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Research Article

Effect of Hydromethanolic Extract of Stem Bark of *Lannea kerstingii* on Anemia and Hepcidin Production

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Abstract

Introduction: Stem bark of *Lannea kerstingii* is used in Togo for its anti-anemic effect. The aim of this study was to verify the efficacy of extracts of *L. kerstingii*'s bark in the treatment of anemia and to explore its mechanism of action. **Materials and Method**: *L. kerstingii* stem bark was collected in the city of Kpalimé, located at 120 km northwest of Lomé. Hydromethanolic extract was obtained by evaporation. Two protocols were carried out and 20 twelve-week-old rats that had an average weight of 175.17±16.8 grams were used. The first protocol consisted of inducing anemia in rats by intraperitoneal injection of phenylhydrazine, then force-feeding them with *L. kerstingii* extract and measuring the effect of this extract on their hemoglobin levels. The second protocol investigated the effect of the extract on the serum hepcidin level, after oral administration of carbon tetrachloride to rats. Hepcidin levels were measured by the ELISA technique. **Results**: *L. kerstingii* stem bark extract significantly corrected the anemia induced by phenylhydrazine use: in fact, the anemia of rats that received *L. kerstingii* extracts was corrected. Control rats that received folic acid had a correction of their anemia too. But, rats that received only saline remained anemic. However, no correlation between the level of extract consumption and the level of hepcidin was established. **Conclusion**: *L. kerstingii* bark hydroethanolic extracts increase hemoglobin levels in anemic rats. The main challenge that needs to be solved is determining the minimum dose to be consumed to ensure its effectiveness.

Keywords: *Lannea kerstingii*, anemia, hepcidin, wistar rat.

INTRODUCTION

Access to a quality health care belongs to important human rights. But that remain a big challenge for low-income countries where traditional medicine remains the dominant medical system due to its affordability for millions of people, both in rural and urban communities. Plants are often used in place of pharmaceuticals becoming increasingly expensive for the indigenous population. In Africa like Asia, extracts of plants are used as the first line of treatment for pathologies such as malaria, diabetes, hypertension, sickle cell disease, anemia and skin diseases [1]. For the wistar rat, anemia is the decrease in hemoglobin level below 11 g/dl [2]. In Togo, a country located in West Africa, the bark of *L. kerstingii* is usually used to treat anemia [3,4]. Plant anti-anaemic virtue may be linked to the induction of osmotic resistance of red blood cells or to the reduction in the synthesis of hepcidin.

In order to contribute for ensuring access to quality health care, especially in rural communities, this study was initiated to evaluate the effect of *L. kerstingii* stem bark on anemic rats through hepcidin synthesis and red blood cells osmotic resistance.

I- MATERIAL AND METHOD

1-1 Material

Raw Material

Stem bark extract of *L. kerstingii* were used. This plant was harvested 120 km northwest of the capital Lomé, in the city of Kpalimé. The botanical identification was carried out in the Botany Department of the University of Lomé and the number is TOG001777.

Laboratory animals

Twenty wistar rats that had in average 12 weeks were used. They had grown in the animal facility of the physiology and pharmacology laboratory of sciences department of the University of Lomé, and their average weight was 175,17±16,8 grams. Those rats who was divided into 5 groups had free access to water and food.

Equipments

The Thomas-Wiley brand grinder Laboratory Mill, model 4 (Philadelphia, USA) was used to grind the dried bark of *L. kerstingii*.

The rotavapor IKA RV 10 Digital (Zhengzhou, China) was used to obtain the extract.

Hematological parameters were evaluated by an auto-hematology analyzer (Mindray®, Model BC-6000, Shenzen, China).

The hepcidin levels were performed on ELISA Equipments (Biorad®, model PW40, Paris, France).

Biological parameters analysis were performed in the laboratory of national research center and sickle cell care (CNRSD) and the immunology laboratory of the Sylvanus Olympio Teaching Hospital Center (CHU Sylvanus Olympio) in Lomé.

1.2- Method

The harvested stem bark was washed and dried under air conditioning for 3 weeks. Then the bark was reduced to powder. The powder was macerated in hydromethanolic solution (20/80) at the rate of 100 grams of powder in one liter of solution during seventy-two hours at laboratory temperature. The macerate underwent a double filtration that are hydrophilic cotton then filter paper. The filtrate was evaporated at 45°C using rotavapor IKA RV 10 Digital until a dry powder was obtained. Before its use to force-feed rats, the extract was stored in the refrigerator at 4±2 degree Celsius.

Two experimental protocols were made with 20 rats that were randomly divided into 5 groups of four labeled rats including one group for positive control and another for negative control. The weight of rats was recorded weekly. Venous blood was collected from the retro-orbital sinus according to the protocol of Joslin Ott [5]. Hb levels were measured using the cyanmethemoglobin method.

1.2.1- Evaluation of the effect of *L. kerstingii* stem bark extract on anemia.

Anemia induction

On first day (D_0), 0.5 ml of blood was drawn from each rat in ethylenediamine tetraacetic acid (EDTA) tube intended to make a basic blood count. Then, 40 g phenylhydrazine (PHZ)/kg body weight was delivered via intra-peritoneal injection for two days (D_2 and D_3) to induce anemia [8,9]. Forty-eight hours after the administration of PHZ that corresponds at D_5, 0.5 ml of blood was again taken from each rat. This sample was used for a second blood count. A rat was anemic if its hemoglobin level was less than 11 g/dl. However, we considered that PHZ induced anemia if there was a decrease of more than 30% in the hemoglobin level compared to its level on D_0 [6-8].

Anemic rats were dispatched in 5 groups. Group 1 served as the negative control and received physiological water (10 mL/kg) orally; group 5 served as the positive control and received folic acid at 0,1 mg/kg. Groups 2, 3, 4 received 100, 200 and 500 g/Kg respectively daily for 14 days from D_6.

A third blood sample was taken on the D_{21} from each rat to perform a complete blood count. This analysis allowed us to evaluate the variation in hemoglobin level induced by force-feeding. We considered that anemia was cared if rat hemoglobin level on D_{21} was equal or greater than its level on D_0 or if the rat had gained more than 30% of its hemoglobin level on D_5.

1.2.2- Evaluation of the effect of *Lannea kerstingii* stem bark extract on hepcidin production.

From each rat, 2 ml of blood were taken in a dry tube, intended for assaying the initial hepcidin level of each rat. After 48 hours of rest of each rat, induction of acute liver lesoin by oral injection in a single bolus of 3 ml/kg/day of carbon tetrachloride (CCl4) was carried out [9]. Twenty-four hours later, 2 ml of blood was collected in a dry tube from each rat to measure the hepcidin level after the creation of liver damage. The induction of liver damage with CCl4 was made on the assumption that the diseased liver produces more hepcidin.

From D_4 to D_{11}, rats in groups 2, 3, 4 were force-fed with *Lannea kerstingii* bark extract. They received respectively 100 mg/kg, 200 mg/kg and 500 mg/kg. At the same period, rats of group 1 (negative control) received physiological water while rats of group 5 (positive control) received Hepafyt® at 10 mg/kg body weight.

A third blood sample was taken on D_{21} from each rat to measure the hepcidin level.. The hepcidin assay was done using the ELISA method [9, 10].

1.2.3- Measurement of *Lannea kerstingii* stem bark extract on osmotic resistance of red blood cells.

After making the rats anemic, they were force-fed with the hydrmethanolic extract of the bark of Lannea kerstingii at different dosages (100, 200 and 500 mg/kg) for 7 days. The experimental protocol consisted of preparing a range of saline solution (NaCl) from 0.1% to 0.9% according to the protocol describe by Redondo in 1995 [11].

1.3- Statistical Analysis

GraphPad ® Prism 5.02 software was used for the analysis of the results that were expressed as mean value with standard error of mean (m ± SEM). One way analysis of variance (ANOVA) was used to compare several groups. $p \leq 0.05$ was considered statistically significant in all analyses.

II – RESULTS

2.1- Changes of hemoglobin level after force-feeding

All rats that received *L. kerstingii* bark extract had an increase in hemoglobin greater than 30% of their hemoglobin level (HL) after induction of anemia. 75% of rats that received folic acid had an increase of more than 30% in their HL. At 21th day, all had a HL higher than their HL at D_0. No increase in hemoglobin level was observed the negative control group. On D_{21}, their hemoglobin level was lower than the HL on D_0.

The variations of averages of HL of the rats at different stages of the protocol are shown in figure 1.

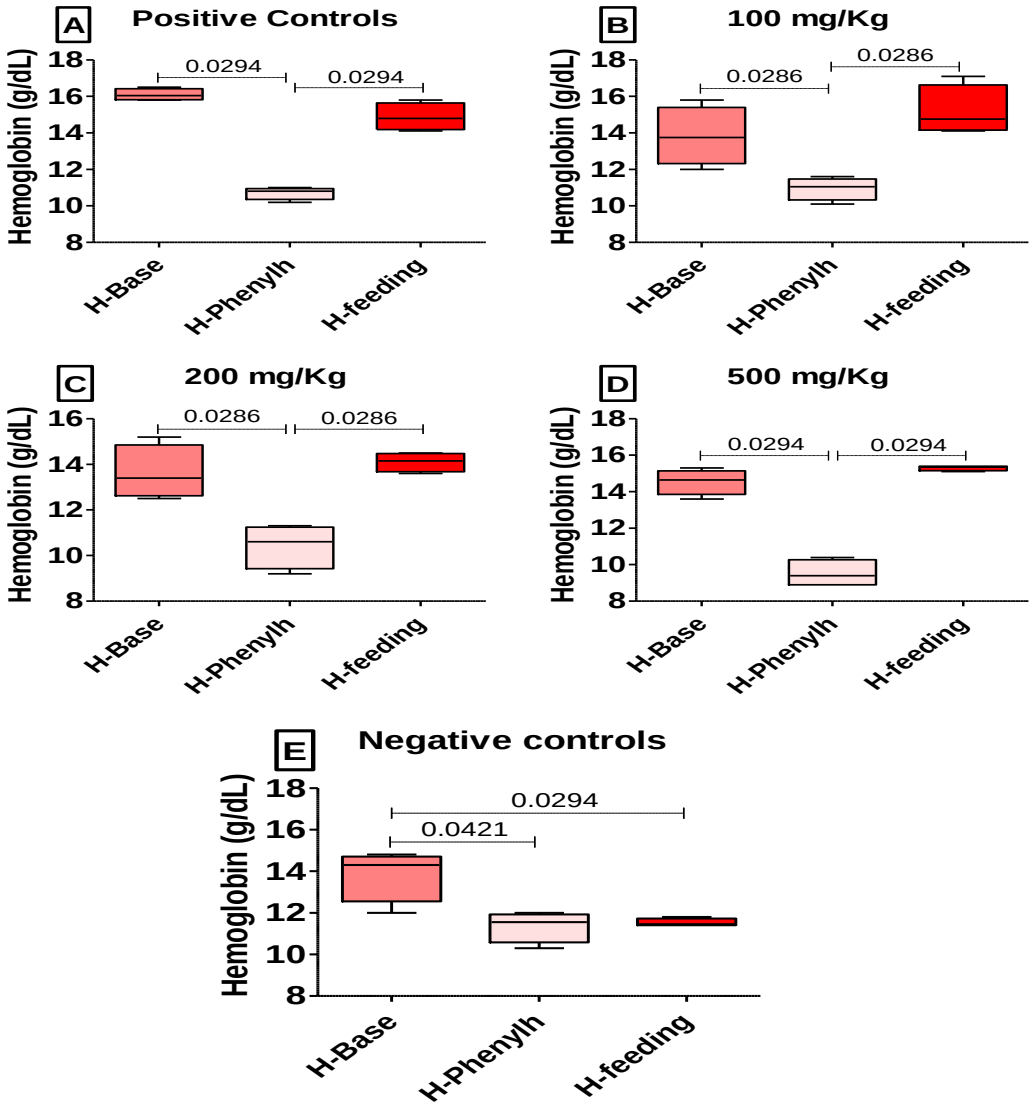

Figure 1 : Variations of averages of hemoglobin levels.

H-Base = initial Hemoglobin Level (HL) ; H-Phenylh = HL after phenyhydrazine administration ; H-feeding = HL after feeding.

2.3- Effect of CCl4 on hepcidin level

The average baseline serum hepcidin level was variable for rats of each group. After administration of CCL_4, the average hepcidin level increased for the rats of groups 2, 3 and 4 while it was decreased for groups 1 and 5 (table I).

Table I : Evolution in mean hepcidin level before and after administration of CCl_4

	Mean ± SEM before CCl4 **10^{-12} g/ml**	Mean ± SEM after CCl_4 **10^{-12} g/ml**
Group 1 (negative control)	1178,92 ± 1274,32	903,16 ± 1615,11
Group 2 (100 mg/kg)	435,53 ± 173,80	533,18 ± 174,33
Group 3 (200 mg/kg)	240,69 ± 12,84	294,79 ± 66,93
Group 4 (500 mg/kg)	243,75 ± 115,41	511,63 ± 223,18
Group 5 (positive control)	1198,05 ± 1415,72	855,50 ± 772,88

2.4- Variation of hepcidin synthesis after force-feeding

Following gavage with physiological saline solution, the extract and Hepafyt®, the average hepcidin level was high for the rats of batches 1 and 3, while it decreased in the rats of groups 2, 4 and 5.

No relation was not established between the hepcidin production and the *L. kerstingii* bark extract consumption due to the unlogical variation in the average of hepcidin levels (figure 2).

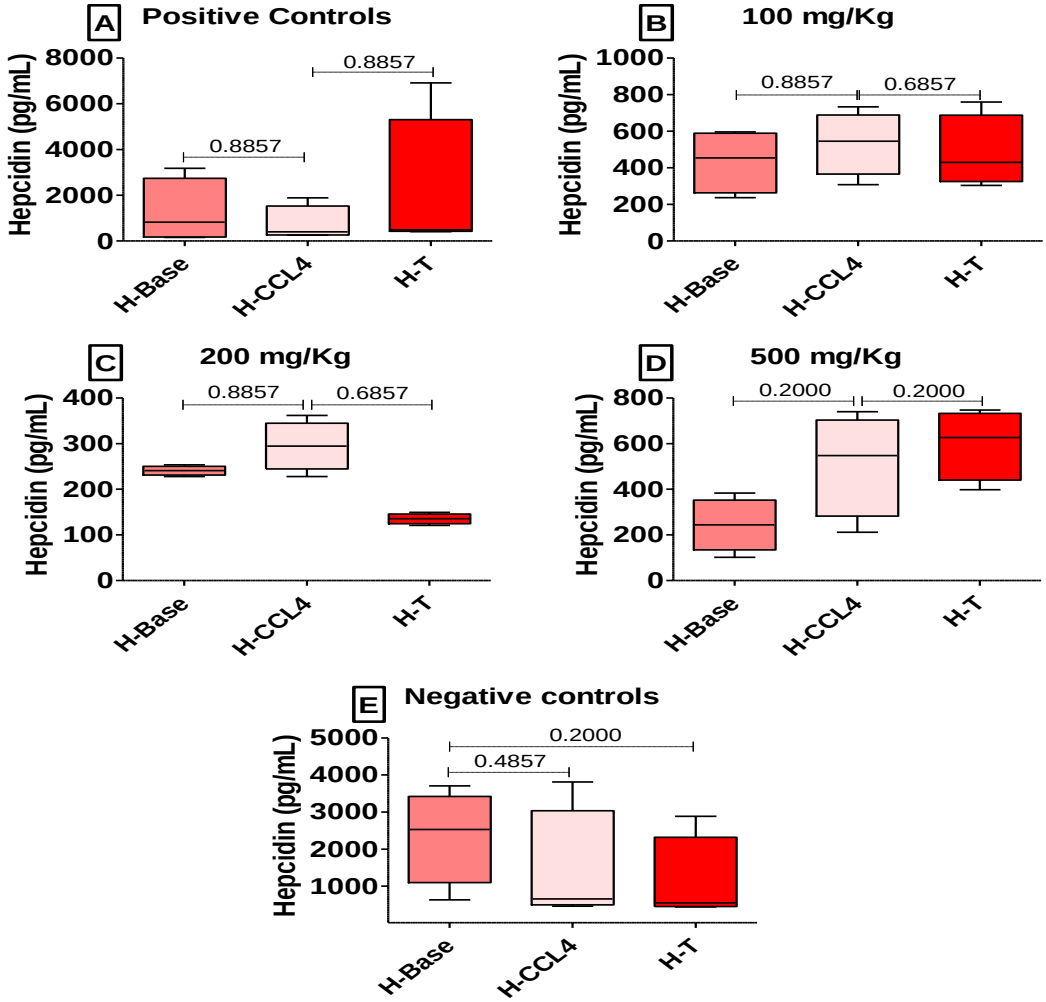

Figure 2 : Variations of averages of hepcidin levels.

H-Base = initial hepcidin level; H-CCL_4 = hepcidin level after CCl_4 administration; H-T = hepcidin level after treatment with the *L. kerstingii* extract.

2.5- Red cell membrane resistance

The extract improves the resistance of the red cell membrane to osmotic shock as illustrated by the data in figure 3.

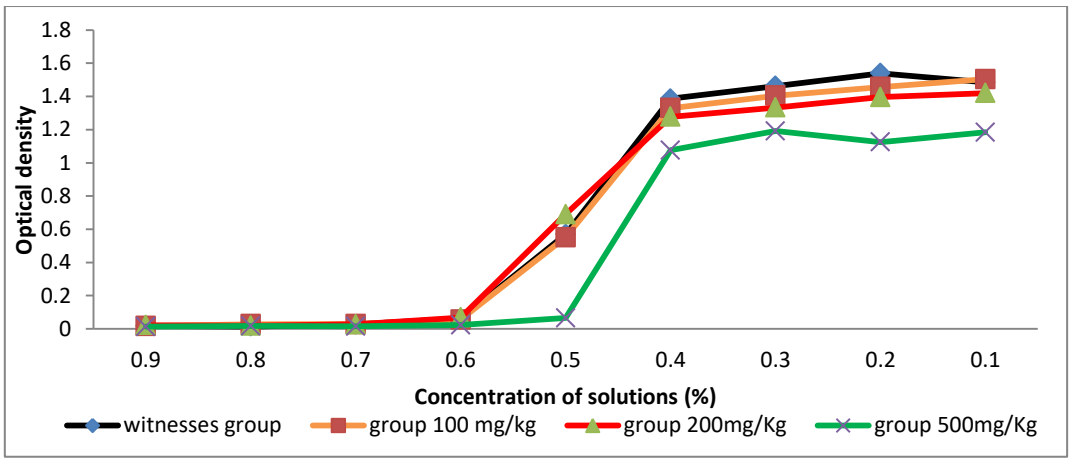

Figure 3 : Curves of osmotic resistance of red blood cells.

Hemolysis is delayed in rats that received L. kerstingii bark extract.

3- DISCUSSION

The decrease in hemoglobin level in all rats treated with PHZ at 40 µg/g body weight confirms the hematological toxicity of this substance [7, 8]. PHZ-induced anemia was cured in all rats given *L. kerstingii* bark extract. The same observation was made in rats that received folic acid which is a known anti-anaemic drug [12]. The mean hemoglobin level of rats given physiological saline did not increase. Moreover, it was lower than the mean of hemoglobin level on D_0. This experiment is proof that the extract was responsible for correcting anemia in rats. Since *L. kerstingii* stem bark extract had the same effect as folic acid on hemoglobin level, it would be likely that this extract would contain vitamin folic acid. This hypothesis must be check.

The correction of anemia was not proportional to the amount of extract received. This means that there would be a minimum dosage to be determined for the treatment of anemia with *L. kerstingii* bark. In addition, perhaps other factors such as sex or hormones are involved.

No rat death related to the *L. kerstingii* bark administration have been observed. It confirms the fact that this extract is a non-toxic plant for humans [13]. The rats of group 1 which received physiological water was anemic on D_{21}. Their hemoglobin level had been belatedly corrected. This same observation was made by Williams who found that Ht and Hb levels return slowly to the initial values [7].

Studies have shown that stem bark of *L. kerstingii* is used to treat anemia. We initially assumed that bark of *L. kerstingii* would have an effect on iron metabolism. We planned to verify this hypothesis by the variation of the hepcidin rate by the consumption of bark extract of L. kerstingii. The choice of hepcidin is related to its role in iron metabolism [9, 10]. The results obtained from the 2nd protocol didn't allow us to draw any conclusion about stem bark of *L. kerstingii* effect on the level of hepcidin in the blood. We could also think that the *L. kerstingii* bark extract would contain a substance which interfered with the determination of the hepcidin level. Perhaps the experience must be conducted on important number of subjects or a longer time for having a scheme that will allow to draw a relation between hepcidin level and of *L Kerstingii* effect.

The optical densities of rats treated with the extract were lower than those of rats in the control group. This reflects a better osmotic resistance in the treated rats. Phytochemical screening revealed the presence of flavonoids, tannins, phenols and coumarins. These metabolites have antioxidant power. They promote tissue regeneration, decrease the permeability of blood capillaries and strengthen their resistance to hemolysis [14].

CONCLUSION

L. kerstingii stem bark extracts increases hemoglobin level in anemic rats. This study confirms the anti-anaemic virtue attributed to the bark of the stem of *L. kerstingii* by the sellers of medicinal plants. It improves the osmotic resistance of red blood cells. It can therefore be recommended as an alternative solution for the treatment of anemia in rural areas, especially in low-income countries. The main challenge that needs to be addressed is the determination of the minimum effective dose.

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