] that have been used in medical treatment since historical times [11]. Several plant species from the pantropical medicinal family, like Combretaceae, have been reported to be used in traditional African medicinal practices to relieve symptoms and treat diseases [12, 13]. Phytochemical studies carried out in the genus Combretum have demonstrated the presence of numerous classes of constituents, including triterpenes, flavonoids, lignans and non-protein amino acids, among others [21]. The properties of many

phenolic constituents of medicinal plants, such as their ability to inhibit enteropoolation and delay gastrointestinal transit, are very useful in the control of diarrhea [22]. For example, Combretum species have been reported to be widely used in traditional medicine for diarrhea and digestive disorders [22]. *Combretum micranthum*G.Don and *Combretum adenogonium*Steud. ex A.Rich. extracts have been reported to have antibacterial activity against antibiotic-resistant diarrheal strains [14]. Diarrheal diseases are among the deadliest infectious diseases, especially among children. In fact, every year there are an estimated 2.5 billion cases of diarrhea in children under five [15]. Ugboko et al.[16] reported that childhood diarrhea affecting children five years and younger accounts for approximately 63% of the global diarrhea burden. 15% of these children die [17].

Benin is one of the developing countries in which diarrheal diseases are among main causes of morbidity [18, 19]. Indeed, they have a direct impact on the costs associated with seeking health care, including several factors such as consultation, medications and, in certain cases, hospitalization which represent a burden on household expenses [19]. The pathogens of diarrheal diseases are mainly bacteria [20]. Over the years, medical therapy based on the use of conventional antibiotics has shown not only its effectiveness but also its limits. Indeed, most of the bacteria responsible for diarrheal episodes develop resistance to the antibiotics used in therapy.
To address this problem, the World Health Organization has supported the idea of diarrheal disease control based on traditional medicine practices and prevention approaches [19]. Indeed, for the proper use of antidiarrheal plants it is important to check their safety. It is therefore important, even essential, to explore the toxicological characteristics of the main plants used by local communities in several African pharmacopoeias in order to secure their use in traditional medicine. It is with this in mind that the paper aim to assess biological activity of *C. grandiflorum* extracts.

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Methods

Preparation of crudes extracts

According to the method described by Klotoé et al. [23], 50 g of powdered leaves and bark of *Combretum grandiflorum*G. Don were macerated each one for 72 h at room temperature in 500 ml of each solvent (distilled water for the aqueous extract and distilled water/ethanol in a proportion 40:60 for the hydro-ethanol extract). The homogenate obtained was filtered three times through hydrophilic cotton and once through Wattman N 1 paper. This filtrate was then dried at 40°C in an oven for the aqueous extract and concentrated in a rotavapor for the hydroethanol extract. The concentrate was placed in the oven at 40°C until complete evaporation. The powder obtained corresponded to the extracts. The obtained extract was weighed in order to evaluate the extraction yield and then stored in the refrigerator at 4°C. The yield (Y) of the crude extract defined as the ratio between the mass of the dry extract obtained and the mass of the treated plant material was calculated by the following formula

$$\mathbf{R} = \frac{\textbf{weight of extract after evaporation}}{\textbf{weight of plant powder used for extration}} \times \mathbf{100}$$

Phytochemical screening:

Phytochemical screening to highlightsecondary metabolites of leaves and bark of *Combretum grandiflorum*G. Don was carried out according to Houghton and Rahman [24].

Antimicrobial activity of *Combretum grandiflorum*G. Don extractson tested microorganisms.

Diarrheal reference strains such as *Escherichia coli* ATCC 12386, *Salmonella Typhi* ATCC 14028 and clinical strains of *Shigella Sonnei* and *Salmonella spp* were used for the antibacterial activity test. . The antifungal activity was carried out on a clinical strain of *Candida albicans*.

Sensitivity test

Thesensitivity test was doneby the disk diffusion method inspired by that described by Lègba*et al*. [25]. Indeed, 1 ml of bacterial culture (adjusted to 0.5 McFarland standard) was used to flood a Petri dish containing Mueller-Hinton agar (Bio-Rad, France). Two to four sterile discs (6 mm) are placed in the Petri dish previously flooded with bacterial culture under aseptic conditions. This disk is inoculated with 50 µl of the tested extracts (100 mg/ml). For each extract, the experiment is duplicated, and a negative control is carried out with the solvent instead of the extract. The plates are then maintained at room temperature for 15-30 min before being incubated at 37°C for 24 h and 48 h. Inhibition diameters were measured using a scale after incubation times of 24 h and 48 h.
The antifungal activity of the active extracts was determined by the solid medium diffusion method according to the method of Alsubhi*et al*. [26]. For the antifungal test itself, Potatose Dextrose Agar (PDA) agar plates were inoculated with the fungal suspension prepared for each type of extract. Wells of 7 mm in diameter were then made sterilely in the agar media using a Pasteur pipette. One hundred (100) µl of each extract (aqueous or hydroethanolic extracts) were placed in the wells. Distilled water was used as a negative control and fluconazole as a positive control. The boxes were left on the bench for two hours for pre-diffusion before being incubated at 37°C for 24 hours. The inhibition diameters observed around the wells after incubation were then measured using a double-decimeter for each type of extract studied. The most active extract corresponds to the one having presented the greatest diameter of inhibition.

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

The Minimums Inhibitory and Bactericidal Concentrations

The Concentrations were determined by the micro dilution test using microplates as described by Lègba*et al*. [25]. To achieve this, a mainsolution of plant extract was setat a concentration of 200 mg/mL of sterile distilled water. In a microplate, 100 µL of Mueller Hinton Broth (MHB) medium was placed in each well. Serial dilution was carried out with the mainsolution from the first well to the ninth well. One hundred (100) µL of the bacterial suspension were deposited in each well. The final concentrations tested vary from 50 mg/mL to 0.19 mg/mL. A positive control and a negative control were added. After determining the MIC using tetrazolium as an indicator of microbial growth, the wells were inoculated on MH agar to determine the CMBs. The CMB corresponds to the small concentration for which there was no bacterial growth.

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Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of extracts.

The Minimum Inhibitory Concentration (MIC) was determined in liquid medium according to the technique of Lavaee et al.[27]. The tests were carried out on successive dilutions of extract prepared at 200 mg/mL. The final concentrations tested vary from 50 mg/mL to 0.19 mg/mL. After the determination of the MIC using tetrazolium as an indicator of microbial growth, the wells were inoculated on PDA agar to determine the MFCs. The CMF corresponds to the small concentration for which there was no fungal growth.

Determination of the antibiotic/antifungal power of active extracts on the strains tested

After determining the MIC, CMB/CMF, the antibiotic/antifungal power of the extracts was determined. For this purpose, the CMB/CMI or CMF/CMI formula was applied to calculate the antibiotic or antifungal power (p.a.) of each extract used.
The results obtained were compared to the standard used to evaluate the antimicrobial activity of plant extracts [28.

Toxicity tests of extracts
Larval cytotoxicity

The cytotoxic effect of the extracted plant was evaluated following an adaptation of the method used by Legba et al. [25]. A serial dilution of 2 in 2 was carried out from 1 mL of the mainsolution of plant extract prepared at 20 mg/mL in 10 tubes. The lethal concentration 50 (LC_{50}) was determined. The standards used to assessthe cytotoxic effect of plants are presented in Table 1.

Table 1. Correspondence between LC50 and toxicity [29].

Valeur de la LC_{50}	**Cytotoxicité de l'extrait**
$LC_{50} \geq$ 0,1 mg/mL	Non toxic
0,1 mg/mL > $LC_{50} \geq$ 0,050 mg/mL	lowtoxicity
0,050 mg/mL > $LC_{50} \geq$ 0,01 mg/mL	Mediumtoxicity
LC_{50} < 0,01 mg/mL	High toxicity

1.1.1 **Acute oral toxicity test**
The acute oral toxicity of the two extracts was explored following the standard protocol for assessing the acute oral toxicity of chemical and natural products of the Organization for Economic Cooperation and Development [30]. Following administration of the extracts, clinical signs of toxicity and the influence of these extracts on hematological, biochemical renal and hepatic parameters were evaluated.

Animal material and batch constitution

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The *in vivo* toxicity study was carried out on nulliparous and non-pregnant female Wistar albino rats weighing on average 120 g and coming from the animal facility of the Applied Microbiology and Pharmacology of Natural Substances Research Unit (URMAPha). Rats were maintained, with free access to standard food and water, under standard conditions (12 h light/12 h dark at 22 ± 2°C).

Administration of extracts

The rats divided into three groups andacclimated were subjected to the administration of the extracts. The plant extracts to be tested being administered orally, the method described in OECD guideline 423, method by toxicity class, was adopted. Given that,*Combretum grandiflorum* plant that we want to test is commonly used by the population and that no toxic effects have been reported, a limit toxicity test, i.e. a single dose of 2000 mg/kg of body weight, was carried out. Twelve hours before carrying out this toxicity test, the rats were deprived of food and water. At the start of the experiment (D0), a blood sample was taken from all rats at the level of the retro-orbital sinus for analyzes of hematological and biochemical constants before administration of the product. Then, the extracts were administered by esophageal gavage in accordance with the selected dose. After administration of the product, a period of 14 days was observed to collect data on clinical signs of toxicity (morbidity, mortality, hair loss, eye color, breathing difficulties, etc.)
At the end of the experiment (D14), a blood sample was taken for analyzes of hematological and biochemical constants.

1.1.2 Sub-acute oral toxicity test

The study was performed according to OECD guideline 407 [31], method by sub-acute oral toxicity class. It was carried out on 30 Wistar rats (15 male rats and 15 female rats) weighing on average 150 g divided according to their weight into six (06) batches of five rats each. Two types of batches were formed: 2 test batches and 1 control batch per sex (Table III). Rats from the test batches were treated with the aqueous and hydroethanolic extracts at the same time (10 hours) for a period of 28 days by esophageal gavage at a single dose of 1000 mg/kg/bw. The control batch is treated with distilled water under the same conditions. The rats from each batch were marked and monitored individually throughout the period of the experiment.

Table 2 Constitution and processing of batches for the sub-acute oral toxicity test

Lot	Type of lot	Number of rats	Substance administered	Dose	Sex
1	Male witness	5	distilled water	1 mL/100g	Male
2	Test	5	aqueousextract	1000 mg/kg	Male
3	Test	5	hydroethanolicextract	1000 mg/kg	Male
4	Test	5	aqueousextract	1000 mg/kg	Female
5	Test	5	hydroethanolicextract	1000 mg/kg	Female
6	femalewitness	5	distilled water	1 mL/100g	Female

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The animals had free access to water and food. During the experimental period, the animals were weighed every seven days (D0; D7; D14; D21, D28) and monitored individually twice a day (11 a.m. and 5 p.m.). An information collection sheet was drawn up for each rat in order to collect possible signs of toxicity (changes in the skin, hair, eyes, appearance of edema, walking backwards, breathing difficulties, morbidity, mortality). At the end of the treatment, the rats were deprived of food the last night before sampling. Blood samples from animals anesthetized with thiopental (60 mg/kg) were taken by puncture from the retroorbital sinus on D29 as well as on D0. The blood sample is collected in two types of tubes, one containing EDTA and the other without anticoagulant (dry tube). The EDTA tube sample was intended for hematological analyses. The dry tubes are centrifuged at 4000 Rpm for 10 min and the serum obtained was used to determine the biochemical parameters. The hematological examinations were carried out using a SYSMEX KX 21N automated system according to the method used by Sodipo et al. [32]. These examinations included the counting of red blood cells and white blood cells, the determination of the hemoglobin level, the hematocrit, the Mean Globular Volume (MCV), the Mean Corpuscular Hemoglobin Content (TCMH) and the Mean Corpuscular Concentration. in hemoglobin (CCMH). The blood biochemical parameters of urea, creatinine and transaminases were quantified.

Data analysis

The data generated from the tests performed were subjected to statistical analysis using SPSS 26.0 software. Quantitative variables are presented as mean and standard deviation. Analysis by the probit method was used to determine the average lethal concentration for the larval cytotoxicity test.
Concerning the acute and sub-acute oral toxicity of the extracts, the Student's t test of paired samples and independent samples was used to compare the mean of the different parameters of the test and control batches. Analysis of variance (ANOVA)was used to compare the mean of the different parameters of the test and control batches on D14 and D28.
ANOVA was also used to compare the data of the different parameters of the efficacy tests of the antidiarrheal effect of plant extracts. The significance threshold was set at 5%.
Concerning the antimicrobial power of the extracts, the student t test was used to compare the effect of the two active extracts on the same strain.

Results
Extraction yield
The extraction yield of the hydroethanolic extract obtained is high compared to that of the aqueous extract.

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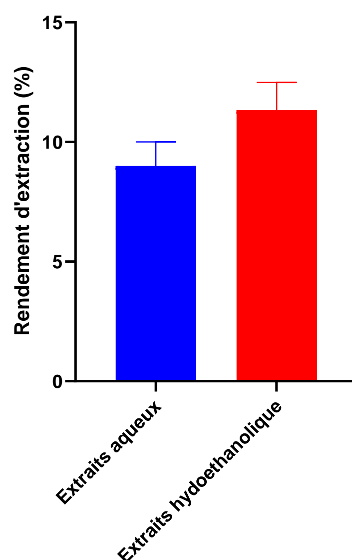

Figure 1: Extraction yield

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Phytochemical screening: Identification of alkaloids and polyphenols in *Combretum grandiflorum* extracts

It is worth noting the presence of polyphenols, tannins, gallic tannins, catechin tannins and flavonoids in the extracts tested (Table 3). Alkaloids and Leuco-anthocyanins are absent in the tested extracts.

Table 3. Highlighting alkaloids and polyphenols in *Combretum grandiflorum* extracts

Extracts/ chemical groups	aqueousextract	hydroethanolicextract
Polyphenols	+	+
Flavonoids	+	+
Tanins	+	+
Catechic tanins	+	+
gallictanins	+	+
Anthocyanes	-	+
Leuco-anthocyanes	-	-
Alcaloïdes	-	-

+: presence; -: Absence

2.4 Antimicrobial assay
2.4.1 Antibacterial activity of extracts

The hydroethanolic extract was active on all the bacterial strains used with a high inhibition diameter compared to the extract aqueous (Figure 2). On strains of *Shigella Sonnei* and *Salmonella spp*, the antibacterial effect of the hydroethanolic extract is significantly ($p<0.05$) better compared to that of the aqueous extract. The aqueous extract produced no antibacterial effectagainst *E. coli* and *Salmonella Typhi*. Among allstrains tested, the largest inhibition diameter was obtained with the hydroethanolic extract on *Salmonella Typhi* strain (12 mm ± 1).

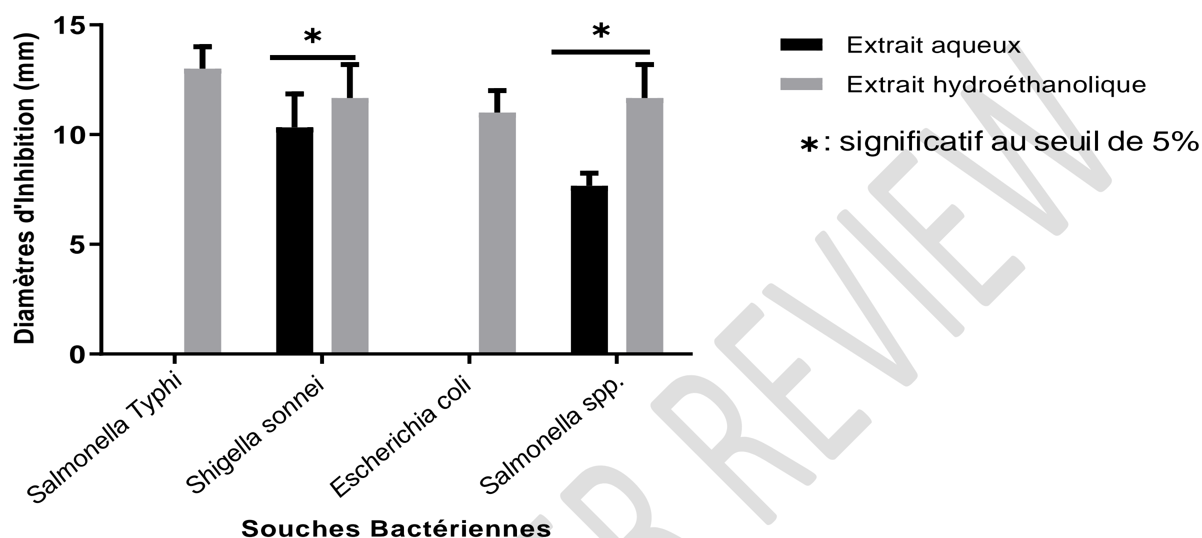

Figure 2: Inhibition diameters of aqueous and hydroethanolic extracts on the bacterial strains tested

Regarding the MIC, CMB and P.a, it appears that the hydro-ethanolic extract had bactericidal power on all strains while the aqueous extract had bacteriostatic power on *Shigella Sonnei* and *Salmonella spp* (Table 4).

Table 4. MIC, CMB and P.a of the extracts studied

Extraits	Paramètres	*E. coli* ATCC 12386	*S. sonnei*	*S.* Typhi ATCC 14028	*Salmonella* spp
aqueousextract	CMI	-	3,12	-	12,5
	CMB	-	12,5	-	50
	P.a	-	**4**	-	**4**
Extrait hydro-éthanolique	CMI	6,25	3,12	1,56	6,25
	CMB	12,5	6,25	3,12	12,5
	P.a	**1**	**1**	**1**	**1**

2.4.1 **Antifungal activity of extracts**

The two extracts were active on the said strain used. The inhibition diameter obtained for the aqueous extract was 12 mm ± 0.66 and that of the hydroethanolic extract was 20 mm ± 0.33.

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The antibiotic power of the hydroethanolic extract was lowerthan that of the aqueous extract (Table 5).

Table 5. Antifungal activity, MIC, CMF and antibiotic power of extracts on Candida albicans

Extraits	**Paramètres**	***Candida albicans***
Extrait aqueux	Diamètre d'inhibition (mm)	12 ± 0,66
hydroethanolicextract	Diamètre d'inhibition (mm)	20 ± 0,33
aqueousextract	CMI	3,12
	CMF	12,5
	P.a	**4**
HydroethanolicExtract	CMI	1,56
	CMF	3,12
	P.a	**1**

Toxicity assayof extracts

Larval cytotoxicity

The LC_{50} obtained is respectively 3.33 mg/mL for the aqueous extract (Figure 3) and 0.420 mg/mL for the hydroethanolic extract (Figure 4). These LC_{50} values reported on the Mousseux scale allow us to conclude that the products tested are not cytotoxic (because $LC_{50} \geq 0.1$ mg/mL) at the concentrations tested.

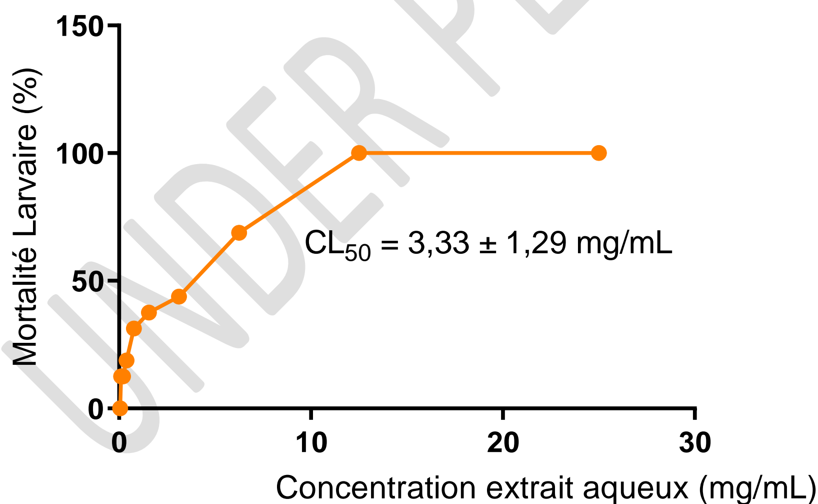

Figure 3: Sensitivity of *Artemia salina* larvae to the aqueous extract of *Combretum grandiflorum*

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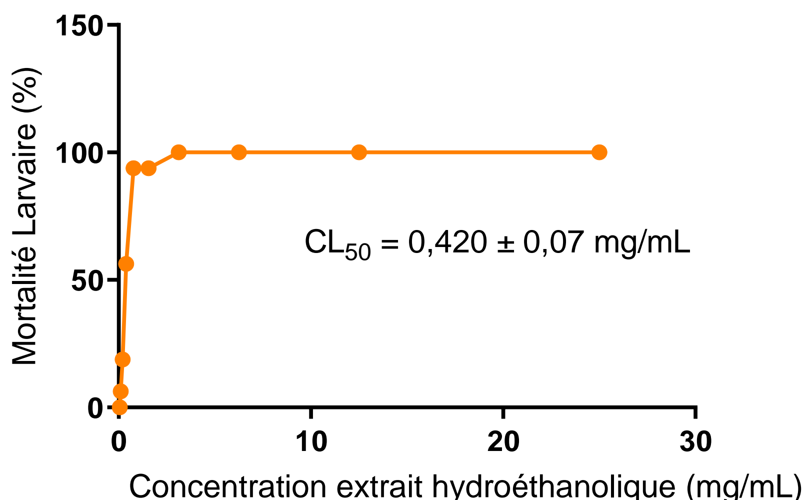

Figure 4: Sensitivity of *Artemia salina* larvae to the hydroethanolic extract of Combretum grandiflorum

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Acute oral toxicity

Mortality

At the end of the 14 days of experimentation, no mortality was observed for the rats for both test and lot of control. Likewise, no clinical signs of toxicity were observed. Oral administration of the tested extracts therefore did not induce rat mortality. Their average lethal dose (LD_{50}) is therefore greater than 2000 mg/kg/BW.

Influence of the tested extracts on the weight evolution of Wistar rats

All treatedWistar rats with extracts showed a significant increase in their body weight during the 14 days of treatment ($p<0.05$) (Table 6). However, no significant difference was noted for the weight gain of rats from the tested groups compared to rats from the control group ($p>0.05$).

Table 6. Effect of products on the weight load of experimental rats

Lot		average(g)	**Test t Student**matched sample of mean cut-off weight at **5%**	**Weightincresed**	**Test t Studentmatchedsample of meancut-off weight at 5%**
WitnessLot	J0	113±16,64	No signicativedifference	5 ± 5	
	J14	118±12,12			
Lot Extrait aqueux	J0	123,67±2,08	significantdifference	7,66 ± 2,88	No signicativedifferencewith the witness lot.
	J14	131,33±2,08			

Lot hydroéthanolique	J0	107,67±4,16	significantdifference	11,33 ± 1,52	noNosignicativedifferencewith the witness lot.
	J14	119±4,35			

- Influence of the tested extracts on the hematological parameters

It appears that the treatments carried out had no significant effect (p>0.05) on the constants of the hematological parameters between Day and D14 and between the lots on D14(Table 7). This allows us to conclude that the treatments carried out did not induce anemia in the experimental rats.

Table 7. Effect of treatments on hematological parameters of experimental rats

Lots	parameters	N	average	Standard deviation	Paired sample Student's t test at the 5% threshold	Independent sample student t test between lot on D14 at the thresholdof 5%
witness	GB J0	3	7,50	0,66	No significantdifference	No significant difference for each of the parameters following the comparison between each of the test lot and the witness lot on D14
	GB J14	3	8,57	2,24		
	GR J0	3	4,60	2,93	No significantdifference	
	GR J14	3	6,29	0,22		
	HB J0	3	9,57	6,14	No significantdifference	
	HB J14	3	13,67	0,21		
	HCT J0	3	25,30	16,24	No significantdifference	
	HCT J14	3	34,23	0,38		
	VGM J0	3	54,57	2,63	No significantdifference	
	VGM J14	3	54,50	2,01		
	MCH J0	3	20,70	0,36	No significantdifference	
	MCH J14	3	21,70	0,98		
	CCMH JO	3	38,00	2,29	No significantdifference	
	CCMH J14	3	39,87	0,42		
	PLT J0	3	765,67	15,18	No significantdifference	
	PLT J14	3	767,00	28,48		
Lotof aqueousextract	GB J0	3	7,10	0,50	No significantdifference	
	GB J14	3	6,67	0,51		
	GR J0	3	6,75	0,80	No significantdifference	
	GR J14	3	6,37	0,49		
	HB J0	3	14,53	1,76	No significantdifference	
	HB J14	3	14,13	0,70		
	HCT J0	3	38,07	3,30	No significantdifference	
	HCT J14	3	36,87	2,37		
	VGM J0	3	56,47	1,91	Aucune différence significative	
	VGM J14	3	57,97	1,22		
	MCH J0	3	21,53	0,78	No significantdifference	
	MCH J14	3	22,23	0,61		

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	CCMH JO	3	38,13	1,85	No significantdifference	
	CCMH J14	3	38,37	0,68		
	PLT J0	3	759,67	15,89	No significantdifference	
	PLT J14	3	723,00	13,45		
LOT of HYDROETHANOLIC EXTRACT	GB J0	3	8,40	0,87	No significantdifference	
	GB J14	3	8,33	1,21		
	GR J0	3	6,63	0,70	No significantdifference	
	GR J14	3	6,40	0,05		
	HB J0	3	17,67	5,17	No significantdifference	
	HB J14	3	14,67	0,21		
	HCT J0	3	43,80	11,96	No significantdifference	
	HCT J14	3	35,73	0,75		
	VGM J0	3	52,47	3,25	No significantdifference	
	VGM J14	3	55,87	1,46		
	MCH J0	3	21,03	1,19	No significantdifference	
	MCH J14	3	22,90	0,40		
	CCMH JO	3	40,13	0,96	No significantdifference	
	CCMH J14	3	41,00	1,42		
	PLT J0	3	761,00	30,64	No significantdifference	
	PLT J14	3	779,67	7,02		

Legend: GB: White blood cell; GR: Red blood cell; HGB: Hemoglobin; MCV: Mean Globular Volume; HCT: Hematocrit; MCH: Mean corpuscular hemoglobin; CCMH: Mean Corpuscular Hemoglobin Concentration; PLT: Blood platelet; D0: Day zero (Start of the experiment); D14: Day fourteen (End of the experiment); N: number of rats

- Influence of the tested extracts on the biochemical parameters of Wistar rats

At the threshold of 5%, no significant influence (p>0.05) favorable to an impairment of liver functions and renal was noted for the biochemical parameters of the kidneys (urea and creatinine) and those of the liver (ALT, AST) (table 8).

Table 8. Effect of treatments on biochemical parameters of experimental rats

Lots	**Parameters**	**number of rats**	**average**	**standarddeviation**	Paired sample Student's t test at the 5% threshold	ANOVA Test between l on D14 a the threshol of 5%
witness	Uremia (mg/dl) J0	3	**0,66**	**0,09**	No	No

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	Uremia (mg/dl) J14	3	**0,64**	**0,02**	significantdifference	significan differenc for each o the paramete following the comparis between the lot
	Creatinemia (mg/dl) J0	3	**5,37**	**0,54**	No significantdifference	
	Creatinemia (mg/dl) J14	3	**6,67**	**1,14**		
	AST/GOT (U/L) J0	3	**82,67**	**2,52**	No significantdifference	
	AST/GOT (U/L) J14	3	**83,12**	**0,93**		
	ALT/GTP (U/L) J0	3	**45,61**	**1,59**	No significantdifference	
	ALT/GTP (U/L) J14	3	**46,50**	**1,44**		
Lot ofaqueousextract	Uremia (mg/dl) J0	3	**0,72**	**0,12**	No significantdifference	
	Uremia (mg/dl) J14	3	**0,63**	**0,03**		
	Creatinemia (mg/dl) J0	3	**6,33**	**0,55**	No significantdifference	
	Creatinemia (mg/dl) J14	3	**6,62**	**0,84**		
	AST/GOT (U/L) J0	3	**81,00**	**13,23**	No significantdifference	
	AST/GOT (U/L) J14	3	**82,83**	**3,90**		
	ALT/GTP (U/L) J0	3	**46,40**	**1,54**	No significantdifference	
	ALT/GTP (U/L) J14	3	**46,12**	**2,58**		
Lot ofhydroethanolicextract	Uremia (mg/dl) J0	3	**0,75**	**0,16**	No significantdifference	
	Uremia (mg/dl) J14	3	**0,68**	**0,04**		
	Creatinemia (mg/dl) J0	3	**4,96**	**1,13**	No significantdifference	
	Creatinemia (mg/dl) J14	3	**5,89**	**0,55**		
	AST/GOT (U/L) J0	3	**85,33**	**8,02**	No significantdifference	
	AST/GOT (U/L) J14	3	**76,84**	**6,60**		
	ALT/GTP (U/L) J0	3	**46,43**	**2,95**	No significantdifference	
	ALT/GTP (U/L) J14	3	**46,37**	**1,51**		

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Sub-acute oral toxicity

- Influence of treatments on the weight growth of female rats.

A weight growth of the rats was noticed in all batches(Figure 5) .

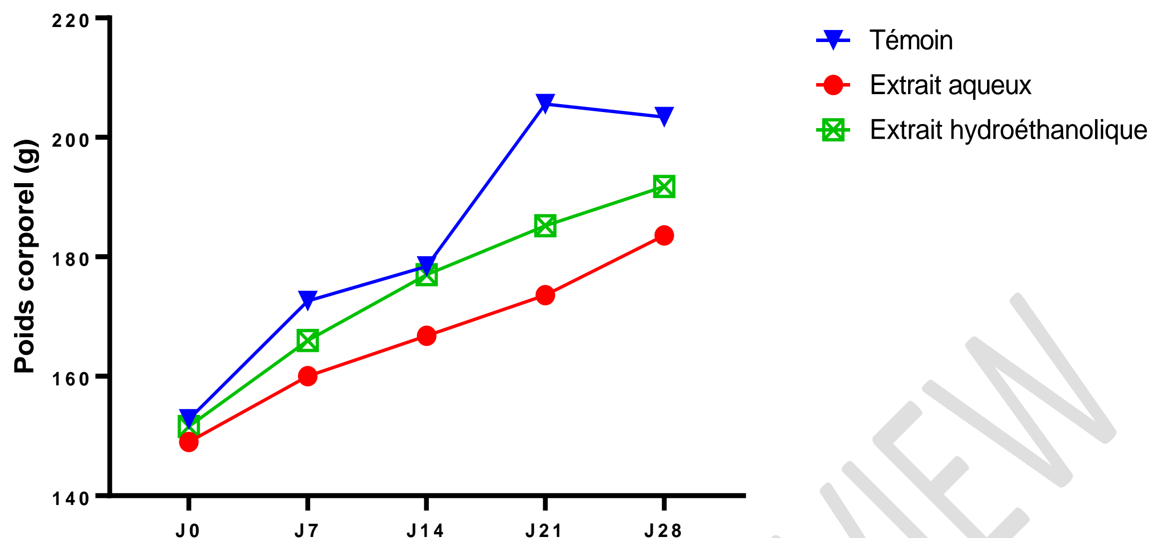

Figure 5: Evolution of the body weight of female rats during the experiment

There is no significant difference in weight growth of rats from the test batches compared to the control batch seven days after treatment. However, from D14 (aqueous extract) and D21 (hydroethanolic extract), the weight growth of rats from the control group is significantly better compared to the test groups (Table 9).

Table 9. Weight gain of experimental female rats from different batches

Lots	witness	aqueousextract	hydroethanolicextract	Comparisonbetweenwitness and test lot of aqueousextract	Compari and test l hydroeth
Gain J7	19,8±4,76	11,00±8,94	14,40±6,42	No significantdifference(P= 0,08)	significar
Gain J14	25,60±4,03	17,80*±5,89	25,4±8,6	significantdifference(P=0,04)	significar
Gain J21	52,8±11,38	24,6*±9,23	33,6*±8,08	significantdifference(P=0,003)	significar

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Gain J28	50,60±8,44	34,6*±10,31	40,2*±4,8	significantdifference(P=0,028)	significa

- Influence of treatments on the weight growth of experimental male rats

Figure 6 presents the weight evolution of experimental male rats. From this figure we notice a weight growth of the rats in all the lots.

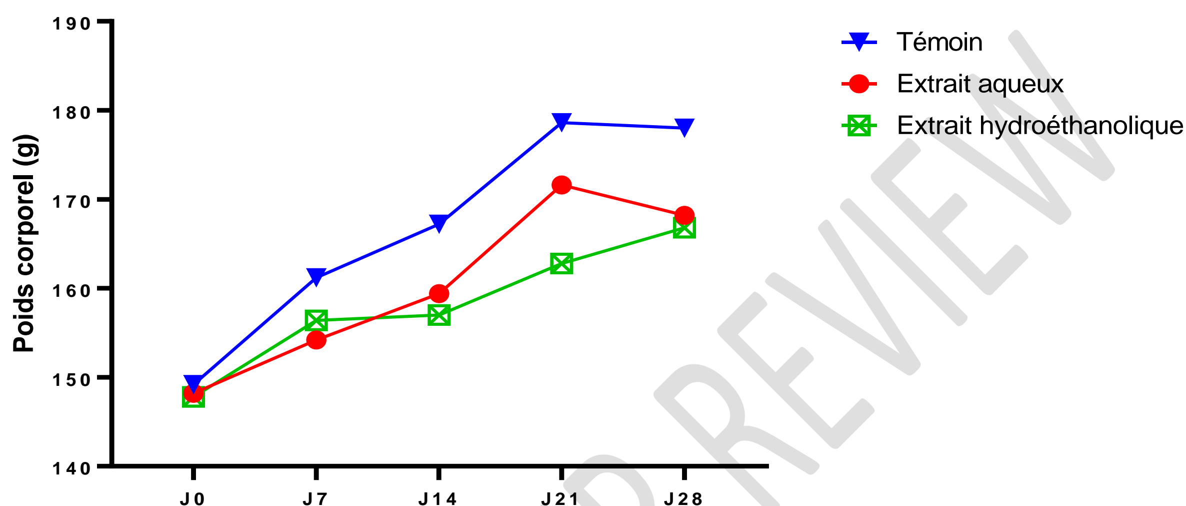

Figure 6: Evolution of the body weight of male rats during the experiment

Table 10 presents the weight gains of male rats from the different batches. From this table, it appears that during the 28 days of treatment the weight growth of the rats in the test batches is statistically identical to that of the control batch with the exception of the Day of weight gain obtained on Day 21 for the hydroethanolic extract.

Table 10. Weight gain of experimental male rats from different batches

Lots	witness	aqueousextract	Hydroethanolicextract	Comparisonbetweenwitness and test lot of aqueousextract	Comparisonbetweenwitness and test lot of hydroethanolicextract
Gain J7	12±4,64	6±5,6	8,60±4,04	No significantdifference(P= 0,103)	No significantdifference(P= 0,251
Gain J14	18,00±6,28	11,20±4,76	9,2±5,85	No significantdifference(P= 0,90)	No significantdifference(P= 0,051
Gain J21	29,40±7,37	23,40±7,47	15*,00± 6,20	No significantdifference(P= 0,237)	significantdifference(P= 0,010)

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Gain J28	28,80±9,17	20,00±5,70	19±5,15	No significantdifference(P= 0,106)	No significantdifference(P= 0,071)

- Influence of treatments on biochemical parameters of female and male rats

Compared to the control lot, the lot treated with the hydroethanolic extract presented a significant reduction in uremia, creatinemia and a significant increase in aspartate aminotransferase (ASAT) (P<0. 05). Concerning the aqueous extract, a significant reduction in uremia compared to the control batch was observed (P<0.05) (Table 11).

Significant increase in aspartate aminotransferase (ASAT) was observed for the treatment with the two extracts tested (P<0.05) (Table 12).

Table 11: Values and comparison of biochemical parameters of female rats from different batches

Lots	**witnesslot**		**Lotof aqueousextract**		**Comparisonbetweenwitness and test lot of aqueousextract at J28**	**Lotof hydroethanolicextract**		**Comparisonbetweenwitness and test lot of hydroétanolic extract at J28**
Parameters	J0	J28	J0	J28		J0	J28	
Uremia (mg/dl)	1,47 ± 0,16	1,15 ± 0,17	1,18 ± 0,24	0,68 ± 0,03*	significantdifference(p=0,003)	1,07 ± 0,14	0,56 ± 0,05*	Différence significative (p=0,001)
Creatinemia (mg/dl)	7,95 ± 0,72	7,00 ± 0,37	8,21 ± 0,41	6,56 ± 0,76	No significantdifference(p= 0,298)	8,15 ± 0,63	5,62 ± 0,54*	Différence significative (p=0,02)
AST/GOT (U/L)	79,41 ± 5,90	81,71 ± 4,63	77,80 ± 1,92	148,60 ± 12,01*	significantdifference(p=0,001)	75,36 ± 7,72	158,20 ± 12,95*	Différence significative (p=0,001)
ALT/GTP (U/L)	60,36 ± 5,47	59,22 ± 1,96	58,16 ± 6,37	47,31 ± 2,30*	significantdifference(p=0,001)	62,97 ± 4,58	53,53 ± 9,43	Pas de différence significative (p= 0,298)

Table 12. Values and comparison of biochemical parameters of male rats from different batches

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Lots	witness lot		Lot of aqueousextract		Comparisonbetweenwitness and test lot of aqueousextractat J28	Lot of hydroethanolicextract		Comparisonbetweenwitness and test lot of hydroethanolic extractat J28
Parameters	J0	J28	J0	J28		J0	J28	
Uremia (mg/dl)	1,34 ± 0,27	1,10 ± 0,23	1,43 ± 0,24	1,33 ± 0,19	No significantdifference(p=0,122)	1,26 ± 0,17	1,17 ± 0,18	No significantdifference(p=0,586)
Creatinemia (mg/dl)	7,96 ± 0,23	6,94 ± 0,23	7,69 ± 0,68	6,17 ± 0,79	No significantdifference(p=0,067)	8,53 ± 0,28	5,95 ± 1,16	No significantdifference(p=0,128)
AST/GOT (U/L)	101,25 ± 2,80	103,51 ± 5,80	101,80 ± 4,97	182,80 ± 29,45*	significantdifference(p=0,001)	102,26 ± 1,58	133,40 ± 14,43*	significantdifference(p=0,003)
ALT/GTP (U/L)	54,32 ± 3,57	54,52 ± 2,14	59,24 ± 2,82	54,22 ± 5,93	No significantdifference(p=0,106)	55,55 ± 2,24	53,29 ± 1,53	No significantdifference(p= 0,326)

- Influence of treatments on hematological parameters of female and male rats

Tables 13 and 14 respectively provide information on the values of the hematological parameters of female rats and male rats. From Table 13, it appears that except for white blood cells, no significant influence was observed for the hematological parameters of female rats. For male rats (Table 14), no significant influence was also noted for the hematological parameters.

Table 13. Values and comparison of hematological parameters of female rats from different batches

Lots	Witness lot		Lot of aqueousextract		Comparisonbetweenwitness lot and aqueousextract lotat J28	Lot of hydroethanolic extract		Comparisonbetweenwitness lot and hydroethanolic extract lotat J28
Parameters	J0	J28	J0	J28		J0	J28	
GB	4,92±1,03	7,08±1,27	6,70±1,19	4,92±1,53*	significantdifference (p=0,041)	5,30±0,98	8,60± 2,40	Aucune différence significative (p=0,247)

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GR	7,53±1,94	6,38±0,36	6,61±1,20	5,64±2,52	No significantdifference(p=0,541)	4,85±1,87	6,37±0,62	No significantdifference(p=0,05)
HB	16,12±3,77	15,46±0,67	14,64±1,17	13,90±3,53	No significantdifference(p=0,361)	12,02±2,63	15,20±0,28	No significantdifference(p=0,449)
HCT	43,52±10,21	38,42±1,31	38,34±0,67	35,48±9,06	No significantdifference(p=0,493)	32,30±10,73	38,06±2,65	No significantdifference(p=0,793)
VGM	58,18±3,29	49,32±8,24	58,12±3,41	53,06±2,12	No significantdifference(p=0,355)	61,10±2,18	53,82±0,32	No significantdifference(p=0,257)
MCH	21,56±0,95	21,46±0,8	22,14±0,80	20,82±1,05	No significantdifference(p=0,310)	27,38±10,35	20,98±1,13	No significantdifference(p=0,461)
CCMH	37,08±1,84	40,24±0,83	38,22±2,92	35,22±9,65	No significantdifference(p=0,280)	44,76±16,62	40,02±2,54	No significantdifference(p=0,280)
PLT	723,20±28,60	773,80±10,33	747,80±28,61	766,80±28,23	No significantdifference(p=0,859)	745,80±26,93	780,60±9,94	No significantdifference(p=0,320)

Table 14. Values and comparison of hematological parameters of male rats from different batches

Lots	**Witness lot**		**Lot of aqueousextract**		**Comparisonbetweenwitness lot and aqueousextract lotat J28**	**Lot of hydroethanolic extract**		**Comparaison entre Lot Témoin-Lot Extrait Hydroéthanolique au J28**
Parameters	J0	J28	J0	J28		J0	J28	
GB	5,88 ± 0,98	7,78 ± 1,06	5,92 ± 1,50	7,34 ± 0,62	No significantdifference(p=0,447)	7,20 ± 1,86	7,82 ± 1,92	No significantdifference(p=0,968)
GR	7,13 ± 0,77	6,64 ± 1,80	7,16 ± 1,04	6,93 ± 0,75	No significantdifference(p=0,748)	7,33 ± 0,98	7,12 ± 0,30	No significantdifference(p=0,570)
HB	14,30 ± 1,42	14,84 ± 0,81	13,00 ± 3,48	13,82 ± 2,23	No significantdifference(p=0,364)	14,68 ± 1,61	13,64 ± 2,62	No significantdifference(p=0,357)
HCT	39,10 ± 4,30	39,24 ± 3,03	40,54 ± 1,43	35,48 ± 4,87	No significantdifference(p=0,181	38,66 ± 3,75	33,04 ± 5,97	No significantdifference(p=0,07)

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					)			
VGM	54,90 ± 2,86	55,60 ± 3,21	54,96 ± 2,79	55,84 ± 2,79	No significantdifference(p=0,903)	53,10 ± 4,84	57,04 ± 1,05	No significantdifference(p=0,368)
MCH	20,08 ± 0,52	21,04 ± 1,46	21,36 ± 2,98	21,66 ± 1,05	No significantdifference(p=0,464)	20,10 ± 1,04	21,90 ± 0,64	No significantdifference(p=0,264)
CCMH	36,66 ± 1,76	37,84 ± 1,00	38,86 ± 4,73	38,82 ± 1,28	No significantdifference(p=0,214)	38,00 ± 1,91	38,42 ± 1,35	No significantdifference(p=0,461)
PLT	764,20 ± 59,92	782,80 ± 7,50	751,40 ± 32,76	766,60 ± 27,39	No significantdifference(p=0,238)	759,20 ± 32,87	772,00 ± 7,18	No significantdifference(p=0,048)

Discussion

The extraction yield of active components from plant materials is influenced by the water/raw material ratio, which is an important factor. Furthermore, the polarity of the extractant plays a key role in increasing the recovery of phenolic and flavonoid compounds [33]. The findings revealed that the hydroethanol solvents gave a significantly higher extraction yield compared to the aqueous extract. The high extraction yield of hydroalcoholic solvents, particularly hydroethanol (40:60), can be attributed to their ability to dissolve polar and nonpolar molecules.

All extracts have a relatively high content of polyphenols and flavonoids, which varies from one extract to another. This variability in chemical composition in polyphenols of the extracts could be explained by the diversity of the different solvents used during the extraction. Indeed, the ability of a herbal remedy to exert inhibitory effects on microbial development is due to its different components such as phenolic compounds, tannins, anthocyanins, coumarins, alkaloids and flavonoids.

Regarding the results of the antibacterial activity of the aqueous and hydroethanolic extracts of the tested plant, it appears that the hydroethanolic extract was active on all the used bacterial strains with a high inhibition diameter compared to the aqueous extract. Of all tested strains, the largest inhibition diameter was obtained with the hydroethanolic extract on the *Salmonella Typhi* strain. Both extracts were active against strains of *Candida albicans*. The inhibition diameter obtained for the aqueous extract was 12 mm ± 0.66 and that of the hydroethanolic extract was 20 mm ± 0.33. The antibiotic power of the hydroethanolic extract was lowerthan of the aqueous extract ones. The inhibitory effect of plant extracts against bacterial pathogens is generally linked to the phenolic composition which can be explained by adsorption on cell membranes, interaction with enzymes or deprivation of substrate and metal ions [34]. Several studies have succeeded in identifying flavonoid glycosides and their aglycones in hydroalcoholic extracts of plants [33]. The existence of flavonoid derivatives in

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water-alcohol mixtures could explain their antibacterial activity. These results are of great importance, especially since the testedextracts inhibit the responsiblestrains of diarrhea. In addition, hydro-ethanolic extracts have bactericidal power on all strains while aqueous extracts have bacteriostatic power.

The cyotoxicity test according to the *Artemia salina* model constitutes a preliminary screening to determine the degree of cytotoxicity of a product. The LC_{50} obtained is respectively 3.33 mg/mL for the aqueous and 0.420 mg/mL for the hydroethanolic extracts. The testedproducts are not cytotoxic (Mousseux, year) at the usedconcentrations. To eliminate any form of doubt and confirm the results of larval cytotoxicity, the acute and sub-acute toxicity testswere carried out.

Concerning the acute toxicity test, the dose was single (2000 mg/kg of body weight) and the animal model used was the Wistar rat. It should be noted that all Wistar rats treated with extracts showed a significant increase in their body weight during the treatment. Our results are contrary to those obtained by Alelign et al. [35]. These authors noted a loss of body weight observed in experimental rats, following extracts of *R. abyssinicus* and *C. Murale* administered at 2000 mg/kg during an acute toxicity study. Weight loss in rats may be due to anorexia and disturbances in carbohydrate, protein or fat metabolism, which may have been affected by extract administration as suggested by Ghelani et al. [36]. Considering the data from this test, it appears that at a dose of 2000 mg/kg, the aqueous and hydroethanolic extracts of *Combretum grandiflorum* induced neither mortality nor alteration of the physiological parameters of Wistar rats (renal, hepatic parameters, hematological constants, body weight). This suggests the normal processing of lipids, carbohydrates and protein metabolism in the animal body, as these nutrients play a major role in different physiological functions of the body [37]. When evaluating the acute toxicity of *Combretum grandiflorum* extracts, the health status of the body was assessed by other biological parameters, including the measurement of serum biomarkers. Liver injury caused by hepatotoxic drugs can result in elevated ALT, AST, and total protein levels [37]. In our study, it is noted that at the threshold of 5%, no significant influence favorable to an impairment of liver and kidney functions was noted for the biochemical parameters of the kidneys (urea and creatinine) and those of the liver (ALT, ASAT). Because hepatocellular damage can lead to an increase in cell membrane permeability and cause the release of amino transferases into the bloodstream [37]. These extracts therefore do not present acute oral toxicity for Wistar rats at the limit dose of 2000 mg/Kg/bw in Wistar rats.

Acute toxicity data generally have limited clinical application. A subacute toxicity study was therefore carried out. Substances administered in chronic diseases may require repeated dose toxicological evaluation (subacute toxicity study), as their daily use may result in accumulation in the body with progressive effects on tissues and organs [38]. Subacute toxicity tests are useful for evaluating the effects of extracts on target organs and hematological or biochemical effects, because these effects are generally not observable in acute toxicity tests. It is also essential to ensure human safety, particularly in the development of pharmaceutical products. Thus, in thestudy, the subacute toxicity profile of the aerial parts of the extract of *Combretum grandiflorum* was evaluated in rats by measuring food and water consumption, body weight, as well as hematological biochemical and histological parameters. The results show that the batch treated with the hydroethanolic extract showed a significant decrease in uremia, creatinemia and a significant increase in aspartate aminotransferase

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(ASAT) (P<0.05). Concerning the aqueous extract, a significant reduction in uremia compared to the control batch was observed in female rats. While in male rats, a significant increase in aspartate aminotransferase (ASAT) was noted for treatment with two testedextracts. .Highlevels of liver enzymes are signs of hepatocellular toxicity, whereas a decrease may indicate enzyme inhibition [39]. In thestudy, it appears that except for white blood cells, no significant influence was observed for the hematological parameters of female rats. For male rats, no significant influence was also noted for hematological parameters. Therefore, it can be inferred that the extract is slightly toxic since it causes elevation of liver enzymes with slight structural changes in the liver of females. Hematological parameters are sensitive markers of physiological changes in response to any environmental pollutant or toxic stress in animals [40]. Blood platelets play an essential role in the blood clotting process.

Conclusion

The aqueous and hydroethanolic extracts of *Combretum grandiflorum* have interesting contents of total polyphenols and flavonoids which justify their antibacterial activity but also their traditional use. Furthermore, the toxicological characterization did not reveal any toxic effects for the concentrations of active extracts. The aqueous and hydroethanolic extracts of *Combretum grandiflorum*, having demonstrated the best *in vitro* activity on diarrheal and fungal strains, it meets the conditions to be used for the formulation of improved traditional medicine for the management of diarrheal diseases.

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