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Evaluation of the toxicological effect of stem bark extracts of *Terminalia superba* Engler & Diels (Combretaceae)

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Key words: *Terminalia superba*, Lethal concentration (LC50), Lethal dose 50 (LD50), Haematological and biochemical parameters

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Abstract

Terminalia superba (*T. superba*) is a medicinal plant used in traditional treatment of several diseases in Africa. In order to establish the safety of *T. superba* extracts, larval cytotoxicity and acute oral toxicity tests were carried out. Lethal concentration (LC50) and lethal dose 50 (LD50) were determined. The hematological and biochemical parameters were analyzed after administration of a single dose of 2000 mg/kg body weight (bw) of ethanolic and hydroethanolic extracts of *T. superba* bark powder to albinos Wistar rats for 14 days. The LC 50 was 1.441mg/ml for the ethanol extract and 2.438mg/ml for the hydroethanolic extract. Acute toxicity study showed no mortality and no toxic effect. There were no significant changes in hematological and biochemical parameters levels when compared to control suggesting that LD50 of *T. superba* bark extract is greater than 2000mg/kg bw. The safety usage of extracts from *T. superba* in traditional medicine vis-à-vis toxicity consideration is discussed and results showed the plant has no marked acute toxic effect in rats.

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Introduction

In Benin, herbal preparations are commonly used as a treatment of diseases. More than 80% of the West African population use traditional medicine to overcome sickness (Booker *et al.*, 2012). This is motivated by the frequent failure of conventional pharmaceutical treatments and high drug prices for developing countries (Eddouks *et al.*, 2007; Jiofack *et al.*, 2010). Traditional care administrators vary plants combination, regardless of their toxicity or their interactions (Deleke *et al.*, 2011).

The pharmacokinetic and pharmacodynamic data of these products are not often known. This could eventually cause treatment failures or accidents. Several studies on traditional treatments revealed insufficient data on plants used in medicine (Pousset, 2006; Yemoa *et al.*, 2008; N'Gues san *et al.*, 2009). Medicinal plants, despite their therapeutic effects, should be used with the utmost caution as they may have a high toxicity risk. The use of a substance as a medicinal product complies with rules. The pharmacological activity of plant extracts should occur at doses where the toxicity is negligible. Toxicity tests therefore accompany tests of biological activities during the selection of new molecules.

Terminalia superba (*T. superba*) is a tree of the Combretaceae family. *T. superba* extracts are used for their antimicrobial (Anago *et al.*, 2006, Tabopda *et al.*, 2009, Kuete *et al.*, 2010), anti-ulcer (Goze *et al.*, 2013), analgesics (Dongmo *et al.*, 2006) and cicatrization (Dougnon *et al.*, 2014) properties. Scientific evidence for their efficacy is widely studied but systemic safety studies are lacking. Therefore, it is essential to access the toxicity of *T. superba* bark extracts in animals to ensure of its safety. This work is part of the valorization of traditional medicine and aims to evaluate the safety of ethanolic and hydro-ethanolic extracts of the *T. superba* bark powder in wistar rats.

Material and methods

Plant material

The plant material consists of dried trunk bark powder of *T. superba*. Bark pieces were colected in Itchèdé - Toffo forest at Adja ouèrè in Benin Republic (West Africa). They were identified and authenticated by the National Herbarium, University of Abomey-Calavi (Benin Republic). The bark were dried at laboratory temperature and crushed into powder that was extracted with freeze-dried ethanol and hydro-ethanol.

Animal material

Female albinos Wistar rats weighting 150 to 200 g at the age of 6-8 weeks were used for the study. Animals were raised in the animal facility of the Institute of Applied Biomedical Sciences (ISBA) under standard conditions with an alternated cycle of twelve hours light and twelve hours dark. They had free access to food and water. Room temperature was maintained at 25°C with a relative humidity of 35-60% and animals were acclimatized at least 5 days before the experiment.

Preparation of the crude extracts

The extracts were prepared according to the method described by Tadeg *et al.* (2005) and modified by Talbi *et al.* (2015). The ethanolic and hydro-ethanolic extracts were prepared from 50g of powder.

The powder were dissolved in 500ml ethanol 96°C for the ethanolic extract and 500ml ethanol 70% (diluted with distilled mater) for the hydro-ethanolic extract.

The mixture was left in maceration for seventy-two hours (72 h) and the resulting macerate was filtered with hydrophilic cotton and filter paper Whatman. The filtrate was evaporated to dryness at 40°C using a rotavapor. The resulting freeze-dried powder was stored in a refrigerator at 8°C until usage.

Larval toxicity

Shrimp larvae were used for cytotoxicity test. The test is based on the survival of larvae (Artemia salina LEACH) in sea water containing the extract. It is a primary non-clinical toxicity test that was proposed by Michael and *et al.* (1956) and later developed by Vanhaecke *et al.* (1981) and modified by Sleet and Brendel (1983). Toxicity is assessed by the lethal concentration (LC50) that is determined using the correspondence table of Sparkler (1995) (Table 1).

Table 1. Correspondence between LC50 and toxicity (Mousseux (1995).

LC_{50} (Letal Concentration 50)	Toxicity
$LC_{50} \geq 0.1$ mg/ ml	(-) Non toxic
0.1 mg/ ml>$LC_{50} \geq$ 0.050 mg/ ml	(+) Low toxicity
0.050 mg/ ml>$LC_{50} \geq$0.01 mg/ ml	(++) Moderate toxicity
LC_{50}< 0.01 mg/ ml	(+++) High toxicity

Lethal concentration (LC50).

Artemia salina eggs were cultured in an Erlenmeyer flask containing sea water taken from the Atlantic Ocean and filtered before use. The medium was left under continuous agitation for 48 hours to allow the eggs give birth to young larvae. An extract stock solution of 50mg/ml concentration was prepared by dissolving 200mg of ethanol extract in 4ml of distilled water. A range of 10 successive dilutions was made with seawater from the stock solution. Extract concentration the dilutions were 25-, 12.5-, 6.25-, 3.12-, 1.56-, 0.78-, 0.39-, 0.19-, 0.09- and 0.04-mg/ml. The same procedure was adopted for hydro ethanol extracts. Using a micropipette, a colony of 16 alive larvae was added to the diluted extract and to Sea water without extract (control). All media were left under stirring and read 24 hours after incubation. The number of alive or deaths larvae 24 hours after incubation with each dilution of extract were determined. The dose-response data were transformed by logarithm and the LC50 was determined by a linear regression study (Hafner *et al.*, 1977).

Oral acute toxicity

The study of oral acute toxicity was conducted in Wistar rats using a unique dose of 2000mg/kg body weight (bw) of extract in accordance with OECD Protocol 425 (OECD 2008). Before the administration of the extracts, the rats were deprived of food but fed with water for 3 hours and then weighed. The extract was administered by gavage. Lot 1 (control) received distilled water, lot 2 received 2000mg/kg bw of the ethanolic extract, and lot 3 received 2000mg/kg of the ethanol extract. The animals were observed individually at least once during the first 30 minutes and at least twice during the first 24 hours after treatment. Particular attention was paid to them daily for 14 days after administration of the substance.

Physical parameters

Clinical observations (mortality, morbidity, healthy or reaction to treatment, behavior pattern, tremors, salivation, diarrhea, sleep disorder and coma) were made once a day.

Body Weight

The individual weight of each rat was determined one hour prior to extract administration (Do) and at the end of the toxicity study (D14).

Blood sample collection

Venous blood samples were collected from all rats after overnight fasting. The samples were collected on Do and D14 from eye vein in a collection tube with and without anticoagulant (Vacutainer System; Becton Dickinson). They were used for haematological and biochemical parameters analysis.

Biochemical analysis

Plasma glucose was measured by Glucose Oxidase and Peroxidase (GOD-POD) method (ELITech Group, Puteaux, France) according to the manufacturer's instructions. Aspartate amino transferase (AST) and alanine amino transferase (ALT) levels were measured using an automated blood analyser Hitachi 705 (Hitachi, Japan), with Dia Sys (Diagnostic Systems GmbH, Germany) reagents. Plasma level of creatinine, urea, potassium (K+), chloride (Cl-), sodium (Na+), were measured using Elitech reagents (ELITech Group, Maizy, France).

Hematological assay

Hematological ananlysis was performed using an automatic hematological analyzer (cell Dyn 3500, Abbott) from blood samples collected in the EDTA tubes. The blood parameters measured were hematocrit (Hct), hemoglobin (HGB), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) lymphocytes (LYM), neutrophils (NEU), eosinophils (EOS), monocytes (MON), basophils (BAS) and platelets (PLT).

Statistical analysis

The values were presented as mean ± standard error of the mean (SEM). The analyzes of variance as well as the "t" test of Student with a post-hoc test of Tukey-Kramer were carried out using the software SPSS 17 and Graphpad Sat. A level of p <0.05 was set as significant.

Results

Larval toxicity

The logarithmic adjusted curves for ethanol extract (Fig. 1A) and hydro ethanol extract (Fig. 1B) showed that the number of dead larvae increases when extracts concentration increases in a dose-response relationship manner.

The LC50 values are 1.441mg/ml for the ethanol extract (Fig. 1A) and 2.438mg/ml for the hydroethanolic extract (Fig. 1B). Both values are greater than 0.1mg/ml which represents the limit of toxicity (Mousseux, 1995).

Artemia salina eggs were cultured in an Erlenmeyer flask containing sea water taken from the Atlantic Ocean and filtered before use. The medium was left under continuous agitation for 48 hours to allow the eggs give birth to young larvae. An extract stock solution of 50 mg/ml concentration was prepared by dissolving 200 mg of ethanol extract in 4 ml of distilled water (Fig. 1A). A range of 10 successive dilutions was made with seawater from the stock solution. Diluted extract concentrations were 25-, 12.5-, 6.25-, 3.12-, 1.56-, 0.78-, 0.39-, 0.19-, 0.09- and 0.04- mg/ml. The same procedure was adopted for hydro-ethanol extracts (Fig. 1B).

Using a micropipette, a colony of 16 living larvae was added to the diluted extract and to Sea water without extract (control). All media were kept under stirring for 24 hours incubation. The number of living or dead larvae was determined 24 hours after incubation in each dilution. The dose-response data were transformed by logarithm and the lethal concentration (LC50) was determined by a linear regression study. The LC50 of ethanol extract (Fig. 1A) was1.441mg/ml and hydro-ethanol extract (Fig. 1B) was 2.438 mg/ml.

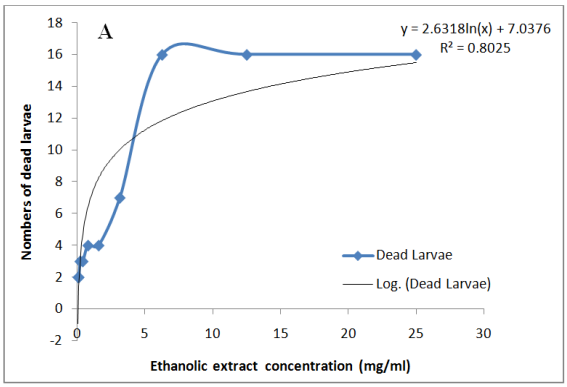

Fig. 1A. Dead larvae number variation in ethanolic extracts**.**

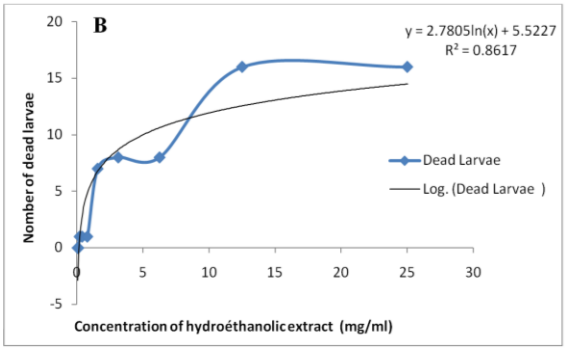

Fig. 1B. Dead larvae number variation in hydro-ethanolic extracts.

Acute Oral Toxicity

Physical parameters

No toxic effect was observed throughout the 14 days study period. No mortality was observed in any group of the rats following the administration of 2000 mg/kg body weight (bw) of ethanolic and hydro-ethanolic extract. Physical observation showed no signs of toxic effect such as changes on behavior pattern, tremors, salivation, diarrhea, sleep and coma in any group of rats throughout the study period. Evolution of the body weight of rats during the toxicity test. The body weight of control and treated rats were shown in Table 2. There was no significant difference in the body weight of the treated rats compared to the control rats.

Determination of biochemical parameters

Serum glucose, urea and creatinine levels did not vary significantly at D14 compared D0, regardless of the type of extract administered (Table 3).

The level of AST and ALAT enzymatic activity level showed no significant changes at D14 compared to Do. Similar results were found for potassium, chloride and sodium (Table 3). The level of each parameter was within the normal ranges when compared to reference values (Table 3).

Hematology

There were no significant changes in packed cell volume, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, monocytes and eosinophils, total leukocyte count, erythrocyte, leukocyte and platelet at D14 compared to Do and to control (not treated group) (Table 4).

Table 4. Hematological parameters in rats.

Venous blood samples were collected from all rats after overnight fasting. The samples were collected on Do and D14 from eye vein in a collection tube with anticoagulant. The blood parameters measured were hematocrit (Hct), hemoglobin (HGB), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) lymphocytes (LYM), neutrophils (NEU), eosinophils (EOS), monocytes (MON), basophils (BAS) and platelets (PLT).

There were no significant changes in packed cell volume, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, monocytes and eosinophils, total leukocyte count, erythrocyte, leukocyte and platelet at D14 compared to Do and to control (not treated group)

Parameters	Lot 1	Lot 2	Lot 3	Normal value
RBC (10^6/mm^3)	6.2 ± 0.5	6.6 ± 0.5	6.8 ± 0,4	7.07 – 9.03
HGB (g/dl)	12.4 ± 1.3	13.2 ± 0.8	13.6 ± 1.1	13.7 – 16.8
Hct (%)	34.2 ± 3.1	36.0 ± 2.6	36.6 ± 2.5	37.9 – 49.9
MCHC (g/dl)	37.5± 0.7	37.8 ± 0.5	37.4 ± 0.0	33.3 – 38.1
MCV (pg)	20.9 ± 0.3	20.7 ± 0.5	20.6 ± 0.4	17.8 – 20.9
MCH (fl)	55.0 ± 1.2	54.6 ± 1.8	54.8 ± 1.6	49.9 – 58.3
WBC (10^3/mm^3)	10.0 ± 1.2	11.7 ± 4.0	10.7 ± 1.2	1.13 – 7.49
% NEU	14.8 ± 4.3	19.6 ± 2.4	17.2 ± 4.1	7.1 – 33.2
% EOS	1.6 ± 1.8	2.8 ± 1.3	2.8 ± 0.8	0.5 – 4.5
% LYM	76.8 ± 5.9	72.4 ± 1.5	73.2 ± 5.8	62.2 – 90 %
% MON	6.8 ± 2.2	5.2 ± 1.8	6.8 ± 2.0	0.8 – 3.9 %
% BAS	00	00	00	0 – 0.8
PLT (10^3/mm^3)	610.6 ± 159.4	645.94± 292.6	673.8 ± 62.4	680 – 1200

RBC: red blood cells, HGB: hemoglobin, Hct: hematocrit, MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular.

Volume; MCH: mean corpuscular haemoglobin, WBC: white blood cells, NEU: neutrophils; EOS: eosinophils; LYM: Lymphocytes; MON: Monocytes; BAS: basophile; PLT: platelets). Lot 1 (control): received distilled water; lot 2 received 2000 mg/kg bw of the ethanolic extract (70%); lot 3 received 2000 mg/kg of the ethanol extract (100%).

Table 2. Effect of extracts on the body weight of rats. The individual weight of each rat was determined one hour prior to extract administration (Do) and at the end of the toxicity study (D14). There was no significant difference in the body weight of the treated rats compared to the control rats.

	Lot 1	lot 2	lot 3
Do	134.2 ± 5.9	137.0 ± 4.5	136.4± 2.7
D14	174.0 ± 8.2	163.0 ± 7.6	165.4 ± 2.9

D: day; Lot 1 (control): received distilled water; lot 2 received 2000 mg/kg bw of the ethanolic extract (70%); lot 3 received 2000 mg/kg of the ethanol extract (100%).

Table 3. Biochemical parameters in rats.

Venous blood samples were collected from all rats after overnight fasting. The samples were collected on Do and D14 from eye vein in a collection tube without anticoagulant (Vacutainer System; Becton Dickinson). Plasma glucose, Aspartate amino transferase (AST), alanine amino transferase (ALT), creatinine, urea, potassium (K+), chloride (Cl-), sodium (Na+) were measured.

Serum glucose, urea and creatinine levels did not vary significantly at D14 compared Do, regardless of the type of extract administered (Table 3). The level of AST and ALAT enzymatic activity level showed no significant changes at D14 compared to Do.

Similar results were found for potassium, chloride and sodium (Table 3). The level of each parameter was within the normal ranges when compared to reference values.

Paramètres	Lot 1	Lot 2	Lot 3	Normal value
Glucose (g/l)	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	0.76 – 1.75
urea (g/l)	0.5 ± 0.0	0.5± 0.1	0.5± 0.0	0.13 – 0.27
Creatinine (mg/l)	6.5 ± 2.1	7.0 ± 0.0	6.8 ± 0.8	2 – 6
AST (UI/I)	129.8 ± 10.3	124.0 ± 4.7	154.2 ± 10.2	74 – 143
ALT (UI/I)	67.8 ± 6.0	73.0 ± 3.9	83.2 ± 8.0	18 – 45
Sodium (mmol/l)	139.2 ± 2.4	136.2 ± 1.5	135.6 ± 3.6	140 – 150
Chloride (mmol/l)	104.6 ± 8.0	112.8 ± 8.2	106.4 ± 4.9	100 – 107
Potassium (mmol/l)	4.0 ± 0.0	4.2 ± 0.4	3.8 ± 0.4	3.31 – 4.9

AST : Aspartate aminotransferase; ALT: Alanine aminotransferase; Lot 1 (control): received distilled water; lot 2 received 2000 mg/kg bw of the ethanolic extract (70%); lot 3 received 2000 mg/kg of the ethanol extract (100%).

Discussion

Plants have played an important role in the development of pharmaceutical compounds (Gilani and Rahman, 2005; Patwardhan, 2005). Despite their beneficial properties, toxic effects related to certain medicinal plants have been reported (Park and *al.*, 2004, Marcus and Snodgrass, 2005). *T. superba* has antimicrobial (Anago *et al.*, 2006, Tabopda *et al.*, 2009, Kuete *et al.*, 2010), anti-ulcer therapy (Goze *et al.*, 2013), Analgesics (Dongmo *et al.*, 2006) and Cicatrization (Dougnon *et al.*, 2014) properties. In this study, we evaluated cytotoxicity and acute oral toxicity of *T. Superb* in order to determine under which conditions the plant can be used with safety.

Larval toxicity test was performed on shrimp larvaeas a preliminary toxicity test (Quignard *et al.*, 2003). There is a positive correlation between the larval toxicity test and the lethal oral dose of medicinal plants in mice (Parra *et al.*, 2001). Our results showed that shrimp larvae remained sensitive to the extracts tested in a dose response relationship. The LC 50 of the ethanolic and hydro-ethanolic extracts are all above 0.1mg/ml, suggesting that *T. superba* is non-toxic (Mousseux, 1995).

Acute oral toxicity tests were conducted in the Wistar albino rat at 2000 mg/kg bw dose limit. Clinical examination revealed that the animals of the different treatment groups did not exhibit significant changes in the body weight when compared to control group. None of the animals died indicating that the lethal dose 50 (LD50) of *T. superba* was much higher than 2000 mg/kg bw. These results suggest that both ethanolic and hydro-ethanolic extracts of *T. superba* have a high tolerance towards rats.

The LD50 of the two extracts is greater than 2000 mg/kg bw. According to the OECD's Globally Harmonized System of Classification (OECD 2001), our extracts can be classified in Category 5 and considered to be a nontoxic oral substance. Our results confirmed those of Kouakou *et al.* (2013) who reported the absence of oral toxicity of aqueous extracts of *T. superba* barks in mice. Our findings are also in agreement with Tom (2011) who showed that the LD50 of the aqueous extract of *T. superba* is greater than 5000 mg/kg and that of the methylene chloride extract is greater than 2000 mg/kg bw.

Furthermore, our results showed that both ethanolic and hydroethanolic extracts of *T. superba* did not lead to significant changes in blood biochemical and haematological parameters. This means that the extracts did not induce changes in the erythrocyte and leukocyte lines. (Yakubu *et al.*, 2003). Transaminases ASAT and ALAT are good indices of liver damage (Martin *et al.*, 1981) while creatinine, urea and electrolyte imbalance signals kidney dysfunction (Kabiru *et al.*, 2013). Our funding that these biochemical parameters were within normal ranges suggests that *T. Superba* ethanolic and hydro-ethanolic extracts, at a unique dose of 2000 mg/kg bw, did cause any damage in liver and kidney functions.

Conclusion

Cytotoxicity and acute oral toxicity studies of ethanolic and hydro-ethanolic extracts of *T. superba* showed no toxic effect on hematological and biochemical parameters that we analyzed. These results support the use of *T. superba* in traditional medicine.

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