Preclinical *in vivo*acute toxicity testing of hyrdoethanolic extracts of *Ficusthiningii*Blume (Moraceae) on Wistar rat models

Comment [M1]: Spellerror

ABSTRACT

Ficusthonningii (Blume) is a medicinal plant whose biological activity has been demonstrated in the management of peptic ulcers. Unfortunately, little scientific data exists on its toxicity. This is why we studied the acute and subacute oral toxicity profile of the hydro-ethanolic extract of the bark of *F.thonningii*and the systemic exposure of the bioactive components of the plant.Acute toxicity was assessed by administering a single dose of 2000 mg/kg to 5-week-old rats and observed for 14 days. The subacute toxicity study was performed in 6-week-old rats. Animals were orally treated with a daily dose of 125 mg/kg, 250 mg/kg, 500 mg/kg extract for 28 days. A satellite group of animals received 500 mg/kg per day for 28 days and after discontinuation of treatment they were observed for 14 days. Hematological and blood biochemical parameters, as well as kidney and liver histology, were recorded at the end of each experiment. For acute treatment, administration of a dose of 2000 mg/kg did not induce any critical behavioral changes or deaths. Following subacute dosing, biochemical analysis revealed a slight elevation of liver parameters in the 500mg/kg hydroethanolic extract, while no significant increase was observed for kidney parameters. This study showed that daily administration of *Ficusthonningii* (Blume) for 28 days resulted in the potential to reverse impairment of liver function.

Keywords:*Ficusthonningii*, Moraceae, hydro-ethanolic extract, stem bark, toxicity, Wistar rat

UNDER PEER REVIEW

INTRODUCTION

Medicinal plants are an important source of food and medicine for humans. So far, about 80 % of the world population depend on medicinal plants primary health interventions [1-3].There has been an increased interest in the use ofmedicinal plants for the treatment of diseases due to their low cost, less toxic and more readily available and environmentally friendly. Medicinalplants are paving a new approach to improvedmedicine and are emerging as alternativetherapeutic options[4, 5].Unfortunately, thelack of enough studies to provide information about their quality, efficacy and safetyremains a major concern to the public.One of the popular plants in Cameroon used in ethnomedicine is the*Ficusthonningii*Blume (Moracea) also known as the strangler or

common wild fig [6-9]. It is extensively used for the treatment of a number of diseases such as diarrhea, stomach pain, peptic ulcer, urinary tract infections, diabetes mellitus, gonorrhea, respiratory infections, and mental illnesses. It is an evergreen tree (about 6-21m tall) with a dense rounded tospreading crown [3, 10] mainlydistributed in the upland forests of tropical andsub-tropical Africa[4, 11-13]. This plant contains various bioactive compounds which include alkaloids, terpenoids, flavonoids, tannins and active proteins, all of which contribute to its curative properties. *In vitro* and *in vivo* pharmacological studies revealed that *F. thonningii*has antimicrobial, antidiarrheal, anthelmintic, antioxidant, anti-inflammatory and analgesic properties [3, 13].

A number of studies [14-16] have recently shown that*Ficusthonningii* has promising biologic activities, for instance,in the management of peptic ulcers. This has motivated the thought that it has the potential to be developed into an improved traditional medicine. Unfortunately, so far, there is still little scientific data in the literature on the possible toxic effects of the plant[16]. This prompted researchers like Tembeet al (2018) [17]to conduct an acute toxicity study on the hydro-ethanolic stem bark extract of the plant in order to contribute information in regards to its toxicity profile and preclinical pharmacology. Therefore, the present study was planned to evaluate the oral acute and subacute toxicity profiles of the hydro-ethanolic extract of the stem bark of *Ficusthonningii*(Blume) as a subsequent study to provide more information concerning the safety of the plant's bioactive components.

The objective of the study was to investigatetheacute toxicity of the bioactive components of the hydro-ethanolic extract of the stem bark of *Ficusthonningii*(Blume) in *Wistar* rats.

UNDER PEER REVIEW

METHODOLOGY

This was an experimental *in vivo* preclinical study conducted in *Wistar* albino rats.This study was conducted from November 2021 to May 2022. The study was carried out at the Animal house affiliated with the laboratory of pharmaco-toxicology and pharmacokineticsof the Faculty of Medicine and Biomedical Sciences in the university of Yaoundé 1. Ethical approval was requested from the institutional review board of the Faculty of Medicine and Biomedical Sciences. An authorization to work in the animal house of the faculty was obtained from the head of department of pharmaco-toxicology and pharmacokinetics. The Organization for Economic Cooperation and Development (OECD) Guidelines 420 for the use of animals inpreclinical studies was applied.

Plant materialselection

The Rationale for the selection of the plant material*Ficus. thonningii*(Blume)wasbased on the fact that this medicinal plant has been documented for use over the centuries by the Western Region of Cameroon for the treatment of several ailments [17].*F. thonningii* remedies are commonly prepared as decoctions, macerations and infusions and the extracts are taken orally.This toxicity studywas a follow upof thestudy previously conducted by ourteam to confirm stability of activity of the plant material as reported by Tembeet al. (2018)[17] on the acute toxicity of *F. thonningii*(Blume). Therefore, the hydro-ethanolic stem bark extract of *F. thonningii* was equally used in the present study. The same doses of the stem bark extract of the plant used by Pougoue (2017) [14] were applied in this study.

Collection and identification of the plant

The powdered stem bark of *F. thonningii*which was previously obtained from the preserved plant material of the work conducted by Pougoue (2017) [5] was used in the present study.Fresh stem barks were collected from the plant growing at Bafoussam on the 03 of January 2017. The barks were then identified taxonomically and authenticated at the National Herbarium of Cameroon by comparison with a sample having the number 44042/HNC by Tadjouteu F.

Animal materials

The animals usedwere white albino rats of the *Wistar* strain less than nine weeks old. Theywere raised in the Laboratory for Preclinical Animal Studies and Pharmaco-Toxicology Research of

FMBS under favorable conditions for their growth and development. The animals were fed with a mixture ofcorn meal (45 %), wheat flour (20 %), fish meal (20 %), soybean meal (10 %), palm kernel (5%), bone flour for calcium intake (0.98 %), cooking salt (0.5 %) and vitamin complex (0.5 %) [17]. The animals chosen for the study were identified using cage card and corresponding bold marker body markings. They were equally subjected to a gross observation to ensure that the selected rats were in a good health condition. Rats were randomly selected with respect to body weight for final allotment to the study. The animal house has natural air-conditioned rooms with optimal air changes per hour, relative humidity, temperature and illumination cycles set to 12h light and 12 hours dark. The animals were grouped and housed in cages with stainless steel grill tops, together with facilities for food, water bottle and bedding of clean paddy husk. The plant extract was administered by oral gavage to each rat with 1 mL per 100 g using an intubation needle fitted onto a syringe of appropriate size [13]

Preparation of the hydro-ethanolic plant extract

Preparation of medicinal plants for experimental purposes is an initial step and key in achieving quality research outcome. It involves extraction and determination of quality and quantity of bioactive constituents before proceeding with the intended biological testing.According to the work done by Pougoueet al.(2017) [14, 17], the hydro-ethanolic maceration of the stem bark of the plant was most active. Hence, a hydro-ethanolic extract was used during this study.

Extraction was done using the Maceration technique which is an extraction procedure in which coarsely powdered plant material, either leaves or stem bark or root bark, is placed inside a container; the menstruum is poured on top until it completely covers the drug material. The container is thenclosed and kept for at least two days.The content is stirred or agitated from time to time to ensure complete extraction.At the end of extraction, the micelle is separated from marc by filtration and from the menstruum by evaporation in an oven[14, 18]. The extract was then weighed in order to determine the percentage yield obtained from theinitial powder quantity and then stored in an air-tight container for subsequent experimental tests.

200 g of the powder was weighed and mixed with several fractions of a 50:50 hydro-ethanolic solution in order to obtain a finalsolution of 2000 mL in a flat-bottomed flask. This mixture was agitated several times within 48 hof maceration, after which the mixture was filtered using Whatman paper number 3. Themacerate was thendried in an oven at 50°C for two days. The

UNDER PEER REVIEW

dried extract obtained wasweighedin order to determine the percentage yield from the initial powder used. The yield (%) was obtained using the following formula below [19]:

$$Percentage\ yield = \frac{mass\ of\ the\ extract\ obtained}{mass\ of\ the\ initial\ powder} \times 100$$

ACUTE TOXICITY FOLLOWING ORAL ADMINISTRATION

The OECD 420 Guideline for the testing of chemicals (Oral toxicity - predetermined dose method) was adopted in 2001 and applied in this study

Treatment groups

Groups of animals of a single sex receive predetermined doses of 5, 50, 300 and 2000mg/kg according to a sequential procedure. The initial dose was chosen on the basis of an orientation study such as that which islikely to cause toxic effects, but without causing severe toxic effects or death. Other groups ofanimals received higher or lower doses depending on the absence or presence of toxic effects ormortality. The procedure was continued until the dose that caused evident toxic effect or the deathof a single animal. The procedure was also interrupted when the highest dose did not give rise toany observed effect or the lowest dose showed no mortality.

Animal extract treatment

The experimental animals, *Wistar* rats, aged six to eight weeks, were randomly allocated and housed in cages, each containing five rats; males and females separately with bedding changes twice a week to maintain hygiene. Feed and water were given *ad libitum.* The animals were randomly selected and marked with a bold marker to permit individual identification and observed for at least 7 days prior to dosing to allow for acclimatization to laboratory conditions. Animals were fasted overnight prior to dosing but water was not withheld. Following the period of fasting, the animals were weighed and the test substance administered on the basis of individual animal weight. Food was withheld after dosing for a further 3-4 hours after dosing.

Preparation of the plant extract doses

Due to the fact that previous acute toxicity studies conducted on *Ficusthonningii*(Blume) by Pougoueet al.(2018) [18] and Stanley et al. (2008) [20]recorded no deaths at the dose of 2000 mg/kg and above, afixed-dose of 2000 mg/kg was applied. We prepared a 100mg/mL concentrated solution. 20 mL of solution was obtained from 2000 mg of the extract.Then, the mixture was homogenized using amagnetic stirrer. From this solution, a dose of 2000 mg/kg of aqueous extract was administeredto the male and female test groups according to their weight, while the control groups receiveddistilled water.The volume did not exceed 1 mL/100 g body weight. The test substance was administered in a single dose using a gastric tube or other suitable

UNDER PEER REVIEW

intubation cannula. For each dose used, the volume administered was calculated using the equation [16];

$$\text{Volume (mL)} = \frac{\text{Dose (mg/kg) x Body weight (kg)}}{\text{Concentration of the extract (mg/mL)}}$$

Physical zootechnicalObservations

Comment [M2]: No referencewasprovided for this section

The animals were observed individually at least once during the first 30 minutes andregularly during the first 24 hours after treatment. Particular attention was required during the first4 hours and daily for 14 days after the administration of the substance. Mortality and various signs of toxicity such as tremor, convulsion, salivation, diarrhea, lethargy, sleep and coma were recorded. The absence of death or death of only one animal implied that the LD_{50}range was more than 2000 mg/kg but considered between 2000 mg/kg and 5000 mg/kg.

All test animals were subjected to a macroscopic autopsy. For each animal, allmacroscopic pathological changes were recorded. In animals that survived 24 hours or more atthe initial dose, microscopic examination of organs (liver and kidneys) with obvious signs of pathology were considered as this examination could provide useful information.After euthanizing the animals with ether blood sampling via the carotid artery was performed forthe determination of biochemical markers of toxicity. The organs were isolated immediately andweighed.

Selection of the animals, accommodation and feeding conditions

Comment [M3]: Reference ??

The preferred rodent species is the rat, although other rodent species may be usedat the beginning of the study, each animal species should be less than nine weeks old. Females must benulliparous and not pregnant. The temperature of the test animal room was maintained at22 °C (± 3°C). Ideally, the relative humidity should be between 50 and 60 %, and should be at least 30 per cent and preferably not more than 70 %, except during the cleaning of thepremises. The lighting sequence should be 12 hours of light and 12 hours of darkness. For thefeeding of animals, the use of conventional laboratory food with drinking water to satiety. The animals are to be grouped per dose. Normally, a total of five animals of each sex is used at each dose level studied.

Preparation of animals and administration of doses

Healthy animals were randomly assigned to the control and treatment groups. Cageswere arranged in such a way that possible effects due to cage placement are minimized. The animals wereidentified uniquely and kept in their cages for at least five days prior to the start of the

UNDER PEER REVIEW

treatment toallow them toacclimatize at the laboratory conditions.At least twelve animals (six females and six males) were used at each dose level. An additional satellite group of twelve animals (six per sex) in the control and in the top dose group were used for the observation of reversibility, persistence, or delayed occurrence of toxic effects, for at least 14 days post treatment.

The animals were administered escalating doses of 125 mg/kg, 250 mg/kg and 500 mg/kg while a further500mg/kg,was administered to a satellite group of animals. Whenthe test substance was administered by gavage, this was done in a single dose to the animals using astomach tube or a suitable intubation cannula. The volume did not exceed 1 mL/100g body weight as demanded by the OECD guidelines [19, 20].

TreatmentObservations

The observation period lasted a total of14 days for the test group. General observations were made at least once a day, at the same time(s)each day and considering the period of anticipated effects after dosing. The health condition of theanimals was recorded. At least twice daily, all animals were observed for morbidity and mortality.

Signs noted include, but not limitedto, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomicactivity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture andresponse to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards) wererecorded.

All animals were weighed at least once daily. Measurements of food consumption weremade daily. On the 14th day, the animals were euthanized with ether.The percentage of weight gain of the animals was obtained from the following formula [19]:

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Blood sampling via the carotid artery was performed forthe determination of biochemical markers of toxicity. The organs were isolated immediately andweighed.The relative weight of the organs was calculated as follows:

$$\text{Relativeorganweight} = \frac{\text{Organweight}}{\text{Bodyweight}} \times 100$$

UNDER PEER REVIEW

Hematological examination

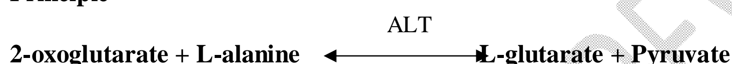
Blood samples were taken from the carotid artery just prior to or as part of the procedure for euthanasia of the animals, and introduced in EDTA tubes. The samples were then sent to the hematology laboratory atUniversity Teaching Hospital (CHU)ofthe University of Yaoundé 1, for analysis. The following hematological examinations were carried out at the end of the test period:hematocrit, haemoglobin concentrations, erythrocyte count, reticulocytes, total and differential leucocytecount, platelet count.

Quantification of Biochemical parameters

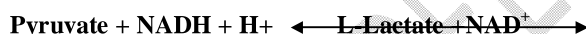
Serum alanine aminotransferase (ALT)

Comment [M4]: Reference ??

Principle



Alanine aminotransferase (ALT) activity was measured by monitoring theconcentration ofpyruvate hydrazine formed by the reaction of 2,4-dinitrophenyl hydrazine andpyruvate. This reaction was measured at 546 nm.



The rate at which the NADH was consumed was measured at 340 nm. This rate isproportional to ALT catalytic activity. The reagents used wereTris Buffer pH 7.8 and L-alanine and NADH, LDH and oxoglutarate

We mixed 1000 µL of reagents with 100 µL of serum and allowed it to incubate for 1 minute. We read the initial absorbance at assay temperature and started the stopwatch simultaneously. We read the absorbance after exactly 1, 2 and 3 minutes. We calculated the ALAT concentration using the formula:

[ALAT] (U/L) = ΔA/min × 1746

Serum aspartate aminotransferase (AST)

Comment [M5]: Reference ??

Principle

UNDER PEER REVIEW

Aspartate aminotransferase (AST) is measured by monitoring the concentrationof oxaloacetate hydrazone formed by the reaction of 2,4-dinitrophenyl hydrazineand oxaloacetate. This reaction is measured at 546 nm.

$$\text{Oxaloacetate} + \text{NADH} + \text{H+} \xleftrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^{+}$$

The rate at which the NADH was consumed is measured at 340 nm. This rate wasproportional to AST catalytic activity. The reagents used were Tris Buffer pH 7.8 and L-alanineand **NADH**, LDH and oxoglutarate

We mixed 1000 µL of reagents with 100 µL of serum and allowed it to incubate for 1 minute. We read the initial absorbance at assay temperature and started the stopwatch simultaneously. We read the absorbance after exactly 1, 2 and 3 minutes. We calculated the ASAT concentration using the formula:

[ASAT] (U/L) = ΔA/min ×1746

Alkaline phosphatase

Comment [M6]: Reference ??

Principle

Under alkaline condition, colorless p-nitrophenol is converted to 4-nitrophenoxide, which develops a very intense yellow color. Its intensity is proportional to the activity of alkaline phosphatase in the sample. The reagents used were Diethanolamine buffer pH 9.8 and magnesium chloride andp-Nitrophenyl phosphate. 1000 µL of working reagent was mixed with 20 µL of sample and incubated for 1 minute at 37°C. The absorbance of the sample (EC) was measured against distilled water decrease per minute during 3 minutes. The average absorbance difference per minute (ΔE/min) was calculated.

ALP [U/L] = ΔE/min sample ×2757

Serum creatinine (Jaffe's reaction)

Principle

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The intensity of the color formed is directly proportional to the creatinine concentration in the sample.The reagents used were Sodium Hydroxide and Picric Acid. 50 µL of the sample was placed in a cuvette and mixed with 1000 µL of reagents. The absorbance (A1) was read after 30 seconds and after 90 seconds (A2) of the sample addition.

UNDER PEER REVIEW

$$\textbf{creatinine (mg/dL)} = \frac{\Delta\textbf{sample} - \Delta\textbf{blank}}{\Delta\textbf{standard} - \Delta\textbf{blank}} \times (\textbf{Standard concentration})$$

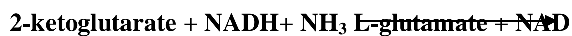
Serum urea

Comment [M7]: Reference ??

Principle

Urea is hydrolyzed by urease in ammonia and CO_2. The ammonia produced in the presence of glutamate dehydrogenase, NADH and alpha-ketoglutarate forms NAD^+ and glutamate.

$CO(NH_2)_2$+ H_2O2NH_3 + CO_2



2-ketoglutarate + NADH+ NH_3 L-glutamate + NAD

The reagentsusedwereGood buffer pH 7.6, ADP, urease and GLDH, andGood buffer pH 10.2, alpha-ketoglutarate and NADH

10 µL of the sample was placed in a cuvette and mixed with 1000 µL of reagents and incubated for 1 minute at 37°C. The absorbance of the sample (E1C1) and standard (E1STD) was read after 30 seconds and after 60 seconds of the sample (E2C) and standard (E2STD) addition.

$$\textbf{Urea[mg/dl]} = \frac{(\textbf{E2C} - \textbf{E1C})}{(\textbf{E2STD} - \textbf{E1STD})} \times \textbf{conc. STD}$$

Uric acid

Comment [M8]: Reference ??

Principle

Uric acid is converted by uricase and hydrogen peroxide which, under the catalytic influence of Peroxidase, oxidizes compound, reacts with 4-aminophenazone and 3-chlorophenolsulphonate giving a red colored compound, whose color intensity is directly proportional to the uric acid concentration in the tested sample.

Reagents used were Good buffer, 3, 5-dichlorophenolsulphonate and ascorbate oxidase, andGoods buffer, 4-aminophenazone, peroxidase and uricase

In the test tubes was introduced 25 µL of the sample with 1000 µL of reagent. The mixture was allowed to incubate at room temperature for 5 min. The absorbance of both standard (ESTD) and sample (EC) were measured against the blank reagent. The reaction remains stable for 60 minutes.

UNDER PEER REVIEW

$$\textbf{Uric acid (mg/dL)} = \frac{\textbf{EC}}{\textbf{ESTD}} \times [\textbf{conc. standard}]$$

Albumin

Comment [M9]: Reference ??

Principle

In the citrate buffer solution, albumin reacts with bromocresol green to form a colored complex. The absorbance is proportional to the concentration of albumin.Reagents used were**,** Bromocresol green,Citrate buffer

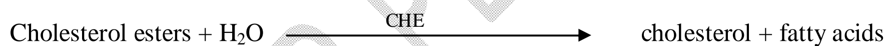
In the test tubes was introduced 10 µL of the sample with 100 µL of reagent. The mixture was allowed to incubate at room temperature. The absorbance was measured against the blank reagent. The reaction remains stable for 30 minutes.

$$\textbf{Albumine (g/L)} = \textbf{conc. standard} \times \frac{\textbf{A(sample)}}{\textbf{A(standard)}}$$

Total cholesterol

Principle

The cholesterol present in the sample originates a colored complex, according to the following reaction:



Cholesterol esters + H_2O $\xrightarrow{CHE}$ cholesterol + fatty acids

Cholesterol + O_2 $\xrightarrow{CHOD}$ 4-cholestenone + H_2O_2

H_2O_2 + Phenol + 4-aminophenazone $\xrightarrow{POD}$ quinonimine + $4H_2O$

The intensity of the color formed is proportional to the cholesterol concentration in the sample.

Reagents used were (buffer): PIPES and phenol, cholesterol esterase (CHE), cholesterol oxidase (CHOD) and peroxidase (POD)

Standard cholesterol

Comment [M10]: Reference ??

Procedure

UNDER PEER REVIEW

In the test tubes was introduced 10 µL of the sample with 1000 µL of reagent. The mixture was allowed to incubate at room temperature for 10 minutes. The absorbance (A) of the samples and standard were measured. The reaction remains stable for 60 minutes.

$$\mathbf{Cholesterol\ (mg/dL)} = \frac{\mathbf{A(sample)}}{\mathbf{A(standard)}} \times \mathbf{conc.\ standard}$$

Triglycerides

Comment [M11]: Reference ??

Principle

The assay is initiated with the enzymatic hydrolysis of the triglycerides by lipase to produce glycerol and free fatty acids. The glycerol released is subsequently measured by a coupled enzymatic reaction system with a colorimetric readout at 540 nm.Reagents used were (buffer): GOOD and p-chlorophenol, (enzymes):lipoprotein lipase, glycerol kinase, glycerol-3-oxidase, peroxidase, ATP and 4-aminophenazone

Triglyceride standard

Comment [M12]: Reference ??

Procedure

The contents of reagent 2 was dissolved in reagent 1 and mixed gently. In the test tubes was introduced 10 µL of the sample with 1000 µL of reagent. The mixture was allowed to incubate at room temperature for 10 minutes. The absorbance of the sample and standard was measured against the blank reagent. The color was stable for at least 30 minutes.

$$\mathbf{Triglycerides\ (mg/dL)} = \frac{\mathbf{A(sample)}}{\mathbf{A(standard)}} \times \mathbf{conc.\ standard}$$

HDL-cholesterol and LDL cholesterol

Comment [M13]: Reference ??

Principle

Low density lipoproteins are precipitated by the addition of phosphotungstic acid in the presence of magnesium ions. The HDL fraction remains the supernatant and this is determined by the cholesterol assay. Reagents used werePhosphotungstic acid, Magnesium chloride

UNDER PEER REVIEW

In the test tubes was introduced 100 μL of the sample with 1000 μL of reagent. The mixture was allowed to incubate at room temperature. The absorbance of the sample and standard was measured against the blank reagent within 60 minutes.

$$\textbf{HDL Conc. in the supernatant (mg/dL)} = \frac{\textbf{Abs. sample}}{\textbf{Abs. standard}} \times \textbf{(Standard concentration)}$$

$$\textbf{LDL} - \textbf{Cholesterol} \left(\frac{\textbf{mg}}{\textbf{dL}}\right) = \textbf{total cholesterol} - \frac{\textbf{triglycerides}}{\textbf{5.0}} \times \textbf{HDL} - \textbf{cholesterol}$$

Total protein

Comment [M14]: Reference ??

Principle

The principle involved in Lowry method is determining the protein concentration by calculating the reactivity of the peptide nitrogen with the Copper ions under alkaline conditions followed by reduction reaction of Folin-Ciocalteuphosphomolybdicphosphotungstic acid to hetero-polymolybdenum blue by copper catalyzed oxidation of aromatic acids.
Reagents used were Solution A: 2% sodium carbonate in 0.1 N NaOH, Solution B: 0.5% copper sulphate solution in 1% sodium potassium tartrate solution, Folin- Ciocalteu reagent

Standard protein solution
Procedure

A test tube was filled with 1mL distilled water to serve as blank. Now to the test tubes add 10 μL of standard and sampleand 1000 μL of reagent. The mixtures were allowed to incubate for10 min.

$$\textbf{Total protein (g/dL)} = \frac{\textbf{A(sample)} - \textbf{A(blank)}}{\textbf{A(standard)} - \textbf{A(blank)}} \times [\textbf{conc. standard}]$$

Total and direct bilirubin

Principle

This assay is based on the Jendrassik-Grof method, in which diazotized sulphanilic acid reacts with conjugated (direct) bilirubin, forming azobilirubin that is detectable at 546 nm.
Reagents used were (total bilirubin): sulphanilic acid, hydrochloric acid, caffeine and sodium benzoate,sodium nitrite, (direct bilirubin): sulphanilic acid, hydrochloric acid

UNDER PEER REVIEW

For total bilirubin, 100 µL of the sample was mixed with 1000 µL of reagent 1 and a drop of reagent 2 and allowed to incubate for 10 to 30 min. The absorbance of the sample and standard was measured against the blank reagent. For direct bilirubin, 100 µL of the sample was mixed with 1000 µL of reagent 3 and a drop of reagent 2 and allowed to incubate for 5 min. The absorbance of the sample and standard was measured against the blank reagent.

$$\mathbf{Bilirubin\ (mg/dL) = (Asample - Ablank) \times 19.0}$$

Pathological examination

Histology allows the preparation of tissues/organs for observation under the microscope.

Fixation by immersion and trimming

Fixation is the first step and the basis of tissue preparation for microscopy. The purpose of fixation is to preserve the cells and tissue constituents in a state as close to living as possible and to allow the tissue to pass through the steps of the histological technique without change or deformation. The fixative used in this study was 10% formalin. The trimming was performed as follows: the different fixed organs were sectioned in a well- defined plane (transverse or longitudinal) and arranged in labeled histological assets. [9]

Dehydration or circulation

It consists of three steps:

- **The dehydration** consists in removing all traces of water from the tissues to be fixed before its inclusion in the kerosene (indeed water is not miscible with this medium of inclusion). For this process, 8 tanks of alcohol, at a rate of 2 hours in each tank, were used successively in the following order: 1 tank of 80% ethanol (1 × 2 h), 4 tanks of 95% ethanol (4 × 2 h) and 3 tanks of 100% ethanol (3 × 2 h).
- **Thinning** consists of replacing the dehydration solution with a miscible solvent in the inclusion medium. It is a transition step carried out by benzene hydrocarbons such as xylene or toluene. Thus, after dehydration, two baths in xylene (2 × 2 h) were enough to carry out this operation.
- **The impregnation** consists in replacing the water of the cells and tissues by kerosene which hardens the samples. In fact, to obtain thin sections, it is necessary to incorporate a neutral substance (kerosene) into the tissues, which hardens the fragments. The cassettes containing the organ fragments were left in molten kerosene (60°C) for 4 hours (2 × 2 h).

Inclusion and sections

UNDER PEER REVIEW

The embedding provides an external support to the tissues. This allows the realization of microtome sections. The tissues are placed in stainless steel molds filled with molten kerosene (60°C) which is set to solidify on a cold surface after appropriate orientation of the tissue in the block. The resulting blocks were cut on LEICA RM 2125 RT hand wheel microtome. The resulting 5 m thick cutting strips were spread in a water bath containing gelatinized water. Using clean, labelled slides, the sections were collected and dried at 45°C for 24 h.

Staining

Staining allows to see the constituents of a cell and a tissue by having them selectively take up and bind dyes. The stain used in this work is the hematoxylin-eosin stain. Hematoxylin stains the acidic constituents of the nucleus blue-violet and eosin stains the basic constituents of the cytoplasm pink-red. The staining was done in two steps: deparaffinization and rehydration on the one hand and the staining itself on the other hand.

The sections were removed from the kerosene (dewaxing) in xylene baths and rehydrated in alcohol baths with decreasing concentration until distilled water; each bath lasting 5 minutes. The sections were then kept in the following baths: xylene (3×5 min), 100% ethanol (3×5 min), 95% ethanol (1 × 5 min), 80% ethanol (3 × 5 min) and distilled water (1 × 5 min). The actual staining was performed by placing the deparaffinized organ sections successively in the following baths: Harris hematoxylin (2 × 5 min), tap water (2 × 5 min), 70% ethanol (1 × 5 min), 95% ethanol (1 × 5 min), 0.5% alcoholic eosin (1 × 5 min) and tap water (1 × 5 min).

Mounting

A thin glass coverslip was used to protect and preserve the sections. The dehydrated sections were mounted using synthetic resins, which is immiscible with water, as the mounting medium. The mounting was done in two steps: dehydration in three absolute ethanol baths (3 × 5 min) and clearing in three xylene baths (3 × 5 min).

Dehydration

For this purpose, the slides were introduced into the following baths: 50% ethanol (3 minutes), 70% ethanol (3 min), 95% ethanol (3 min) and 100% ethanol (3 × 5 min).

Xylene exposure and mounting

Xylene exposure was used to perform the thinning. For this purpose, the slides were introduced in three xylene baths (3 × 5 min). finally, a thin glass coverslip was used to protect and preserve the sections. The dehydrated sections were mounted using the synthetic resins, which is immiscible in water, as the mounting medium.

UNDER PEER REVIEW

Statistical analysis

The results were expressed in terms of mean ± standard deviation. The comparison between the groups were analyzed using one-way analysis of variance, the ANOVA test followed by *Dunnett's* post-hoc multiple comparison testusing the GraphPadInstat version 5.0 software. A p-value of less than 0.05 was considered statistically significant

RESULT

Acute toxicity

Evaluation of the zootechnical parameters

Behavioral observations following the administration of the plant

None of the animals died during the acute toxicity study, hence the LD_{50} is more than 2000mg/kg. Table 1 shows the behavior of the animals after the administration of a single dose of 2000 mg/kg of the extract of *F. thonningii*.

Table 1: Observation of the behavior of the rats after the administration of the plant extract

Observations		**Day 1 (30min after induction)**	**Day 1 to day 14**
Appearance	Piloerection	++	-
	Abnormal posture	-	-
	Hypo-activity	-	-
Neuromuscular	Hypotonia	-	-
	Convulsion	-	-
	Tremors	-	-
	Myoclonia	-	-
Behavior when opening of the cage	Vocalization	-	-
	Aggressiveness	-	-
	Hypo-reactivity	-	-

UNDER PEER REVIEW

Kinetics of weight change

Figure 1shows the kinetics of weight change of the animals after the administration of the hydro-ethanolic extract of *F. thonningii*(Blume) at a dose of 2000 mg/kg. A positive evolution of the test groups in comparison to the control groups was observed.

Weight gain

Figure 2 shows the weight gain of the animals subjected to the administration of a dose of 2000 mg/kg of the hydro-ethanolic extract of *F. thonningii* in comparison to the control groups. The administration of the plant leads to a non-significant decrease in weight gain in both males and females with a *p-value*> 0.05.

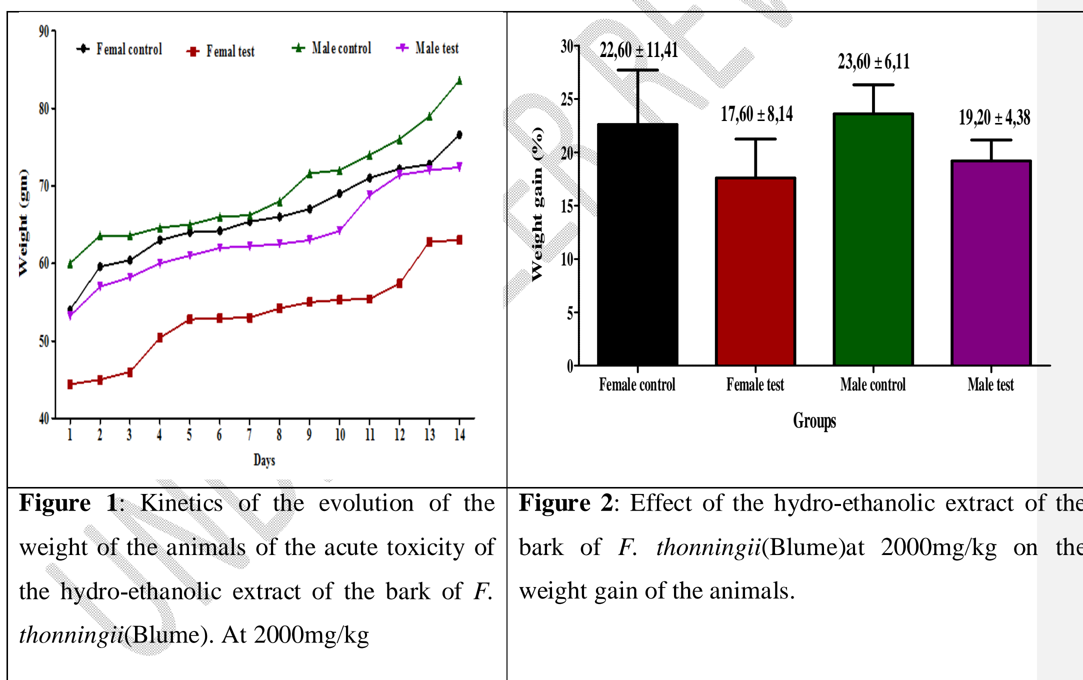


Figure 1: Kinetics of the evolution of the weight of the animals of the acute toxicity of the hydro-ethanolic extract of the bark of *F. thonningii*(Blume). At 2000mg/kg	**Figure 2**: Effect of the hydro-ethanolic extract of the bark of *F. thonningii*(Blume)at 2000mg/kg on the weight gain of the animals.

Food intake

Figure 3 shows that the administration of the hydro-ethanolic extract of *F. thonningii*(Blume) at the dose of 2000 mg/kg leads to a non-significant increase in food intake in both males and females with a *p-value*> 0.05.

Water intake

Figure 4 shows that the administration of the plant leads to a non-significant decrease in water intake in both males and females with a *p-value*> 0.05.

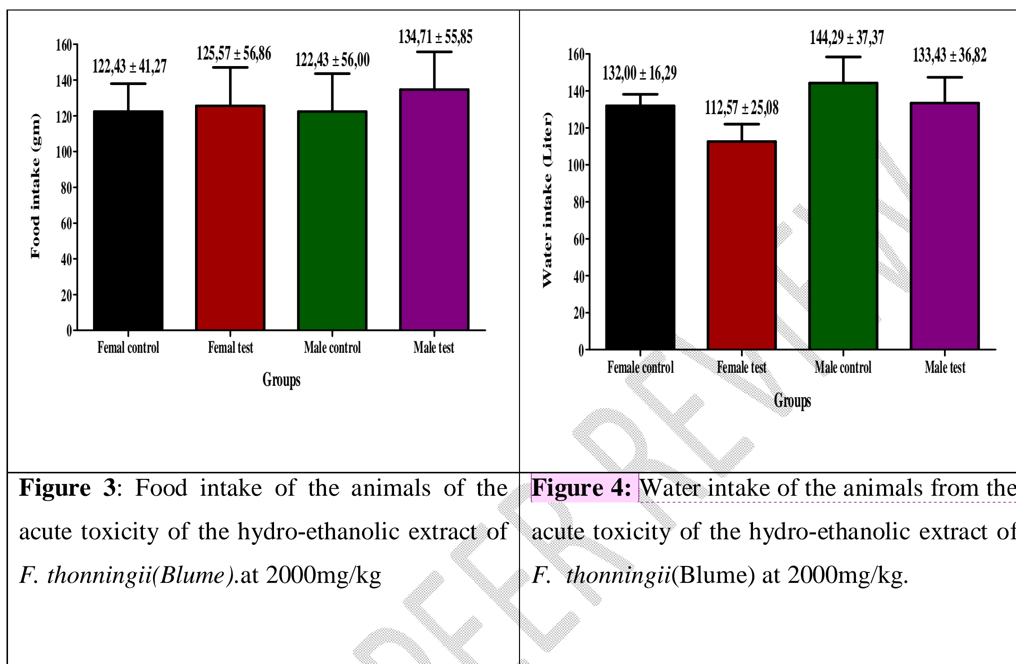


Figure 3: Food intake of the animals of the acute toxicity of the hydro-ethanolic extract of *F. thonningii(Blume)*.at 2000mg/kg

Figure 4: Water intake of the animals from the acute toxicity of the hydro-ethanolic extract of *F. thonningii*(Blume) at 2000mg/kg.

Comment [M15]: Figure 3 and 4 canbecombinedinto a single figure

Comparison of zootechnical parameters

The zootechnical parameters of the different study groups used in the acute toxicity. revealed that the administration of the plant extract to the animalsshowed a non-significant change in food intake, water and weight change as shown in table 2.

Table 2: Comparative table of the zootechnical parameters of different study groups.

Parameters	Female control	Female test	Male control	Male test
Food intake (g)	122,43 ± 41,27	125,57 ± 56,86	122,43 ± 56,00	134,714 ± 55,85
Water intake (mL)	132,00 ± 16,29	112,57 ± 25,08	144,29 ± 37,37	133,43 ± 36,82
Weight intake (%)	23,60 ± 6,11	22,02 ± 7,13	24,60 ± 2,70	23,41 ± 4,05

Comment [M16]: Error in data

The results are expressed as mean ± SEM with n = 5; Data analysis was performed using the ANOVA test, followed by Dunnett's post hoc multiple comparison test. The differences were

considered significant from the *p-value* $*p<0.05$ compared to the rats of the healthy control groups.

The relative weight of organs

The administration of the plant extract did not significantly affect the relative weight of the animals with a *p-value* > 0.05. The plant did not affect the macroscopy through visual inspection of the internal organs of the animals comparing the test and control animals (Table 3).

Table 3: The effect of the extract (2000mg/kg) on the relative weight of the organs of the animals.

Organs		Female Control	Female Test	Male Control	Male Test
Brain		1,09 ± 0,20	1,17 ± 0,17	1,30 ± 0,12	1,07 ± 0,19
Lungs		0,56 ± 0,10	0,59 ± 0,14	0,76 ± 0,27	0,61 ± 0,11
Liver		2,97 ± 0,57	1,96 ± 0,31	3,09 ± 0,34	2,67 ± 0,73
Spleen		0,26 ± 0,07	0,29 ± 0,07	0,54 ± 0,39	0,37 ± 0,26
Heart		0,34 ± 0,11	0,28 ± 0,08	0,36 ± 0,07	0,29 ± 0,09
Kidney	**Left**	0,37 ± 0,05	0,28 ± 0,05	0,38 ± 0,06	0,30 ± 0,08
	Right	0,37 ± 0,05	0,27 ± 0,05	0,37 ± 0,04	0,31 ± 0,08
Ovary/ Testes	**Left**	0,05 ± 0,05	0,05 ± 0,04	0,50 ± 0,25	0,49 ± 0,33
	Right	0,06 ± 0,04	0,07 ± 0,06	0,46 ± 0,30	0,48 ± 0,31

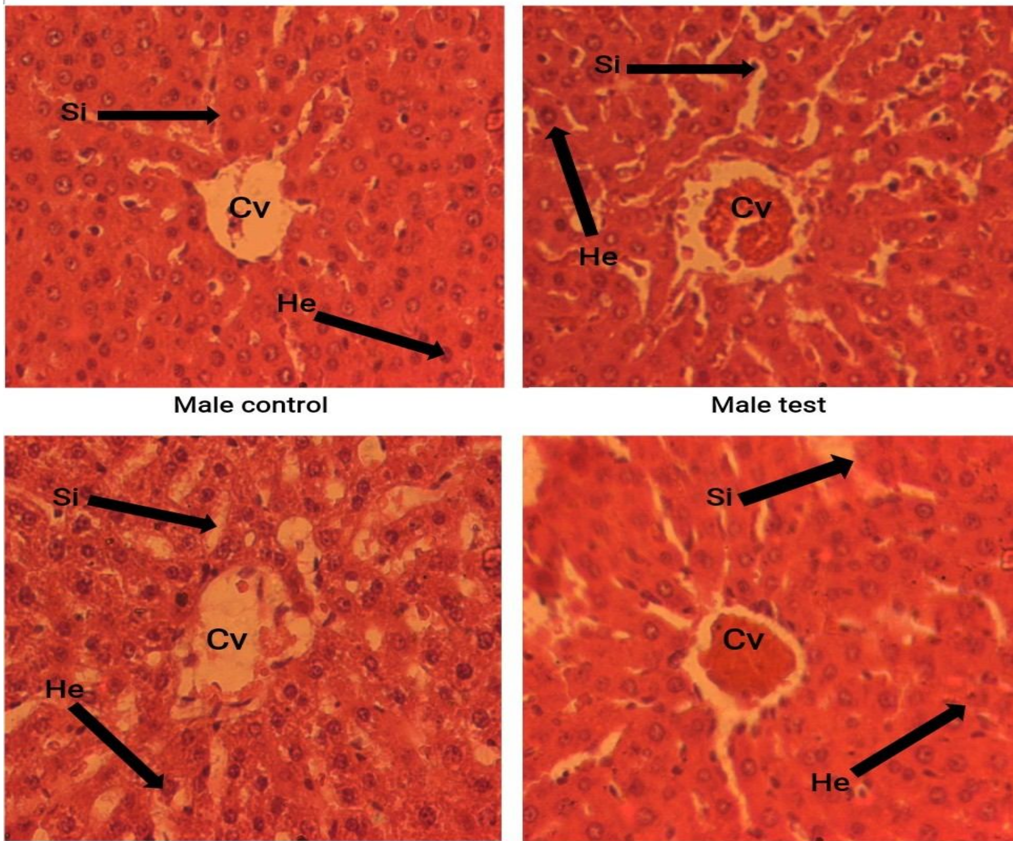
The results are expressed as mean ± SEM with n = 5. Data analysis was performed using the ANOVA test, followed by Dunnett's post-hoc multiple comparison test. The differences were considered significant from the p-value $*p<0.05$ compared to the rats of the control groups.

UNDER PEER REVIEW

Histological analysis

Liver histology analysis

Analysis of the sections (Figure5) showed no changes in the structure of the liver in the control groups, with the centrilobular vein, hepatocytes, and sinusoids well identified. However, an obstruction of the centrilobular vein was observed in the test groups, indicating possible hepatic cytolysis.



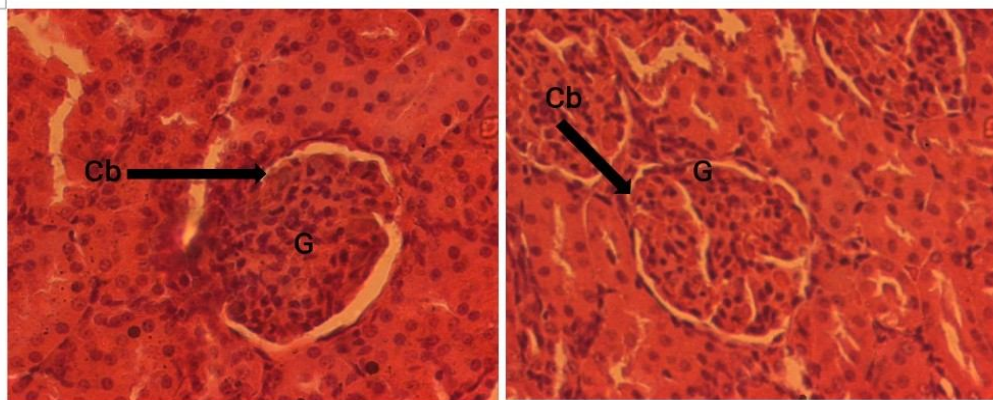
Female Control Group **Female treatment Group**

Cv = Central vein; **He** = Hepatocytes; **Si** = Sinusoids.

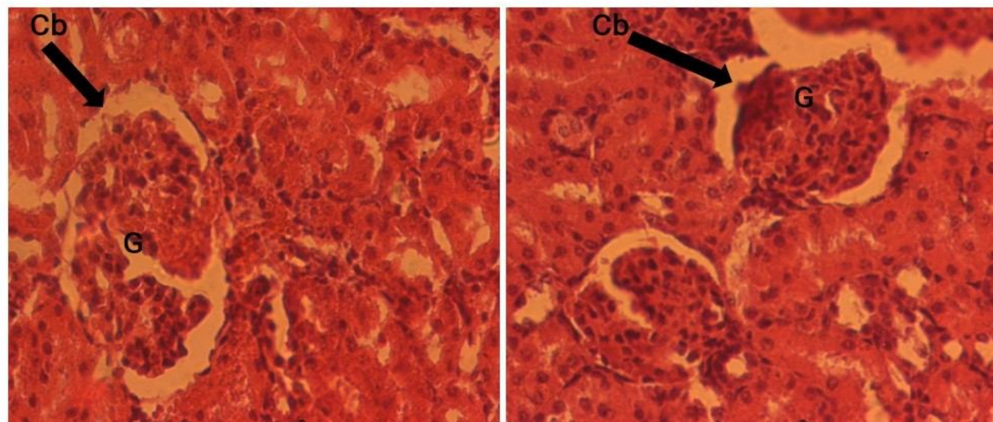
Figure 5: Histology of liver in acute toxicity.

Kidney histology analysis

Analysis of the sections (Figure6) did not reveal major changes in the structure of the kidney in all groups. The well-structured glomerulus was observed as well as Bowman's capsule with its two well-identified leaflets, the urinary space well represented and the podocytes well identified.



Male control Male test



Female Control Group **Female Control Group**

GB = Capsule de Bowman ; **GL** = Glomerulus.

Figure 6: Histology of the kidneys in acute toxicity.

Biochemical analysis

Effect of plant extract on liver parameters

The administration of the hydro-ethanolic extract of the bark of *Ficusthonningii*(Blume) at a dose of 2000mg/kg with a *p-value* > *0.05*did not significantly affect liver enzyme activity (AST and ALT) in both male and female groups. In addition, the AST/ALT ratio was close to 1 indicating the absence of hepatic cytolysis.The administration of the extract led to a non-significant increase

with a *p-value > 0.05*, in the activity of alkaline phosphatase. The slight rise in total protein levels indicates possible inflammation following administration of the extract (figure 7).

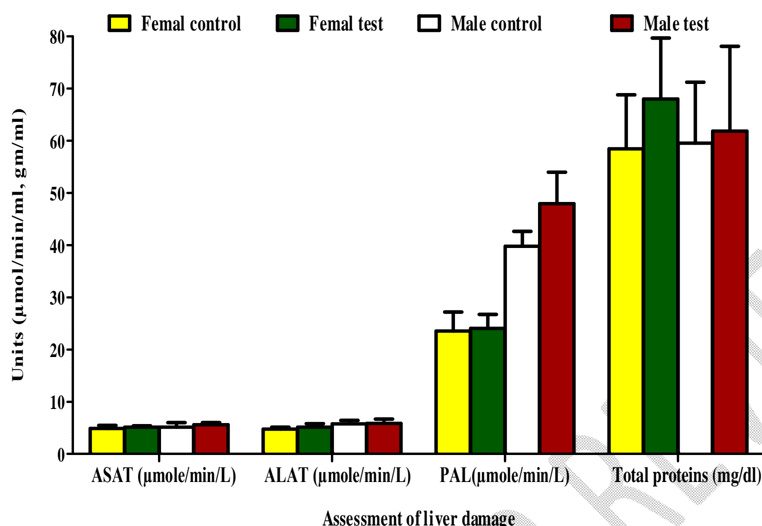


Figure 7: Effect of the *F. thonningii* extract (2000mg/kg) on liver parameters in acute toxicity.

Effect of plant extract on kidney parameters

The administration of the extract leads to a non-significant increase in the concentration of urea with a *p-value > 0.05*. With regard to uric acid and creatinine, no significant change was observed table 3.

Table 3: Biochemical parameters of the plant extract in acute toxicity.

Parameters	Male Control	Test male	Female control	Test Female
ASAT (µmol/min/mL)	5,12 ± 1,94	5,58 ± 0,98	4,89 ± 1,30	5,14 ± 0,50
ALAT (µmol/min/mL)	5,75 ± 1,53	5,86 ± 1,88	4,74 ± 0,85	5,13 ± 1,45
PAL (µmol/min/mL)	39,80 ± 6,31	47,94 ± 13,46	23,53 ± 7,34	24,06 ± 5,96
Total Proteins (mg/dl)	59,54 ± 26,18	61,85 ± 36,35	58,46 ± 23,15	68,00 ± 26,09
Creatinine(µmol/l)	3,45 ± 0,32	3,60 ± 0,14	3,69 ± 0,11	3,38 ± 0,19
Urea (mmol/l)	12,19 ± 5,14	12,50 ± 8,84	10,94 ± 5,98	12,50 ± 7,22

UNDER PEER REVIEW

Uric acid x 10^4 (µmol/l)	4,18 ± 0,0001	4,18 ± 0,0004	4,18 ± 0,0003	4,18 ± 0,0002

The results are expressed as mean ± SEM with n = 5; Data analysis was performed using the ANOVA test, followed by Dunnet's post hoc multiple comparison test. The differences were considered significant from the *p-value* $^*p<0.05$ compared to the rats of the healthy control groups.

DISCUSSION

Despite the popularity of medicinal plants, few scientific studies have been undertaken to determine the safety of traditional medicinal plants. It is argued, that plants are not only effective but also very safe as compared to conventional drugs for similar indications. The claim that natural plant products are safe should be accepted only after the plant product has passed through toxicity testing using modern scientific methods [21].

Ficus genus belongs to the Moraceae family and includes extremely different plants, from trees exceeding 30 m high in their countries of origin, to dwarf, crawling species.*F. thonningii*(Blume) has been used in several countries as a medicinal plant in the treatment of several ailments such asurinary tract infections, diabetes mellitus, gonorrhea, respiratory infections, mental illnesses and peptic ulcers [6, 22]. This study was done to evaluate the toxic effects of *F. thonningii* stem bark considering the fact several researchers [15, 22, 19] have shown that it has promising biological activity in the management of peptic ulcers, a globally prevalent disease, with little attention accorded to the study of its toxicity.

In the acute toxicity assay, oral treatment with the stem bark extract of *F. thonningii*was well tolerated. A single dose of 2000 mg/kg of the plant administered orally to both male and female rats did not cause signs of toxicity, changes in behavior, or mortality.The administration of the hydroethanolic extract at different dose ranges led to a non-significant decrease in weight gain in both males and females. The administration of the hydro-ethanolic extract of *F. thonningii*(Blume) at the dose of 2000 mg/kg leads to a non-significant increase in food intake and a non-significant decrease in water intake in both males and females.The administration of the plant extract did not significantly affect the relative weight of the animals as well as the macroscopy of the internal organs of the animals. Histopathological analysis of the liver revealed signs of hepatic cytolysis whereas there was no sign of toxicity to the kidneys. Biochemical

UNDER PEER REVIEW

analysis of the liver damage parameters revealed that there is no significant change in liver enzymes (AST and ALT) and alkaline phosphatase indicating that this plant had no real damage to the liver tissue. Also, analysis of kidney damage parameters shows that there is no significant change in creatinine, urea and uric acid levels.This is in agreement with the work done by Tembe et al. (2018)[17] and that which was done on the leaves of *F. thonningii* by Stanley (2008)[23, 24]. The similarity in the results from the oral acute study after five years of preparation of plant material confirms the bridging of the toxicology data at a single dose.

In general, changes in body weight of treated animals, as well as the vital organs (liver,kidneys, lungs, adrenal glands, testicles, ovaries, spleen, stomach and heart), are indicators of toxicity[20, 21].

CONCLUSION

Our study showed that, when a single dose of 2000 mg/kg of *F. thonningii* stem bark extract was administered, there was no death and no significant effects on liver and kidney function.It also showed that the three dose levels (125 mg/kg, 250 mg/kg, 500 mg/kg) administered daily to the animals for a period of 28 days caused a reversible alteration of liver function and no significant impact on kidney function.

UNDER PEER REVIEW

REFERENCES

Ondigo DA, Munyendo WLL, Andala D, Maima AO, Mosweta JM, Odhiambo KW Enhancement of antibacterial activity through phyto-fabrication of silver nanoparticles with Ficus thonningii aqueous extracts.IETNanobiotechnol. 2022 Sep;16(7-8):250-258. doi: 10.1049/nbt2.12093. Epub 2022 Aug 9. PMID: 35942698

1. Adane H, Atnafie SA, Kifle ZD, Ambikar D. Evaluation of *In Vivo* Antiulcer Activity of Hydro-Methanol Extract and Solvent Fractions of the Stem Bark of *Ficus thonningii* (Moraceae) on Rodent Models. .Biomed Res Int. 2021 Apr 9;2021:6685395. doi: 10.1155/2021/6685395. eCollection 2021.PMID:

2. Dangarembizi R, Erlwanger KH, Chivandi E Effects of Ficus thonningii extracts on the gastrointestinal tract and clinical biochemistry of suckling rats.Afr J Tradit Complement Altern Med. 2014 Jan 28;11(2):285-91. doi: 10.4314/ajtcam. v11i2.10. eCollection 2014.PMID: 25435610.

3. Koukouikila-Koussounda F, Abena AA, Nzoungani A, Mombouli JV, Ouamba JM, Kun J, Ntoumi F In vitro evaluation of antiplasmodial activity of extracts of Acanthospermumhispidum DC (Asteraceae) and Ficus thonningii Blume (Moraceae), two plants used in traditional medicine in the Republic of Congo. .Afr J Tradit Complement Altern Med. 2012 Dec 31;10(2):270-6. eCollection 2013.PMID: 24146450

4. Balehegn M, Eik LO, Tesfay Y. Replacing commercial concentrate by Ficus thonningii improved productivity of goats in Ethiopia.TropAnim Health Prod. 2014 Jun;46(5):889-94. doi: 10.1007/s11250-014-0582-9. Epub 2014 Apr 9. PMID:

5. Ango PY, Kapche DW, Fotso GW, Fozing CD, Yeboah EM, Mapitse R, Demirtas I, Ngadjui BT, Yeboah SO.Z Thonningiiflavanonol A and thonningiiflavanonol B, two novel flavonoids, and other constituents of Ficus thonningii Blume (Moraceae).Naturforsch C J Biosci. 2016 Mar;71(3-4):65-71. doi: 10.1515/znc-2015-0147.PMID: 26959540.

6. Ahur VM, Adenkola YA, Saganuwan SA, Ikye-Tor JT. Vet Res Forum. 2013 Fall;4(4):207-12. PMID: 25568673 Ameliorative properties of aqueous extract of Ficus thonningii on erythrocyte osmotic fragility induced by acetaminophen in Rattus norvegicus.

7. Falade MO, Akinboye DO, Gbotosho GO, Ajaiyeoba EO, Happi TC, Abiodun OO, Oduola AM.J In Vitro and In Vivo Antimalarial Activity of Ficus thonningii Blume (Moraceae) and Lophiraalata Banks (Ochnaceae), Identified from the Ethnomedicine of the Nigerian Middle Belt.Parasitol Res. 2014; 2014:972853. doi: 10.1155/2014/972853. Epub 2014 May 14. PMID: 24955248

8. Alturkistani HA, Tashkandi FM, Mohammedsaleh ZM. Histological stains: A literature review and case study. Global J health Sci. 2016, *(3): 71-79. Doi10.5539/gjhs.v8n372.PMCID: PMC4804027.

9. World Health Organization (WHO). (2008). Traditional medicine; Fact sheet number 134.

10. Ali S., Nasreen S., Safeer S., Andleeb S., Ejaz M. and Shakir H. A. Medicinal plants as therapeutic agents for cancer treatment.Punjab Univ. J. Zool. 31(2). 2016;295–305.

11. Egharevba O.H., Carew O., Kunle O.F. Phytochemical and Pharmacognostic Analysis of *Ficus thonningii* Blume Leaves for Monograph Development. Int. j. basic appl. 4(2). 2015;94–100.

12. Dangarembizi R., Kennedy H.E., Davison M., and Eliton C. Phytochemistry, pharmacology and ethnomedicinal uses of *Ficus thonningii* (Blume Moraceae): a review. Afr J Tradit Compl Altern Med. 2013;10(2):203–12.

13. Pougoue K.J. Investigation of the antiulcer, and toxicity of the stem bark extract of *Ficus thonningii* Blume Linn. (Moraceae) on Wistar rats. university of Yaounde 1; 2017.

14. Adane H., Atnafie S.A., Kifle Z.D., Ambikar D. Evaluation of In Vivo Antiulcer Activity of Hydro-Methanol Extract and Solvent Fractions of the Stem Bark of *Ficus thonningii* (Moraceae) on Rodent Models. Biomed Res Int. 2021;6685395.

15. Usman H., Abdulrahman F.I., Usman. Qualitative phytochemical screening and in vitro antimicrobial effects of methanol stem bark extract of *Ficus thonningii* (Moraceae). Afr J Trad. 6 (3). 2009;289–95.

16. Tembe F.E., Pougoue K.J., Ngoupayo J., Njunkio B.N., Nguidjoe E., Tabi Y.O., et al. Evaluation of the Toxicity of Secondary Metabolites in Aqueous Extracts of *Ficus thonningii* (Blume) in Wistar rats. AM j ethnomed. 5(2). 2018;13.

17. World Health Organization. Regional Office for South-East Asia. A compilation of technical papers presented during regional meeting, Bangkok, Thailand. 16-18 December, 2014. In New Delhi:

18. World Health Organization. Regional Office for South-East Asia; 2014 [cited 2021 Nov 13]. Available from: https://apps.who.int/iris/handle/10665/272986

19. Galati G., O'Brien P.J. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radic Biol Med. 2004;37(3):287–303.

20. Christophe M, Longo F, Nkenfou C, Sando Z, Ndeme E, Tan P. Evaluation of acute and subacute toxicity of stem bark aqueous extract of *Anthocleista schweinfurthii* (Loganiaceae). World J Pharm Pharm Sci. 2015 Mar 4;4:197–208.

21. Blanchard D.C., Fukunaga-Stinson C., Takahashi L. K., Flannelly K.J., Blanchard R.J. Dominance and aggression in social groups of male and female rats. Behav Processes [Internet]. 1984 [cited 2022 May 29];9(1):31–48. Available from: https://linkinghub.elsevier.com/retrieve/pii/0376635784900068.

22. OECD Guidelines for the testing of chemicals. Test No. 420: Acute Oral Toxicity - Fixed Dose Procedure | OECD Guidelines for the Testing of Chemicals, Section 4 : Health Effects | OECD iLibrary [Internet]. [cited 2022 Mar 14]. Available from: https://www.oecd-ilibrary.org/environment/test-no-420-acute-oral-toxicity-fixed-dose procedure_9789264070943-en.

23. Fongang Y.S.F., Bankeu J.J.K., Ali M.S., Awantu A.F, Zeeshan, Assob C.N., et al. Flavonoids and other bioactive constituents from *Ficus thonningii* Blume (Moraceae). PHYTOL [Internet]. 2015 Mar [cited 2022 Jun 15];11:139–45. Available from: https://linkinghub.elsevier.com/retrieve/pii/S187439001400233X..

24. OECD Guidelines for the testing of chemicals. Repeated dose 28-day oral toxicity study in rodents. In 2008..