Journal of Diseases and Medicinal Plants
2023; 9(2): 40-48
http://www.sciencepublishinggroup.com/j/jdmp
doi: 10.11648/j.jdmp.20230902.12
ISSN: 2469-8202 (Print); ISSN: 2469-8210 (Online)

Anti-Anemic Potential of a Herbal Recipe Against Phenylhydrazine-Induced Anemia in Rats

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To cite this article:

Tatiana Kangah Mireille Kple, Joel Akakpo-Akue, Konan Armand Marcelin Kouassi, Sitapha Ouattara, Gbe Kouakou N'Dri Ange Konan, Diane Koulai, Larissa Adjoua Kouadio, Chiaye Claire Antoinette Yapo-Crezoit, Adou Koffi Mattieu kra, Allico Joseph Djaman. Anti-Anemic Potential of a Herbal Recipe Against Phenylhydrazine-Induced Anemia in Rats. *Journal of Diseases and Medicinal Plants*. Vol. 9, No. 2, 2023, pp. 40-48. doi: 10.11648/j.jdmp.20230902.12

Received: March 8, 2023; **Accepted**: April 6, 2023; **Published**: April 27, 2023

Abstract: Anemia is characterized by an abnormal decrease in the level of hemoglobin or the number of red blood cells in the blood. It is a public health problem that mainly affects pregnant women and young children. In the present study, the objective was to evaluated the anti-anemic potential of a recipe composed of three medicinal plants, *Xylopia aethiopica* (Dunal) A. Rich, *Zanthoxylum leprieurii (GUILL)* and *Harungara madagascariensis (LAM),* used to treat sickle cell anemia in rats. Aqueous and hydroethanol extracts of recipe composed were first prepared and quantitative phytochemical tests were carried out. Then, the anti-anemic activity of the recipe extracts was assessed. The anemia was induced in animals by intraperitoneal injection of phenylhydrazine at a repeated dose of 40 mg/kg bw for two days. Treatment was carried out with the aqueous and hydroethanol extracts of the plant recipe at doses of 400 and 800 mg/kg bw. The obtained results indicated that recipe composed contains total phenols (43.31±0.92 mg GAE/g and 33.82±0.6 mg GAE /g of dry weight), total flavonoids (17.07±0.89 mg QE and 19.76±1.15 mg QE/g of dry weight) and total tannin (8.65±0.01 mg QE and 26.5±0.95 mg Cat E/g of dry weight) in decocted and hydroethanol extracts respectively. The anemia was effectively corrected after 21 days of oral administration of the extracts to the rats. The extracts significantly increased hemoglobin ($P < 0.05$), red blood cell count ($P < 0.001$), and hematocrit ($P < 0.01$) as early as the 7th days of treatment compared with untreated controls. These results show that the plant recipe composed of *Xylopia aethiopica, Zanthoxylum leprieurii* and *Harungara madagascariensis* possess antianemic properties.

Keywords: Anemia, Antianemic Potential, Phenylhydrazine, Medicinal Plants

1. Introduction

Anemia in general, due to their frequency, constitute a public health problem in the world and particularly in developing countries [1]. They are characterized by a decrease in circulating hemoglobin levels with normalization of plasma volumes to age-appropriate values [2]. Two billion people worldwide are affected, and developing countries pay the highest price with prevalences of around 60% in pregnant women, 50% in children under 4 years of age and 45% in school children [3]. The clinical signs of anemia are pallor and functional symptoms such as asthenia, exertional dyspnea, dizziness, headache, etc. Several factors explain the appearance of anemia. Iron deficiency is the most common cause, but micronutrient deficiencies (folic acid, vitamin B12, vitamin A) are also involved. Inflammation, whether acute or chronic, and intestinal parasitosis are also common causes of anemia. Hereditary or acquired disorders affecting hemoglobin

synthesis, red blood cell production and survival can also lead to anemia [2]. Dietary changes, iron supplementation and blood transfusions are commonly preferred for the management of anemia. Oral iron therapy has many disadvantages such as insufficient absorption and lack of compliance [4]. Furthermore, consumption of high quantity of these iron supplements can lead to serious health-related complications such as some neurogenic disorders or cancer [5].

As for blood transfusions, they often present risks of infection and compatibility [6, 7]. Although various drugs are available for the treatment of anemia, they are often not affordable for resource-poor people. In addition, as in many remote areas, people still do not have access to quality medicines [8]. Because anemia has a multifactorial cause [2], it is important to turn to traditional medicine to address this public health problem.

It is within this framework that our research team was interested in three medicinal plants, *Zanthoxylum leprieurii (GUILL), Harungara madagascariensis (LAM)* and *Xylopia aethiopica* (Dunal) A. Rich, which are used in the composition of a recipe used in the traditional environment in the management of sickle cell anemia in Côte d'Ivoire. *Zanthoxylum leprieurii* and *Harungara madagascariensis* are two Rutaceae commonly used by populations in the treatment of anemia, pain and bacterial diseases [9, 10]. These two plants are also used to relieve symptoms of sickle cell disease [11, 12]. As for *Xylopia aethiopica,* it is an Annonaceae that increases hemoglobin levels and relieves pain [13]. In rural areas, it is used more in remedies as an ingredient. These plants, taken individually according to the literature, have their LD_{50} higher than 5000 mg/kg body weight (bw), and even administered to rats for 60 days, do not alter their biochemical parameters, but increase the hemoglobin level which is the key parameter of anemia [9, 14]. The present study was undertaken to verify the anti-anemic activity of this recipe based on these three medicinal plants.

2. Materials and Methods

2.1. Plant Material

Fruits of *Xylopia aethiopica* (Dunal) A. Rich*,* barks *of Zanthoxylum leprieurii* (GUILL), and leaves of *Harungara madagascariensis* (LAM) were collected in January 2018 from the Indenie-Djuablin region of Eastern Côte d'Ivoire. For each batch of a given species, not only were foreign species removed, but also the harvests were cleared of samples that were soiled, rotted, burned by fire, or contaminated by mold or any other pest. Except for the bark, the fruits and leaves were rinsed, shaken dry and cut into small pieces. These plant pieces were then dried in the shade at room temperature (25-30°C) for four weeks. The drying was done in a well-ventilated room. After drying, the plant parts (of each individual plant species) were finely ground using an IKA-Labortechnik electric grinder (Type MFC /Janke & Kunkel). Based on the information collected from the traditional healers, the recipe was compiled. The powders obtained after pulverization of these plants were assembled in the quantities of 33.33 ± 1 g per plant and the new composition obtained was coded ZHm.

2.2. Animal Material

Nulliparous and non-pregnant Wistar rats weighing between 104 and 130 g from the animal house of the Higher Normal School of Abidjan (Côte d'Ivoire) were used. These animals were placed in conditions where the ambient temperature was between 26 and 30°C, humidity between 40% and 60%, and lighting of 12 hours of light and 12 hours of darkness. The rats had free and continuous access to water and food. All procedures and techniques used in this experiment were performed according to the National Institute of Health guidelines for the care and use of laboratory animals [15].

2.3. Preparation of Extracts

2.3.1. Preparation of the Aqueous Extract

Following the method of Konkon *et al*. [16], one hundred grams (100 g) of the plant powder mixture was boiled for 20 min in 2 L of distilled water. The decoctate was cooled to room temperature (25°C) and filtered three times through absorbent cotton and once through Whatman 3 mm filter paper. The filtrate was then dried at 50°C using a Venticell® type oven. The powder obtained was the total aqueous extract coded DZHm.

2.3.2. Preparation of the Hydroethanol Extract

The hydroethanol extract was prepared according to the method of Zirihi *et al*. [17]. One hundred grams (100 g) of the plant powder mixture was dissolved in one liter of hydroalcoholic solvent comprising 70% ethanol and 30% distilled water. The mixture was then homogenized using a Severin ® brand blender. The homogenate obtained was wrung out in a cloth square and then filtered three times on hydrophilic cotton and once on whatman paper (3 mm). The filtrate was evaporated at 45°C using a Venticell® type oven for 24 hours. The dry powder obtained was the hydroethanol extract coded EZHm.

2.4. Determination of Phenolic Compounds

2.4.1. Determination of Total Phenols

Total phenols were determined according to the method of Folin-Ciocalteu [18]. One (1) mL of extract from the mixture of the three plants was introduced into a test tube. To the contents of the tube, 1 mL of Folin-Ciocalteu reagent was added. The tube was allowed to stand for 3 min and then 1 mL of 20% (w/v) sodium carbonate solution was added. The contents of the tube were made up to 10 mL with distilled water. The tube was placed in the dark for 30 min and the absorbance was measured at 745 nm against a blank. A standard range established from a solution of gallic acid (1 mg/mL), under the same conditions as the assay, was used to determine the amount of phenols in the sample.

2.4.2. Determination of Total Flavonoids

Flavonoid assay was performed as described by Meda *et al*.

[19]. Extract from the mixture of the three plants (0.5 mL) was introduced into a test tube. Then, 0.5 mL of distilled water, 0.5 mL of aluminum chloride, 0.5 mL of potassium acetate and 2 mL of distilled water were successively added. The tube was allowed to stand for 30 min in the dark and the absorbance was read at 415 nm against a blank. A standard range established from a solution of quercetin (0.1 mg/mL), under the same conditions as the assay, was used to determine the amount of flavonoids in the sample.

2.4.3. Determination of Tannins

Tannins contents was determinate according to the method described by Bainbridge *et al*. [20]. One (1) mL of extract from the mixture of the three plants was introduced into a test tube. Then, 5 mL of vanillin was added. The tube was allowed to stand for 30 min in the dark and the absorbance was read at 500 nm against a blank. The amount of tannins in the samples was determined using a standard range established from a solution of tannic acid (2 mg/mL) under the same conditions as the test.

2.5. Study of Anti-Anemic Properties

Principle

The method used is those described by Naughton *et al*. [21] and Berger [22]. It consists of testing the inhibitory action of plant extracts on induced anemia by intraperitoneal injection of 40 mg/kg/day of phenylhydrazine (PHZ) for two days (D0 and D1). Animals treated with phenylhydrazine whose hemoglobin concentration is below 13 g/dL are considered anemic.

Treatment of animals

Thirty-five (35) rats of different sexes, divided into seven groups of 5 rats, with a body weight of 150-170 g and aged 4-5 months, were used. Prior to induction of anemia, blood was collected from the rats of the different groups by tail incision (D0). Anemia was then induced by intraperitoneal injection of phenylhydrazine (40 mg/kg/day) for two days (D_0 and D1) to rats of groups 2, 3, 4, 5, 6 and 7. Rats in group 1 (normal) received distilled water. Before treatment with the different products (vitamin B12 and plant extracts), the hemoglobin concentration was determined to retain only those rats that are anemic (hemoglobin concentration < 13 g/dL).

The selected animals were used for further work. The rats of group 2 (anemic) received distilled water and those of group 3 received vitamin B12 for 21 days. As for the rats of groups 4 and 5; 6 and 7, they were respectively treated with the aqueous and hydroethanol extracts of the recipe at doses of 400 and 800 mg/Kg bw for 21 days.

Blood sampling and determination of hematological parameters

A small portion of the anesthetized rat tail (less than 5 mm) was cut. To facilitate collection, the tail was previously immersed in warm water to induce vasodilation, and then gently massaged from the base to the tip. The draining blood was collected directly into an EDTA tube. This method was rapid and easily obtained 1 mL of blood in less than 1 min [23]. Hematological parameters were assayed on days D7, D14 and D21 in the seven groups of rats using an automatic blood cell counter (Sysmex KX 21). Rats from all seven groups were weighed before any sampling on days D0, D2, D7, D14 and D21.

2.6. Statistical Analysis

The results were expressed as means with standard errors on the mean. The graphical representation of the data was done using Graph Pad Prism 9.0 (Microsoft U.S.A). Statistical analysis of the results was performed using analysis of variance (ANOVA ONE WAY). Differences between means were determined according to the DUNCAN and TURKEY comparison test to which the t-Student test was added. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Phenolic Compound Content of the Extracts of the Recipe

Table 1 shows the phenolic content of the aqueous (DZHm) and hydroethanol (EZHm) extracts of the recipe. The total phenolic content of each plant extract is expressed as milligram gallic acid equivalent per gram (mg GAE/g) of dry matter. Flavonoids are expressed as milligram quercetin equivalent per gram (mg QE/g) of dry matter and tannins as milligram catechol equivalent per gram (mg Cat E/g) of dry matter. Each content was determined from the equation of the calibration curve of the phenolic compound. Thus, the content of total phenols was obtained by the equation y= 0.0863x; R2 = 0.9977; that of flavonoids was obtained by the equation y=1.3574x – 0.0181; R2 = 0.9976, and finally, the standard curve of equation y=3.92x; R2 = 0, 9727 allowed to determine the content of tannins.

Table 1. *Phenolic compound content of ZHm recipe extracts.*

Extracts	Total Polyphenols (mg GAE/g)	Total Flavonoids (mg QE/g)	Total Tannins (mg Cat E/g)
Aqueous extract (decocted) DZHm	43,31 ± 0,92[a]	17,07 ±0,89[a]	8,65 ± 0,01[a]
Ethanolic extract (70%) EZHm	33,82±0,6[b]	19,76 ± 1,15[a]	26,57 ± 0,95[b]

DZHm: aqueous extract of the recipe; EZHm: hydroethanol extract of the recipe.
Means in the same column that carry the same letter are not significantly different.

The total phenol contents obtained are 43.31 ± 0.92 and 33.82 ± 0.6 mg GAE/g of extract for DZHm and EZHm respectively. Total flavonoids contents were 17.07 ±0.89 mg QE/g for DZHm and 19.76 ± 1.15 mg QE/g for EZHm, and total tannins contents were 8.65 ±0.01 mg Cat E/g for DZHm and 26.57 ± 0.95 mg Cat E/g for EZHm. Analysis of these

results reveals that the aqueous extract (DZHm) is richer in total phenols ($P < 0.01$) than the hydroethanol (EZHm) one. The total flavonoid contents of the two extracts are statistically identical, unlike the tannin content of EZHm which is significantly higher ($p< 0.0001$) than that of DZHm.

3.2. Effect of ZHm Recipe Extracts on Hematological Parameters

Administration of phenylhydrazine (PHZ) over a period of two days to rats caused a significant ($p<0.05$) decrease in the number of erythrocytes (red blood cells) in all seven groups of animals. The hemoglobin level of the treated animals was 7.23 g/dL; this value is half that of normal rats (13.37 g/dL) (Figure 1A). As for the number of red blood cells, the values decreased from 7.23×10^6/µL in normal rats to 3.85×10^6/µL in the phenylhydrazine-treated rats (Figure 1B). In addition, hematocrit decreased from 43.12% in normal rats to 23.1% in anemic rats (Figure 1C). Phenylhydrazine decreased both red blood cell count, hemoglobin level, and hematocrit level.

Administration of the different recipe extracts and vitamin B12 to rats induced a significant increase in hemoglobin (Hb), red blood cell (RBC) count and hematocrit (Hte) from the 7th day of treatment compared to untreated controls. The hemoglobin level of the untreated rats was 9 g/dL, those of the treated rats were 11.43 g/dL for the rats given vitamin B12; 10.53 g/dL and 11.4 g/dL for the rats given the aqueous extract at 400 and 800 mg/kg bw, respectively. No significant difference was observed between the hemoglobin levels of the vitamin B12-treated rats and those of the rats treated with the different doses of the aqueous extract (Figure 1B).

With regard to red blood cell count, a significant difference ($p<0.0001$) was observed at 7th days of treatment between the values of anemic controls and those of rats treated with the aqueous extract of the recipe. Red blood cell counts were 4.46×10^6/µL; 5.74×10^6/µL; 5.44×10^6/µL; and 5.52×10^6/µL for anemic and untreated rats, vitamin B12-treated rats, and rats treated with the aqueous extract at doses of 400 and 800 mg/kg bw, respectively (Figure 1A).

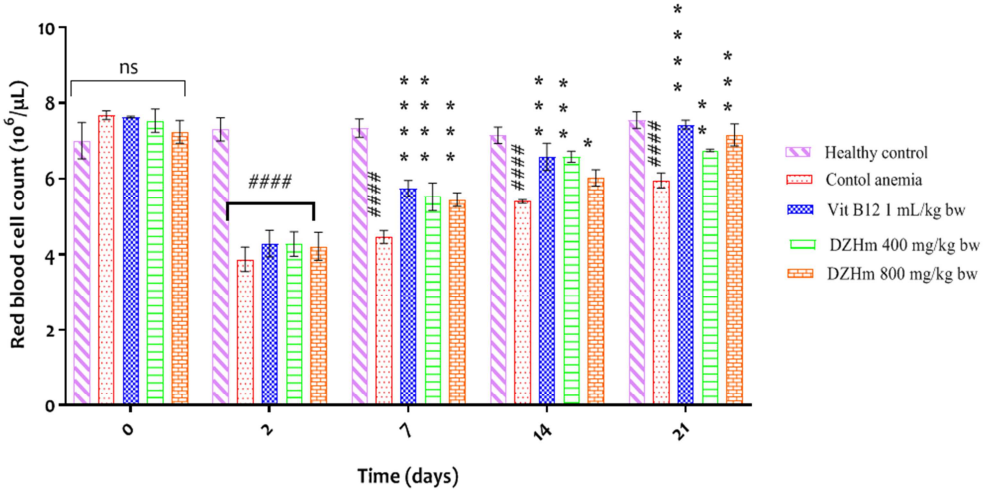
As for hematocrit, the values recorded in rats treated with vitamin B12 and aqueous extract of ZHm recipe were significantly ($p<0.01$) increased after 7 days of treatment compared to those of anemic and untreated rats. These values were 26.93%; 38.7%; 36.57% and 38.07% for anemic controls, rats treated with vitamin B12 and those treated with aqueous extract at doses of 400 and 800 mg/kg bw, respectively (Figure 1C).

With the hydroethanol extract of the recipe, a significant difference ($p < 0.01$) in red blood cell count and hemoglobin level was observed on 7th day of treatment between untreated anemic control rats and treated rats. The hemoglobin level was 11.50 g/dL and 11.93 g/dL for rats treated with 400 and 800 mg/kg bw of hydroethanol extract respectively (Figure 2A). The red blood cell count was 5.71×10^6/µL and 5.60×10^6/µL for rats treated with 400 and 800 mg/kg bw, respectively (Figure 2B). For hematocrit, the values were 41.77% and 41% for rats treated at 400 and 800 mg/kg bw, respectively (Figure 2C).

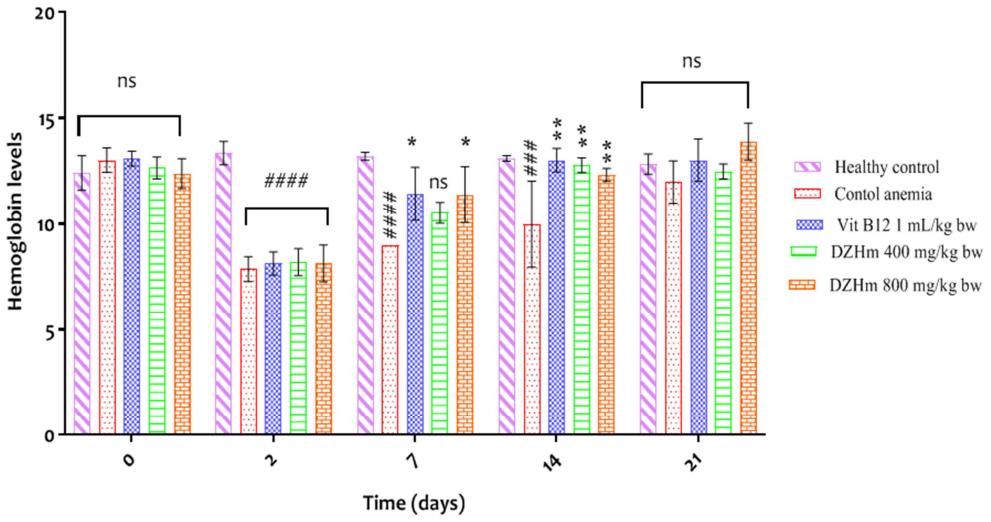
In general, all rats treated with the extracts (DZHm and EZHm) at the different doses tested and with vitamin B12 had a progressive recovery of the hematological parameters until 21st day of the treatment.

3.3. Effect of Aqueous and Hydroethanol Extracts of ZHm Recipe on the Weight of Rats

Figures 3A and 3B show the weight changes of rats before induction of anemia (D0), after induction of anemia with phenylhydrazine (PHZ) (D2) and at 1st, 2nd and 3rd weeks of treatment with the different products. Administration of phenylhydrazine resulted in weight loss in rats from groups 2 to 7 on 2nd day (4.88%). A progressive increase in weight was observed after treatment of rats with vitamin B12, aqueous extract (Figure 3A) and hydroethanol extract (Figure 3B) of ZHm recipe. The weight gain was greatest between the 2nd and 4th days of treatment. Overall, all rats had a progressive increase in body weight. The dose of 400 mg/kg bw of extract induced the greatest increase in weight gain, with a better effect for the aqueous extract.



A



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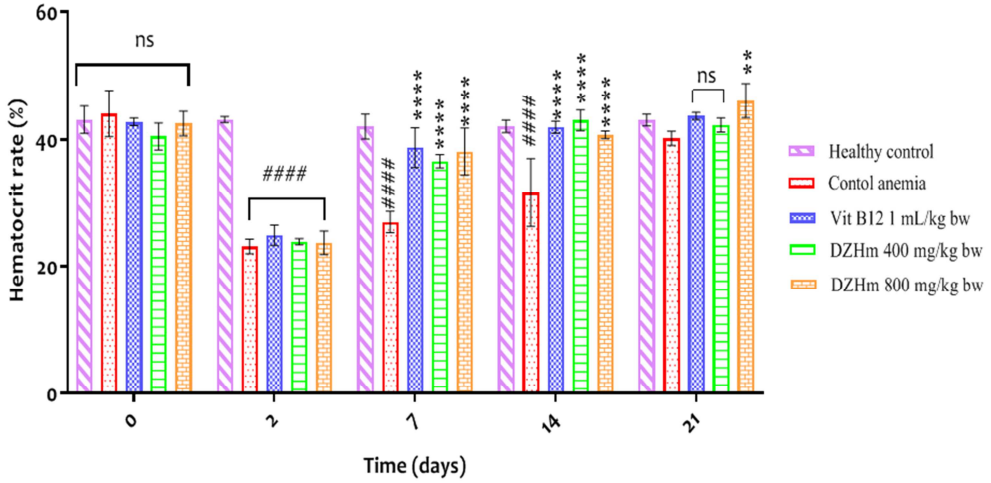
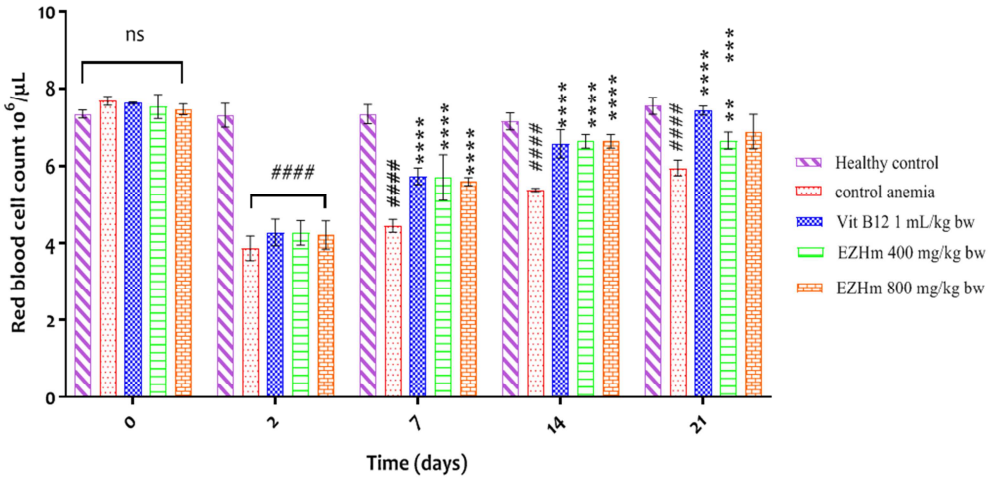


C

Figure 1. *Effect of aqueous extract of the recipe on red blood cell (A), hemoglobin (B) and hematocrit (C) level before and after induction of anemia by phenylhydrazine in rats.*

Values are presented as mean ± standard deviation
(###) ($p<0.001$); (####) ($p<0.0001$) represents significant differences between healthy rats and anemic controls rats.
(*) ($p<0.05$); (**) ($p<0.01$); (***) ($p<0.001$); (****) ($p<0.0001$) represents significant differences between anemic rats controls and treated rats.



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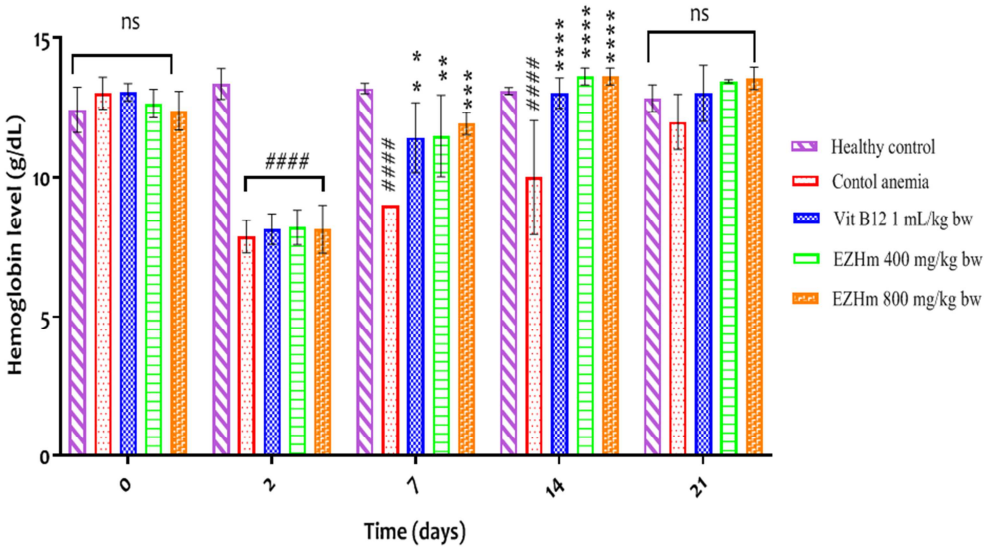

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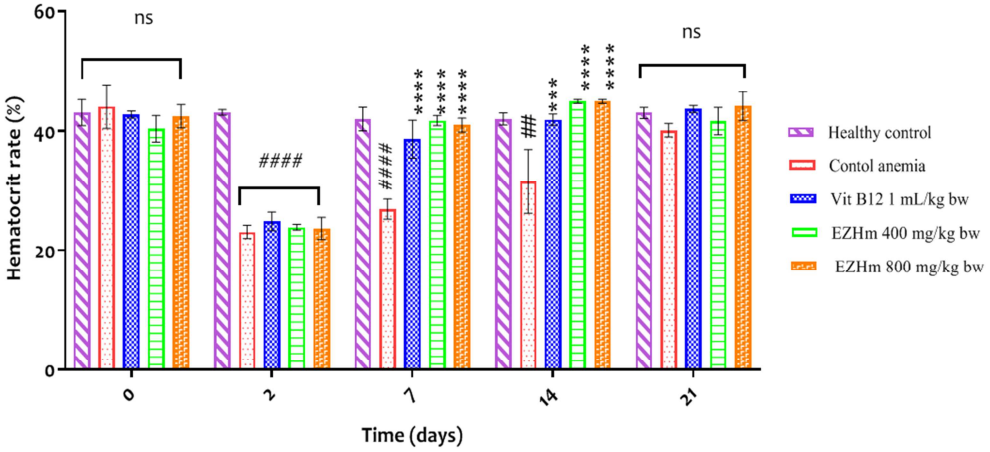
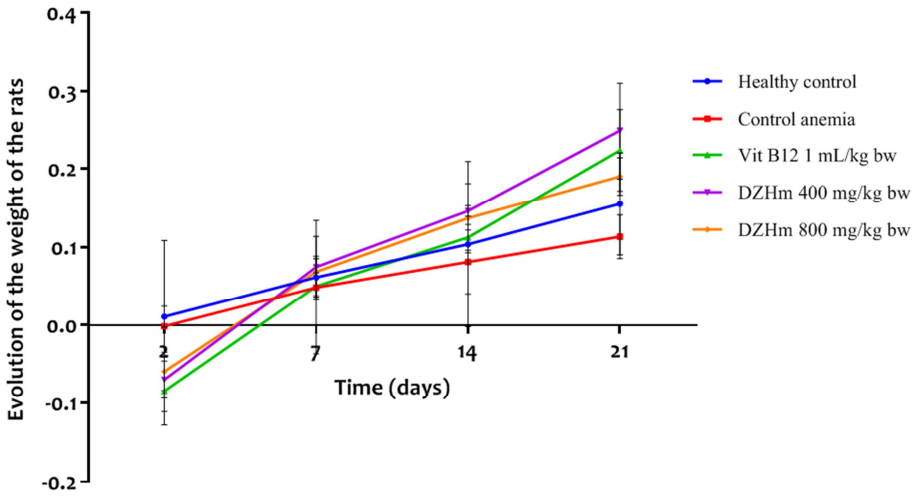


C

Figure 2. *Effect of hydroethanol extract of the recipe on red blood cell (A), hemoglobin (B) and hematocrit (C) level before and after induction of anemia by phenylhydrazine in rats.*

Values are presented as mean ± standard deviation
(##) ($p<0.01$); (###) ($p<0.001$); (####) ($p<0.0001$) represents significant differences between healthy rats and anemic controls rats.
(*) ($p<0.05$); (**) ($p<0.01$); (***) ($p<0.001$); (****) ($p<0.0001$) represents significant differences between anemic rats controls and treated rats.



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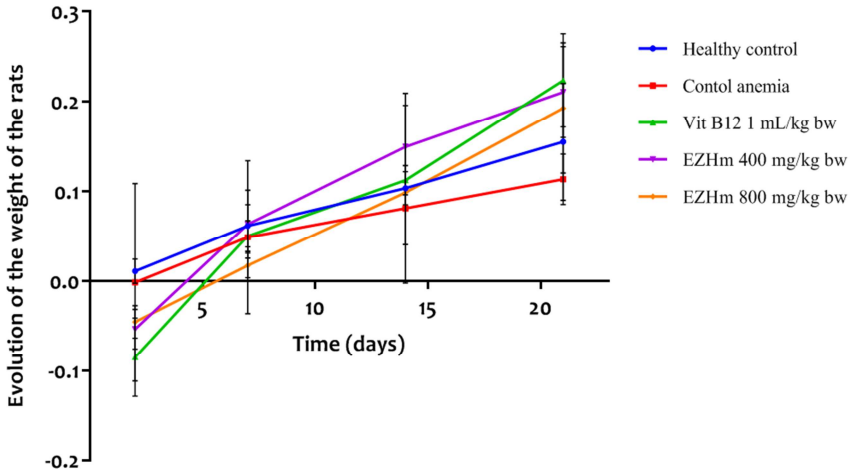

B

Values are presented as mean ± standard deviation

Figure 3. *Effect of aqueous extract (A) and hydroethanol extract (B) of the recipe on weight levels before and after induction of anemia by phenylhydrazine in rats.*

4. Discussion

The objective of this work was to study the antianemic properties of aqueous and hydroethanol extracts of a plant recipe composed of *Xylopia aethiopica* (Dunal) A. Rich, *Zanthoxylum leprieurii (GUILL) and Harungara madagascariensis (LAM).* Quantitative analysis of phenolic compounds revealed that the aqueous and hydroethanol extracts of this recipe contain flavonoids and tannins in different proportions. The total flavonoid contents of both extracts were statistically identical while the hydroethanol extract was richer in tannins.

The results of the anti-anemic effect show that in normal (healthy) control rats, the blood parameters did not change significantly throughout the experiment. The average red blood cell count in these rats was $7.40x10^6$ uL. The hemoglobin level was 13.37 g/dL and the hematocrit was 43.12%. These results are close to those of Yenon *et al.* [24], Gui *et al.* [25] and Karamoko *et al.* [26] and attest that the blood parameters of the normal rats remained constant over time and are similar to the norm.

Administration of phenylhydrazine resulted in a significant decrease in hemoglobin, red blood cell count and hematocrit. Phenylhydrazine induced hemolytic anemia in rats by decreasing the values of these parameters. These results are close to those of Adebayo *et al.* [27], Karamoko *et al.* [26] and Sheth *et al.* [14] who observed a decrease in red blood cell count and hematocrit after induction of anemia in rats. According to Pandey *et al.* [28], the decrease in hemoglobin level may be associated with hemolysis or disturbances in heme biosynthesis as a result of inhibition of iron binding with heme and decreased activity of enzymes involved in heme biosynthesis.

In contrast to the normal control rats, the values of the hematological parameters of the treated rats increased gradually over time until a complete restoration of normal levels. This shows that without a new administration of phenylhydrazine (during the experiment), the blood parameters are gradually regenerated by the body and normal values were gradually restored around the 14^{th} day. These results would attest that phenylhydrazine, administered over 2 days, does not interfere in the regeneration mechanism and does not permanently alter the body's ability to regenerate blood parameters [29, 30]. This assertion is supported by the results of the work of Goorani *et al.* [31].

Regarding the animals treated with vitamin B12, the analysis of the data collected shows that, normal values of blood parameters are quickly restored. This reveals that vitamin B12 is effective in treating phenylhydrazine damage [28, 32].

As for the rats treated with the ZHm recipe extracts, the results indicate a significant and progressive improvement of the values of the hematological parameters at 7^{th} day of the treatment compared to the control rats. These results could be due to the presence of alkaloids and phenolic compounds in these extracts. Indeed, alkaloids and flavonoids are potent antioxidants that prevent and repair damage to red blood cells by free radicals or highly reactive oxygen species [33, 34]. James *et al.* [35] and Sheth *et al.* [14] reported that most anti-anemic compounds are known to be free radical inhibitors, which reverse anemic conditions. Alkaloids inhibit cyclic adenosine monophosphate (AMP_c) phosphodiesterase, thus accumulating AMP_c. This effect stimulates protein phosphorylation and synthesis, which improves erythropoiesis [36]. These phytochemicals may have contributed to the anti-anemic activity of the plant recipe observed in the present study by stimulating erythropoiesis in the bone marrow.

The results of this study are consistent with those of Etame *et al.* [9], Okot-Asi *et al.* [37], Olusayo and Monsi [38] and Imo *et al.* [13]. These authors all showed that the different extracts of *H. madagascariensis* and *X. aethiopica* did not reduce hemoglobin levels over time. Thus, the presence of *H. madagascariensis* leaves and *X. aethiopica* fruits in this recipe would have contributed to the antianemic activity of the ZHm recipe extracts.

Anemia is one of the clinical signs of sickle cell disease [39]. One of the therapeutic means used is transfusion therapy. It is an effective treatment for most complications, as it increases the hemoglobin level and thus the oxygen-carrying capacity of the blood, while decreasing the sickle cell count [40, 41]. However, repeated blood transfusions expose patients to the risk of infection. ZHm recipe extracts may protect sickle cell patients from these infections and prevent the anemia associated with sickle cell disease by increasing hemoglobin and white blood cell counts.

The body weight of anemic rats treated with ZHm recipe extracts of and vitamin B12 was also monitored during the 21 days of treatment. Analysis of the results reveals that phenylhydrazine caused a reduction in body weight of the rats after induction of anemia. This observation is in agreement with Luka *et al.* [42]. Loss of body weight is one of the clinical signs of anemia, and this would be due to the lack of appetite in anemic rats. During treatment, rats fed normally, thus promoting body weight gain. The decrease in body weight in anemic rats could be explained by a reduction in the activities of di-saccharidases that catalyze the final step of carbohydrate digestion [43]. The aqueous and hydroethanol extracts of the ZHm recipe improved the weight of treated rats compared to anemic rats. The weight gain of rats treated with the aqueous and hydroethanol extracts was similar to that of rats given vitamin B12, the reference antianemic. These results are similar to those obtained by Olusayo and Monsi [38] and Imo *et al.* [13] with the aqueous extracts of *H. madagascariensis* and *X. aethiopica* respectively.

5. Conclusion

The present study aimed to evaluate the anti-anemic potential of a recipe based on three medicinal plants traditionally used in the management of sickle cell anemia. At the end of this work, it was found that the aqueous and hydroethanol extracts of this recipe, at doses of 400 and 800 mg/kg bw, significantly increased the hemoglobin level, the number of red blood cells and the hematocrit as early as the 1st week of treatment of anemic rats. The anti-anemic activity of this plant-based recipe is similar to that of vitamin B12 and justifies its use against sickle cell disease in traditional settings by the populations of the Indenie-Djuablin region.

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