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Evaluation of Subacute Toxicity of Hydroethanolic Extracts Combinations from *Gnetum africanum* (Welv.) and *Gnetum buchholzianum* (Engl.) (Genetaceae) Leaves: Two Botanical with Antiproliferative and Antioxidant Potential

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Abstract

This study was conducted to assess the toxic effect of hydroethanolic extract combinations of *Gnetum africanum* Welv. leaves and *Gnetum buchholzianum* Engl. (Gnetaceae) in experimental rats to test the validity of the treatment of liver disorders related to oxidative stress. The Combinations (m/m) 50-50 for E2 and 75-25 for E3 of ethanol-water extracts from plant leaves at the respective doses of 100, 200, and 400 mg/kg of body weight were used for 32-day toxicity. They were obtained after harvesting leaves, sorting, drying in the air cover for three weeks, and grinding. The resulting powder was doubly macerated with 70% ethanol for 48 hours and filtered. The filtrate was concentrated with the Heidolph-brand rotary rotavapor and each extract obtained was preserved. The administrations were carried out by gavage to wistar, male and non-pregnant female albino rats. In the end, the animals were sacrificed. The serum and organ homogenates were obtained for biochemical,

tissue, and histopathological analyses respectively. The analyses revealed insignificant variations at the 5% probability threshold of the weight growth of experimental animals. These variations were found to be statistically significant at the same probability for biochemical and tissue parameters based on the dose of plant extracts and compared to control animals. Histopathological analysis of liver tissue showed leukodate infiltration that indicates extract-induced inflammation of the hepatocytes at the 400 mg/kg dose of body weight in females. However, this infiltration of the cells would have improved the regeneration of hepatocytes justified by the normal rate of transaminases. These results showed that combinations of hydroethanolic extracts of *G. africanum* and *G. buchholzianumare* non-toxicand may be potential candidates in the Cameroonian flora medicinal plant database shown in the monitoring of oxidative stress-related diseases.

Keywords

Gnetum africanum, *Gnetum buchholzianum*, Subacute Toxicity, Oxidative Stress, Albino Rats

1. Introduction

From ancient times to the present day, science has always been concerned with optimizing the pharmacological effect of medicinal plants and their interactions with the environment [1]. Also, humans have always been in a permanent quest to search for new natural substances to prevent and cure diseases these new substances provide the basic use for primary health care for populations, especially those living in tropical and subtropical areas [2] [3]. However, there is a paradox about these substances. Despite their importance and daily use as Enhanced Traditional Medicines (ATMs), little information is available on their toxicological value involving the regulation and management of (MT) [4].

Several studies have shown the pharmacological efficacy of a large number of medicinal plants. These studies have demonstrated antiproliferative, antimicrobial, anti-inflammatory, anti-tumor, anti-cancer, antioxidant activity, and significant effects in the healing process or in the treatment of cardiovascular disease [5]. Among these plants, the genus Gnetum which two species are present in Africa; *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. These plants which are heavily used as food are also thought to be potential sources of antioxidants with strong pharmacological properties [6]. Ethnobotanical study shows the *Gnetum* spp. has medicinal properties and has been used traditionally to treat different ailments. In Nigeria, for example, leaves known locally as afang are used to treat spleen, angina, and cathartic. In the Democratic Republic of Congo, where they are known as fumbwa, they are used to fight nausea, as well as being an antidote against certain poisons, the leaves are used to fight warts, hemorrhoids, and boils. Due to it high fibre content, it is used to treat constipation in Mozambique [7]. It also eases the pain of contractions and therefore facilitates

childbirth [8].

In Cameroon, and particularly in English and French parts, these leaves are known respectively as "eru" and "okok" and are used in traditional medicine in the treatment of anaemia in children. A study based on the anti-diarhic activity of extracts from these leaves has been reported by [9]. Literature reveals abundant therapeutic efficacy of these plants although they should be free of adverse side effects for the patient since the main criterion for the selection of medicinal plants is above all safety [10]. In this regard, the literature provides insufficient information on the toxicological effects associated with the use of these plants. Our study aims to assess the subacute toxicity of ethanolic extract combinations of *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. Wistar rats. To achieve this, we proceeded by obtaining crude extracts of the two plants, followed by phytochemical screening. Finally, the induction of subacute toxicity in Wistar rats and the control of biochemical and histological parameters were carried out.

2. Material and Methods

2.1. Plant Material

The leaves of *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. were harvested from Dibombari, Coastal Region of Cameroon (**Lat: 4.3179; Lon: 9.7209**) and Limbe Southwest Region of Cameroon (**Lat: 4.1796; Lon: 9.2825**) respectively. They were identified at the National Herbarium Center (HNC), Cameroon and voucher specimen number assigned; **NA 55068HNC** for *Gnetum africanum* and **NE 52338HNC** for *Gnetum buchholzianum*.

2.2. Preparing Combinations of Extracts

After harvest, they were transported to the laboratory where they underwent successive tap water washing, dripping and drying in the shade for three weeks. They were then ground until a fine powder was obtained. The resulting powder was stored in a glass container and stored away from air and moisture until the extraction stage.

2.3. Extraction

The powder (2.72 Kg) of *G. africanum* and *G. buchholzianum* leaves was used to extract bioactive compounds by ethanol-distilled water (70-30) maceration for 48 hours. After filtration, the residue was then rinsed with the same solvent and extracted again by the previous system and under the same conditions. The different filters were evaporated each time to the Heidolph brand rotary rotavapoor under reduced pressure and each hydroethanolic extract was obtained and then preserved.

2.4. Obtaining the Combinations of Hydroethanolic Extracts

A fraction of each hydroethanolic extract was used to obtain combinations of

extracts in proportions (m/m) of (25-75), (50-50) and (75-25) referred to respectively as E1, E2, E3, 25%, 50% and 75% of the extract of *G. africanum* Welv. and 75%, 50%, 25% the extract of *G. buchholzianum* Engl.

2.5. Phytochemical Screening

The purpose of phytochemical screening is to detect the presence of large classes of secondary metabolites present in each extract. Thus, the presence of secondary compounds such as flavonoids, coumarines, anthraquinones, tannins, terpenoids, saponosides and alkaloids was sought using the methods described by [11].

2.6. Animal Material and Experimentation

42 non-gravitated males and females Wistar albino rats weighing an average of 140 ± 35 g were enrolled. These rats were obtained in August 2019 from the Research Laboratory in Food and Nutrition Sciences of the Faculty of Science of the University of Douala. They are raised in cages lined with renewable wood chips every two days, with free access to water and food in an environment subject to 12 hours of light and 12 hours of alternating darkness. During the acclimatization and multiplication period, they are fed a reference food called the whole food of the food sells with a supplement of 50 g/kg of protein from the fish *Ethmalosa* sp. and vitamin C supplementation to be able to withstand experimentation (**Table 1**) [12].

2.7. Subacute Toxicity

This phase was conducted in line with OECD Guidelines 407 [14]. It was conducted on 42 Wistar albino rats divided into seven equal batches of 3 males and 3 females as follows: lot 1 (controls), receiving distilled water at a rate of 1 ml/100

Table 1. Rat feeding composition [13].

Ingredients	Quantity (g/kg)
Casein	150
Corn flour	397.5
L-Cysteine	3
Cellulose	50
Sucrose	100
Maltose	132
Mineral salt mixture	35
Vitamin Mix	10
Choline bi tartrate	2.5
Soybean oil	70
Fishmeal (Ethmalosa sp)	50

mg of body weight (control lot), lot 2 (E2-100), lot 3 (E2-100) lot 4 (E2-400), lot 5 (E3-100), lot 6 (E3-200), and lot 7 (E3-400), receiving a solution of the E2 and E3 extract combinations at a rate of 100, 200, 400 mg/kg body weight. The treatment lasted 32 days. Water intake, food intake and weight growth were assessed every 2 days.

2.7.1. Sacrifices of Rats and Collects Samples

At the end of the treatment, the rats were fasted for 24 hours and then sacrificed after ketamine administration, at a rate of 50 mg/kg. Blood samples for biochemical tests were taken, followed by organ sampling. The organs taken were the liver, kidneys and heart. These were rinsed with a salty solution at 0.9% and then weighed [15].

3 mg of each organ were weighed kept at −20˚C for grinding and obtaining homogenates. The grinding obtained using a porcelain mortar was used for further dosage of tissue stress parameters (SOD, CAT, MDA and GSH). The remaining organ was preserved after labelling in 10% formaldehyde for histopathological investigation.

2.7.2. Analysis of Biochemical Parameters

The measurement of transaminase activities (ALAT, ASAT) and quantification of total proteins, creatinine and urea was carried out using methods taken from the literature with slight modifications. This activity and dosage was measured using the LABKIT kit.

2.7.3. Analysis of Tissue Parameters

The measurement of the activities of CTU, SOD, GSH and MDA were carried out respectively using the modified method of [16] [17] [18] [19].

2.7.4. Histopathological Investigation

Liver and kidney samples stored in 10% formaldehyde were and treated with Conventional techniques. These tissues were then dehydrated at high alcohol levels, then inserted into the paraffin, and cut into 4 - 5-m sections. These paraffin cuts (5 m thick) were coloured with hematoxyline-eosine prior to microscopic examination [20].

2.8. Statistical Analysis

The data was entered into a 2010 Excel sheet—USA and subsequently exported to GraphPad version 7.0.1 (GraphPad, San Diego, USA) for statistical analysis. The data were presented as an average ± Average Standard Error (ESM) in graphs. Non-parametric tests of Mann-Whitney and Kruskal-Wallis were used to make comparisons. The significance threshold has been set at $p\text{-value} < 0.05$.

2.9. Ethical Consideration

Ethically, an application for ethical clearance was filed and obtained from the Institutional Ethics Committee of the University of Douala (IEC-UD) under the

registration code **No 1748CEI-UDo/08/2019/T.**

3. Results

3.1. Phytochemical Screening

The different ethanol extracts obtained after maceration were dark green in colour and patous in appearance. The combined extracts revealed the presence of groups of chemical compounds such as saponins, sterols and flavonoides while alkaloids were absent (Table 2). The yield on the extract was 40.2%.

Table 2. Phytochemical screening of combinations of hydroethanol extracts from *G. africanum* (EBa) and *G. buchholzianum* (EBb).

Secondary metabolites	Observations	E1	E2	E3
Saponins	Plenty of moss	+	+	+
Cathechic tannins	Appearance of a green blue color	−	+	+
Alkaloids	No appearance of a white precipitate	−	−	−
Sterols	Forming a red ring	+	+	+
Flavonoids	Presence of red coloration	+	+	+

Caption: −: Negative; Positive: Positive.

3.2. Subacute Toxicity

3.2.1. Change in Settings Based on Duration of Follow-Up

1) Evolution of weight growth

Male and female animals subjected under the same experimental conditions do not have the same weight growth. Also at the probability threshold $P < 0.05$, there is no statistically significant difference between the different concentrations of extracts administered and the sex (Figure 1 and Figure 2). The daily administration of leaves hydroethanolic extract combinations did not cause any weight loss in experimental animals compared to male and female control lots (Table 3).

Table 3. Varaition of animal weight during experimentation.

Sex	Males (Weight (g))			Females (Weight (g))		
Animals	Pi	Pf	ΔP	Pi	Pf	ΔP
control	166.4	204.6	38.2	144.8	172.25	27.45
E2-100	168.2	207.6	39.4	143.4	164	20.6
E2-200	163.2	214	50.8	138.6	156.25	17.65
E2-400	168.8	207	38.2	139.4	168.75	29.35
E3-100	156	214.5	58.5	136	170.5	34.5
E3-200	178.4	212.8	34.4	151.2	180	28.8
E3-400	175.4	212.2	36.8	134	143	9

Caption: Pi: Initial Weight; Pf: Final Weight; ΔP: Weight Variation.

a) Combination E2 in female rats

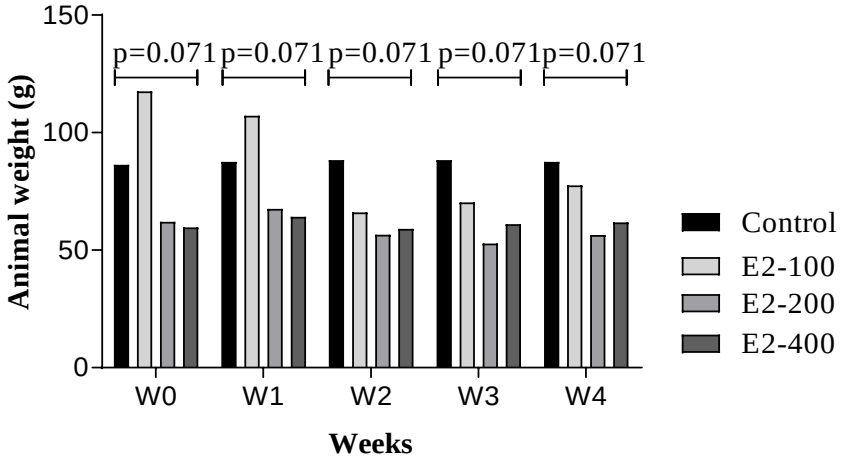


Figure 1. Evolution of weight growth based on the E2 combination and follow-up weeks in females. Each bar represents the average of the parameter, the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

b) Combination E3 in male rats

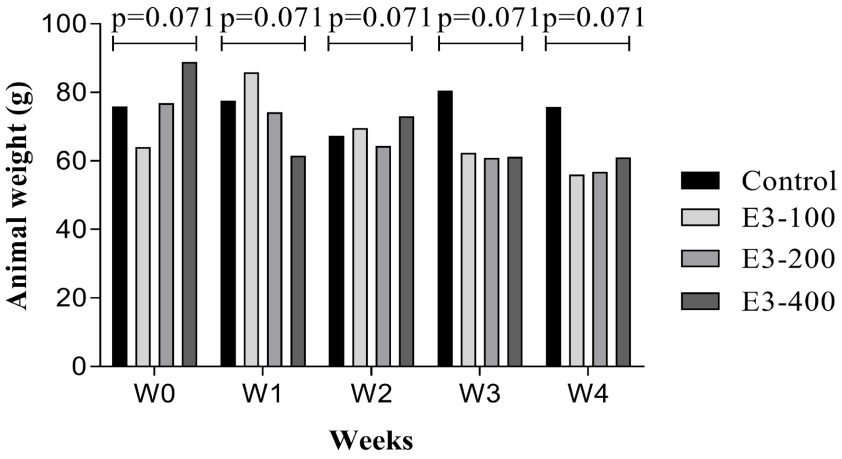


Figure 2. Evolution of weight growth based on the E3 combination and follow-up weeks in males. Each bar represents the average of the parameter, the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

2) Water intake

At varying concentrations and combinations, there is a statistically significant difference at $p < 0.5$ between the combinations of E2 and E3 extracts respectively for the 100 and 200 mg/kg body weight concentrations of experimental female animals. In male animals, this difference is statistically to the concentration of 200 mg/kg of body weight at P −0.001 (Figure 3 and Figure 4). The daily administration of concentrations of 200 and 400 mg/kg of body weight resulted in a decrease in water consumption in females compared to males.

a) Combination E2 and E3 in female rats

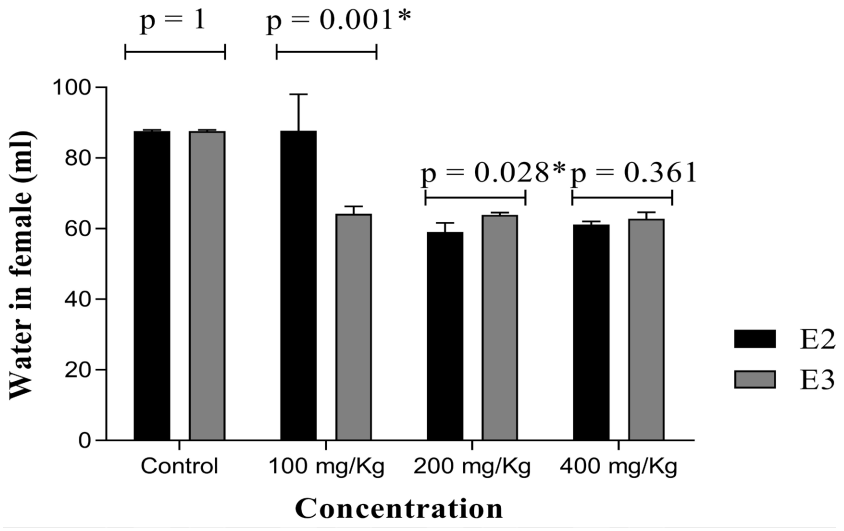


Figure 3. Change in water intake based on dose of different leaf extracts in females. Each bar represents the average of the parameter, the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

b) Combination E2 and E3 in male rats

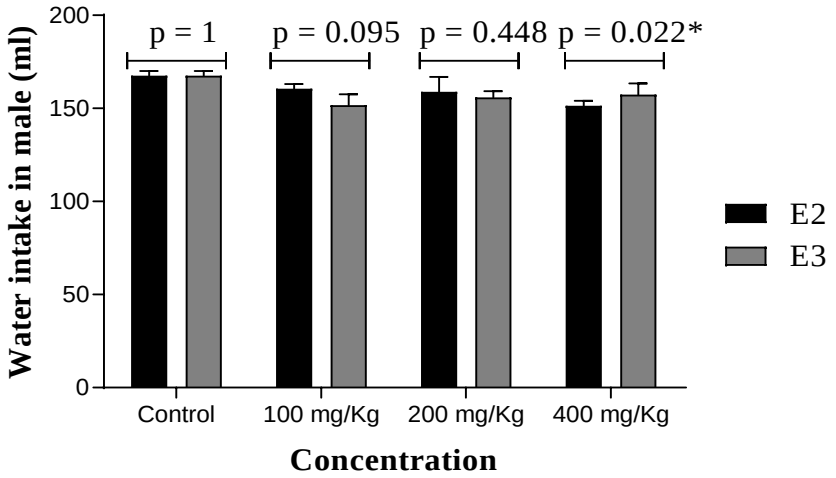


Figure 4. Change in water intake based on dose of different plants in males. Each bar represents the average of the parameter; the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

3) Food intake

a) Combination E2 and E3 in female rats

In female animals, the difference is statistically significant at $p < 0.5$ between the combinations of E2 and E3 extracts respectively for the 100 and 200 mg/kg body weight concentrations of the experimental female animals. In male animals, however, there is no statistical difference to $p < 0.05$ regardless of the concentration of extract administered as animals during the experiment (**Figure 5** and **Figure 6**). The daily administration of concentrations of hydroethanolic extract combinations did not result in any decrease in food consumption in males.

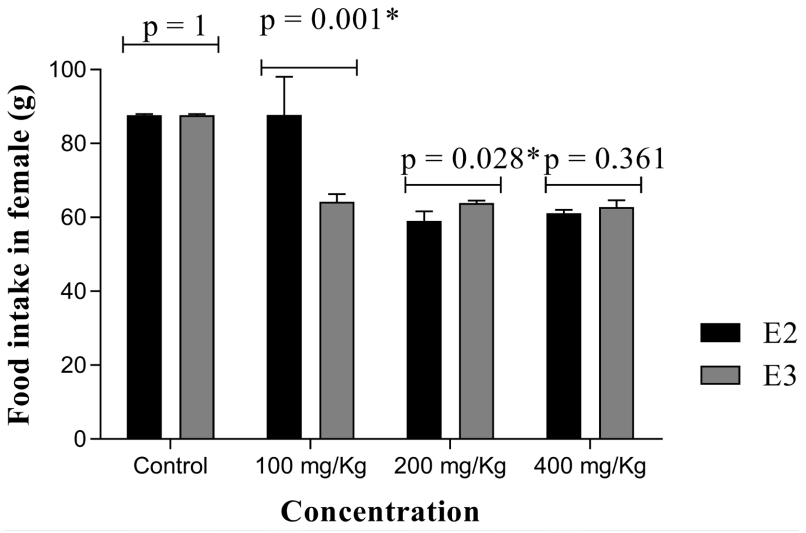


Figure 5. Change in food intake based on dose of different plants in females. Each bar represents the average of the parameter, the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

b) Combination E2 and E3 in male rats

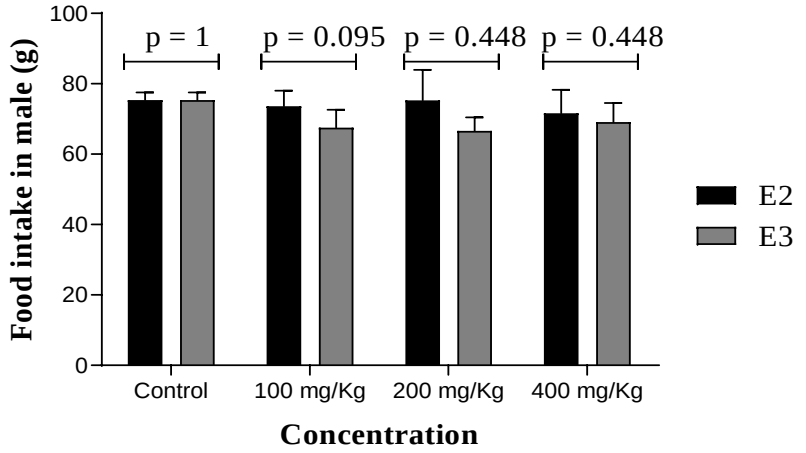
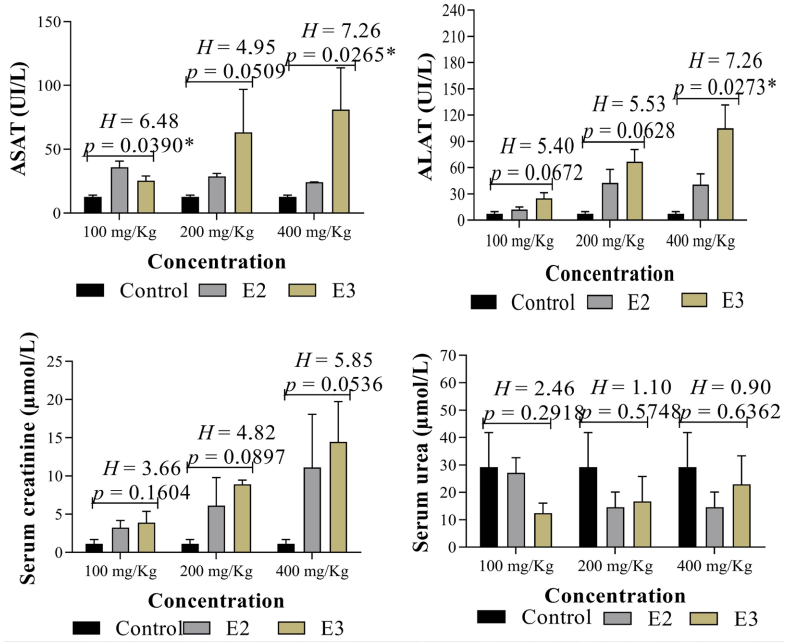


Figure 6. Change in food intake based on dose of different plants in males. Each bar represents the average of the parameter, the Mann-Whitney test was used to make the comparisons; *: Statistically significant at p-value < 0.05.

3.2.2. Effects of E2 and E3 Extract Combinations on Tissue Parameters in Male and Female Experimental Rats

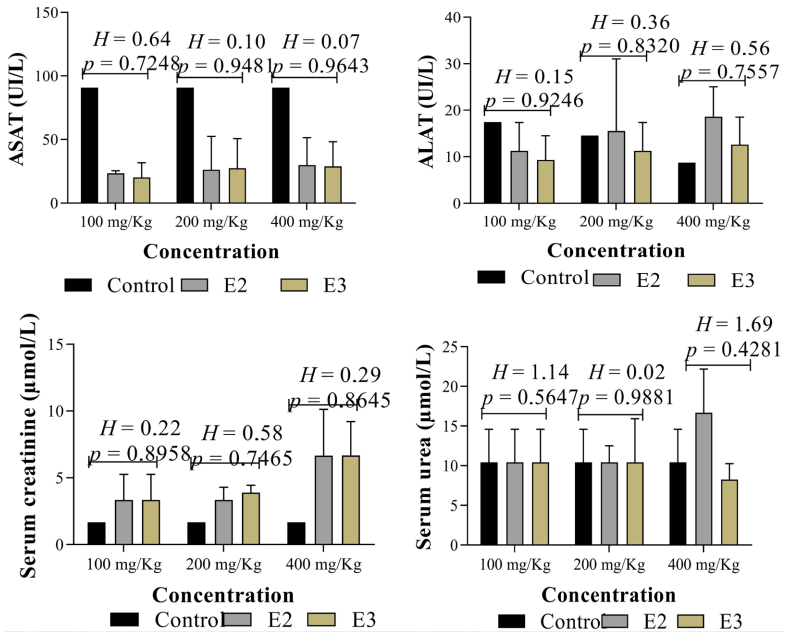
The figures below represent the values of the biochemical parameters of the combinations of E2 and E3 extracts in male and female animals based on the different concentrations administered during the experiment. At the E2 and E3 concentration of 400 mg/kg of body weight, there is a statistically significant difference between the levels of ALAT, uremia and creatininemia in males at the 5% threshold. This difference is statistically significant at the threshold of 0.0273% in females for ALAT levels, with 0.039% ASAT suggesting possible alteration (**Figure 7(A)** and **Figure 7(B)**).

a) In female's rats



(A)

b) In male's rats



(B)

Figure 7. (A) Effects of E2 and E3 extract combinations on biochemical parameters in female experimental rats. (B) Effects of E2 and E3 extract combinations on biochemical parameters in male experimental rats. The data are presented as an average ± Average Standard Error (ESM). The Kruskal-Wallis non-Parametric test was used to make comparisons: Statistically significant at the 5% threshold.

3.2.3. Effects of E2 and E3 Hydroethanolic Extract Combinations on Tissue Parameters in Male and Female Experimental Rats

The parameters studied are MDA, GSH, SOD and catalase in female and male rats alternatively (Figures 8-15). The difference is more statistically significant for liver catalase in both sexes according to the variations of lettres a, b and c.

a) MDA in experimental animals

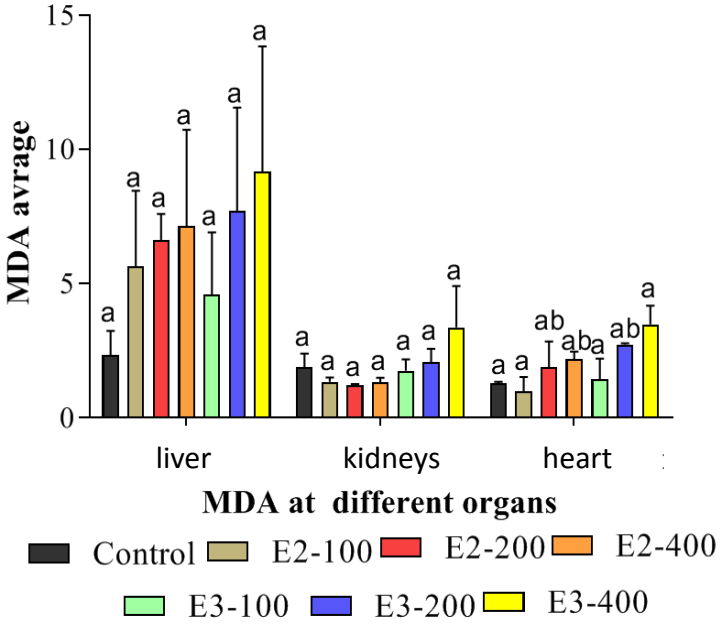


Figure 8. Effects of E2 and E3 hydroethanolic extract combinations on MDA in female experimental rats.

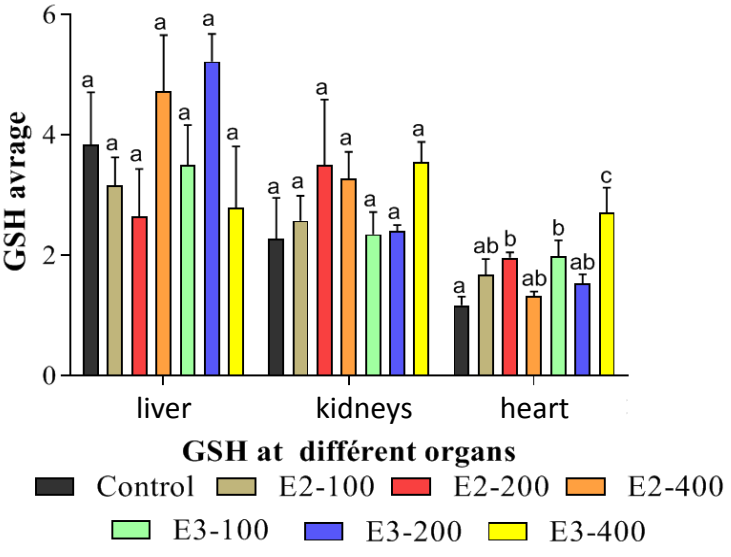


Figure 9. Effects of E2 and E3 hydroethanolic extract combinations on MDA in male experimental rats.

b) GSH/SOD

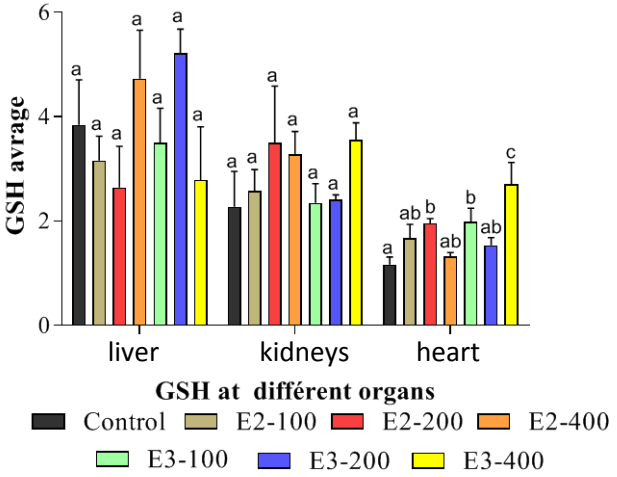


Figure 10. Effects of E2 and E3 hydroethanolic extract combinations on GSH in female experimental rats.

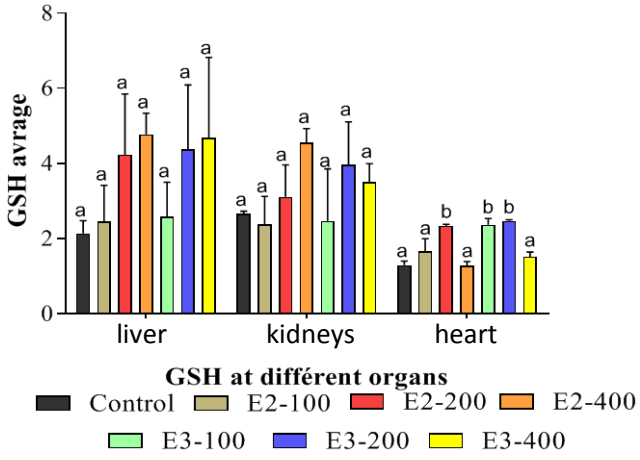


Figure 11. Effects of E2 and E3 hydroethanolic extract combinations on GSH in male experimental rats.

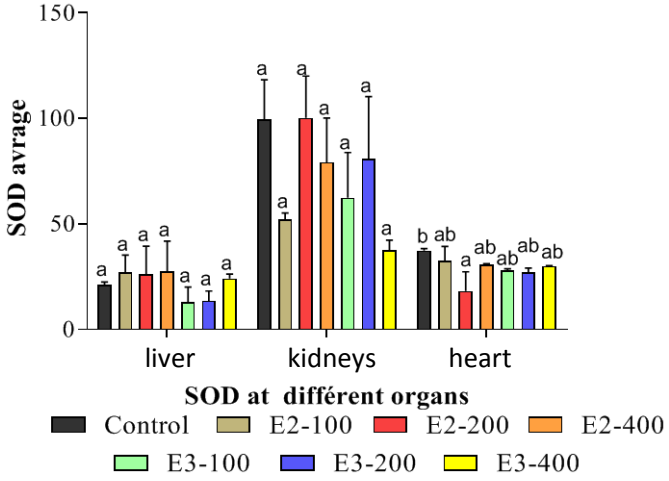


Figure 12. Effects of E2 and E3 hydroethanolic extract combinations on SOD in female experimental rats.

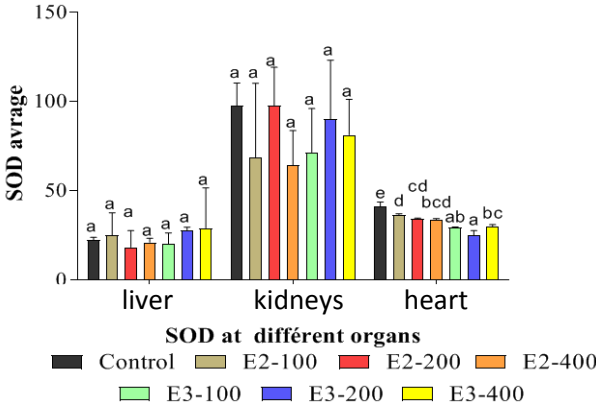


Figure 13. Effects of E2 and E3 hydroethanolic extract combinations on SOD in male experimental rats.

c) Catalase

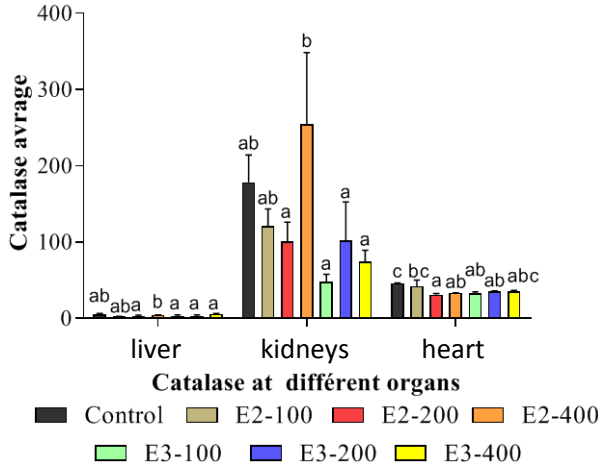


Figure 14. Effects of E2 and E3 hydroethanolic extract combinations on CAT in female experimental rats.

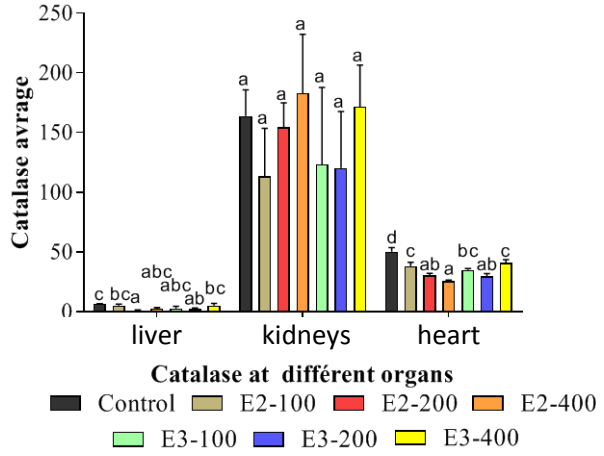


Figure 15. Effects of E2 and E3 hydroethanolic extract combinations on CAT in male experimental rats.

3.2.4. Effects of E2 and E3 Hydroethanolic Extract Combinations on Histological Parameters in Male and Female Experimental Rats

Daily administration of the combinations of E2 and E3 hydroethanolic extracts showed no noticeable effect on the liver and kidney tissues of the treated rats (Figure 16) compared to control rats (Figure 17). The damage observed in females, on the other hand, shows inflammatory foci with a strong infiltration of leukocytes at the highest dose (400 mg/kg) (Figure 18).

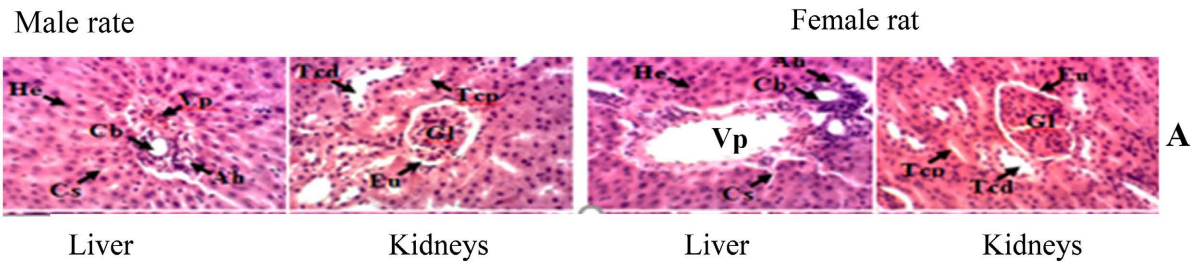


Figure 16. Microphotographs at HE of Liver (×100) and Kidneys (×200) of male and female rats controls (A).

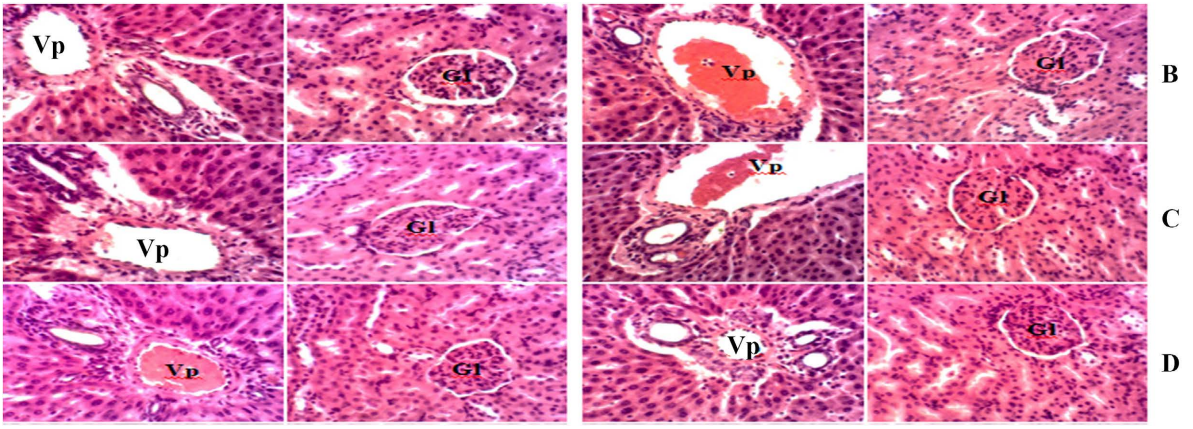


Figure 17. Microphotographs at HE of Liver (×100) and Kidneys (×200) of male rats at E2-100 (B), E2-200 (C), E2-400 (D).

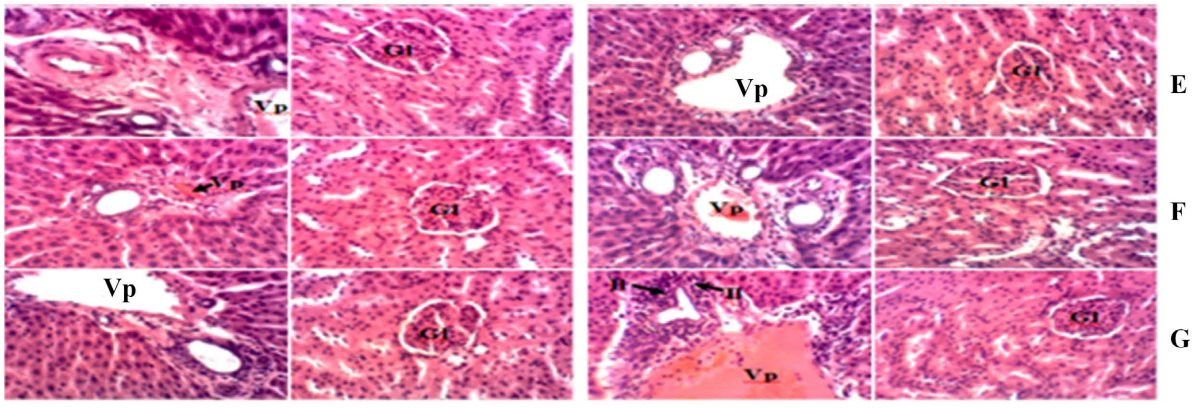


Figure 18. Microphotographs at HE of Liver (×100) and Kidneys (×200) of female rats at E3-100 (E), E3-200 (F) et E3-400 (G). **Legend:** Liver: Vp = Hepatic portal vein; He = Hepatocyte; Cs = Sinusoidal capillary; Ah = hepatic artery; Cb = Gallic canaliculus; It = Leukocyte infiltration; **Kidney:** G = Glomerulus; Eu = urinary space; Tcd = Distal convoluted tubule; Tcp = Proximal convoluted tubule.

4. Discussion

4.1. Phytochemical Screening

The extraction of the leaves of *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. showed a high yield. This high yield could be explained by the choice of solvent used and the amount of plant matter. This result is, respectively, contrary to those found by [21] [22] for methanol extraction [6] for methanol extraction of the same leaves. The phytochemical screening (**Table 2**) of the extracts studied highlighted groups of chemical compounds such as saponins, cathechic tannins, steroids and Flavonoids. These contain more secondary metabolites than ethanolic extract or watery extract from the roots of *Carica papaya* Linn [23]. Flavonoids are phenolic phytochemicals that represent substantial components of the human diet and are believed to keep the individual healthy. They are endowed with various biological activities, including antioxidant effects, lipid peroxidation inhibitors, protective anti-inflammatory [24] [25] and hepatotoxic effects.

4.2. Monitoring Subacute Toxicity

This study was conducted with the aim of evaluating the toxicological effect in order to prevent human exposure to potential risks associated with the use of extract combinations of *G. africanum* and *G. buchholzianum* leaves. In order to assess the effects that could result from the repeated use of these combinations of plant extracts, subacute toxicity was performed, body weight was assessed and decreases were less observed in both sexes. This parameter is important because it can control the health of an animal. Weight loss is frequently used as a first indicator of the harmful effects of drugs [26]. A substance is considered toxic if it results in a mass reduction of more than 10%, and this condition can be considered a sign of toxicity even if other changes do not occur [27]. This weight loss of animals during the experiment could be attributed to antinutritional substances such as tannins and saponins although highlighted in phytochemical screening. The work carried out by [28] has reported that these substances cause a malabsorption of nutrients in the body of animals. These antinutrients are thought to be responsible for the low consumption of food and water and thus the reduction of the body weight of animals treated at different doses of plant extracts compared to controls. These results cannot corroborate for those of Onyekwe et *al.* [29] who showed a weight loss in animals treated with ethanolic extract of P. guava roots for 90 days. However, in this study, no weight loss was recorded (**Table 3**), showing that these combinations of leaf extracts of *G. africanum* and *G. buchholzianum* were not toxic. Transaminases are enzymatic biomarkers that may indicate tissue damage caused by secondary metabolites that can be observed before structural damage could be demonstrated by histological techniques [30]. These enzymes are synthesized in the cytoplasm and released into the general circulation when cells are damaged [31] and at some level can be used to check the extent of hepatocellular damages [32]. In females, daily ad-

ministration of leaves hydroethanolic extract combinations selectively altered transaminase activities at 400 mg/kg body weight (b.w) ALAT and ASAT levels increased significantly at the respective probability thresholds of 0.026 and 0.027 compared to $p < 0.05$, this rate is higher for ALAT activity because more sensitive to hepatocytes [33], This increase in the level of transaminases in the serum may be due to alteration of another organ as the levels of MDA and CAT being raised non-statically significantly in control rats and those with 400 mg/kg and significant between controls and all other concentrations. However, in male animals, there was no statistically significant difference at the probability threshold of 0.05. Rather, a decrease in ALT and AST activities at the respective concentrations of E2-400 and E3-400 mg/kg of b.w. This decline suggests that these combinations of leaf extracts have an effect on the liver. Investigations conducted by Uboh et *al.* [30] showed a decrease in transaminase levels in males treated with *P. guajava* leaf extract and an increase in AST levels in females; they also reported a non-significant effect of watery *P. guajava* leaf extract on liver function in male animals. The decrease in transaminase levels may also reflect the hepatoprotective function of these leaf extract combinations. The work of Roy *et al.* [34] also showed the hepatoprotective effect of the watery solution of *P. guajava* leaf extract, with a more pronounced effect at a dose of 500 mg/kg in both sexes. The liver and kidneys are the target organs of toxic chemicals because of their essential functions in the detoxification and excretion process; these organs are very useful in toxicity studies because of their sensitivity to dangerous compounds [35]. In this work, the parameters of evaluated kidney function were creatinine and serum urea. The kidney regulates the excretion of urea, creatinine and the reabsorption of electrolytes in the blood; in cases of glomerular alteration, these substances accumulate in bodily fluids [36]. Abnormally high serum levels of creatinine, uric acid and urea are biomarkers of possible kidney dysfunction [37]. In our study, although serum levels of urea and creatinine increased, urinary levels of these two parameters are normal compared to those of control. In addition, on the histological level, sections of the animal's kidneys showed no alteration of this organ, indicating its proper functioning. On the other hand, leukodist infiltrations were observed in female animals followed by a sharp rise in ALAT activity. These leukotic infiltrations would indicate acute hepatocellular insufficiency by centro-lobular hepatic necrosis. These results corroborate with those found by [33] and [32] (**Figure 18**) at higher doses of 400 mg/kg of extracts combinations.

5. Conclusion

The toxicological profile of this study based on combinations of hydroethanolic extracts of *Gnetum africanum* and *Gnetum buchholzianum* at different doses showed liver damage in female rats while renal organs were not altered at dose level of 400 mg/kg body weight administered orally and daily. The low dose of these extract combinations is expected to be the potential candidate in the pro-

duction of traditionally improved drugs to fight against oxidative stress and inflammatory diseases. In view of the classes of secondary metabolites present and the safety at low doses, it will be of importance to isolate and purify the chemical compounds for pharmacological purposes.

Data Availability

All data generated or analyzed during this study was included in this Original Research Article.

Authors' Contributions

Bertin SONE ENONE designed and carried out the study. Gisèle LOE ETAME, Jean-Pierre NGENE, Charles Christian NGOULE and Angèle FOYET FONDJO carried out the laboratory material and histologic analysis. Bertin SONE ENONE, Ronald BIDINGHA, and Jules Christophe MANZ KOULE wrote the Original Research Article. Loick Pradel KOJOM FOKO and François SIEWE carried out the statistical analyzes, edited the language, and revised the Original Research Article for intellectual content. Josiane ETANG, and Albert MOUELLE SONE supervised the work. All authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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