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EVALUATION OF THE ABILITY OF A RECIPE EXTRACT COMPOSED OF THE LEAVES OF THREE FOOD PLANTS TO REVERSE PHENYL HYDRAZINE-INDUCED ANEMIA IN WISTAR RATS: *CEIBA PENTANDRA, IPOMEA BATATAS* AND *SPINACIA OLERACEAE*

Assi Anicet AGNISSAN[1], Joel AKAKPO-AKUE[2], Patrick Aubin DAKIA*[1], Yvan- Andréa Yahaut KONAN[1], Alico Joseph Djaman[2], Kouakou BROU[1]

[1]Nutrition and Food Security Laboratory, UFR of Food Sciences and Technologies, Nangui Abrogoua University, 02 BP 801 Abidjan 02, Côte d'Ivoire.
[2]Biology and Health Laboratory, UFR Biosciences, Félix Houphouët-Boigny University (UFHB), 22 BP 582 Abidjan 22, Ivory Coast.

***Corresponding Author: Patrick Aubin DAKIA**
Nutrition and Food Security Laboratory, UFR of Food Sciences and Technologies, Nangui Abrogoua University, 02 BP 801 Abidjan 02, Côte d'Ivoire.

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ABSTRACT
*Anemia is a public health problem that mainly affects people in various countries. It would be interesting to identify food plants that, while meeting food needs, restore at the same time, the anemic state of populations. This present study was therefore conducted to evaluate the ability of a recipe extract composed of the leaves of three food plants (Ceiba pentandra, Ipomoea batatas and Spinacia oleracea) to reverse phenyl hydrazine-induced anemia in Wistar rats. So, the phenolic compounds, mineral profile, acute toxicity and antianemia activity of recipe extract were evaluated. The results showed that the yield of extraction was 20.7 ± 2.1 % and revealed the presence of polyphenols (821.6 mg/100 g), flavonoids (99.8 mg/100 g), tannins (108 mg/100 g) and iron (*190.14 mg/100 g). *The acute toxicity test did not cause any mortality, nor any sign of toxicity at the dose of 2000 mg/Kg bw. Antianemia activity showed that the oral administration of the recipe extract at the doses of 200, 400 and 800 mg/Kg bw and standard treatment with folic acid resulted in anemic rats returning to normal levels of red blood cells, hemoglobin and hematocrit compared to non-anemic control rats. The red blood cell count increased from 3.4 ±1.70 to 7.65±3.58.10^6/µL; hemoglobin increased from 6.7±3.61 g/dL to 13.6±7.13g/dL and haematocrits from 23.2±2.53 % to 45.5±3.09 %. This study highlighted the antianemic activity of the recipe extract. This antianemic property could be due to the presence of polyphenols, flavonoids, tannins and iron in the recipe extract.*

KEYWORDS: Antianemic activity, mineral profile, Phenolic compounds, food plants, *Ceiba pentandra, Ipomoea batatas, Spinacia oleracea.*

INTRODUCTION
Anemia is characterized by a decrease in the concentration of circulating hemoglobin below threshold values defined by the **Apouey, (2017)**These values take into account age, sex, gestational status, ethnicity, smoking and altitude (**Kaelin, 2014).** They are estimated at 13 g/dL in men and 12 g/dL in women. Anemias can be grouped into two groups: central anemias and peripheral anemias. Central anemias follow a defect in bone marrow production. As for peripherals, they result from a shortening of the life of red blood cells (RBCs), by abundant hemorrhage or significant destruction due to inflammatory diseases such as sickle cell disease (**Wuillemin, 2017).** They often result from a deficiency of factors essential to erythropoiesis such as iron. Iron deficiency is the most common nutritional deficiency, affecting almost 700 million people (**Van, 1992).** In tropical countries, less resorption of iron present in plants is an unbalancing factor.

Deficiencies in the intake of B vitamins are well known, especially folic acid **(Van 1992).** In addition to deficiencies due to specific nutrients, general infections and certain diseases including malaria and sickle cell anemia are also listed as causes of anemia (**Van, 2000).** According to WHO estimates, 1.62 billion people are anemic in the world, or 24.8% of the world's population. Africa and South-East Asia are the most affected continents where the anemia disease is a public health problem, with major negative consequences on human health and economic development (**Mason, 2005).** Indeed, prevalences are 46% for Africa and 57% for

South-East Asia (**Touré, 2019).** It affects all age groups with a predominance in children under five years of age and women of childbearing age. 47.7% of preschool children are affected. The lowest prevalence is among men with 12.7%. Children with anemia have a 4.3 times higher risk of death than non-anemic children (**Brabin, 2001).** An anemic woman is five times more likely to die from pregnancy-related causes than a woman who is not anemic (**Boni-Cissé & Aké-Assi, 2007).** In Côte d'Ivoire, according to WHO statistics, the prevalence of children aged 6 to 59 months and pregnant women aged 15 to 49 years suffering from anemia is 72.2 and 54.4% respectively (**Touré, 2019**). There are certainly treatments offered by modern medicine ranging from iron supplementation, folic acid to blood transfusion depending on the severity of the case. But anemia continues to plague our countries either because these treatments cannot overcome it because the examinations are not always thorough, or because they are expensive for the populations. In alternative medicine, nutrition often uses food plants in the treatment of anemia. Thus, plants such as *Ceiba pentandra* (**Alexandre et al., 2019)**, *Ipomoea batatas* and *Spinacia oleracea* are three species of food plants that could be used in the treatment of anemia because of their nutritional values (**Dongmo, 2009).** In addition meeting the nutritional needs of populations, the benefit of using them would be certain if they effectively reversed anemia. This present study aims to evaluate the anti-anemic potential of a recipe based on the leaves of the three food plants mentioned above.

MATERIAL

Plant material

The plant material used in this work consisted of the leaves of *Ceiba pentandra*, *Ipomoea batatas* and *Spinacia oleracea*. *Ceiba pentandra* leaves were collected in the tropical rainforest of «Yabi kan », in the department of Akoupé, located at 142 km from Abidjan, in Côte d'Ivoire. Those of *Ipomoea batatas* and *Spinacia oleraceae* were purchased from market gardeners in Akoupé.

Animal material

The animals used in our experiments were male and female rats of the species *Rattus norvegicus* of wistar strain. These animals were obtained at the animal shop of the «Ecole Nationale Supérieure», Félix Houphouët Boigny University. These animals were composed of 36 female rats used in the antianemic test and 10 male and female rats used in the acute toxicity test . They were 8 weeks old. The rats were nulliparous, non-pregnant with an average body mass of 200 g. All procedures have been approved by the Ethics Committee of the Félix Houphouët-Boigny University and in accordance with the principles of the Scientific Ethics Committee of Biology for the use of laboratory animals for experimental tests **(Aworet-Samseny et al., 2011)**. As part of this work, they were used for anti-anemic tests.

Chemical material

Folin-ciocalteu, sodium carbonate, methanol, gallic acid, potassium acetate**,** aluminum chloride**,** sulfuric acid, tannic acid, quercetin, vanillin, phenyl hydrazine, vitamin B9.

Preparing the recipe extract

The leaves were washed, cut and then dried separately, away from the sun, at room temperature. They were then reduced to powder using an electric grinder (Retdch RM 200). The shredded material of each plant species (100 g) was mixed in a stainless-steel jar to make up the leaf recipe. Three hundred grams (300 g) of recipe powder were dissolved in 3 liters of water and then boiled for 8 minutes. The decoction obtained was wrung out using a square of white cotton cloth. The solution was successively filtered 3 times on absorbent cotton and once on 3 mm filter paper. The filtrate obtained was evaporated in a Venticell type oven at 50°C, for 48 hours. The refined brown powder (decocted or concentrated) obtained constitutes the extract of the recipe. It was stored in the refrigerator at 5°C in an airtight jar.

Determination of total phenols of the recipe extract

The content of total phenols was determined by the method of **Singleton et al. (1999)** using the Folin-Ciocalteu reagent. A standard range derived from a stock solution of gallic acid (1 mg/mL) was used to determine the amount of phenols in the sample.

Dosage of tannins of the recipe extract

The determination of tannins was carried out according to the method described by **Bainbridge et al. (1996).** The quantity of tannins in the samples was determined using a standard range derived from a stock solution of tannic acid (2 mg/mL) under the same conditions as the test.

Determination of flavonoids of the recipe extract

The determination of flavonoids was carried out according to the method described by **Meda et al. (2005)**. A standard range, derived from a stock solution of quercetin (0.1 mg/mL) under the same conditions as the test, determined the amount of flavonoids in the sample.

Determination of mineral profile of the recipe extract

The ash obtained from the samples was used to make the mineral profile of the recipe using the D.C.AR Variable Pressure Scanning Electron Microscope (SEM). (SEM FEG Supra 40 VPZeiss), equipped with an X-ray detector (OXFORD Instruments) connected to an EDS (Inca Dry Cool, liquid alcohol-free) microanalyzer platform. The mineral profile of the samples was performed in four steps. First, the analysis area was chosen and then the magnification was fixed (x 50) so as to observe a maximum of particles. Then, for the calibration the diameter (30nm to 120nm) and the energy of the probe (20KeV to 25KeV) were chosen and then

the working distance (WD = 8.5mm) was fixed. For the acquisition of the chemical composition, it is carried out over three repeats and then an average with a standard deviation is established. Finally, the data obtained is transferred to a file that can be used, in particular on Word or Excel.

Assessment of the acute toxicity of the recipe extract

The acute toxicity study was carried out according to the European guideline of the **OECD (2001).** This is a method by class of acute toxicity allowing to assess the dose levels that can lead to the death of a laboratory animal, to know the symptoms of acute poisoning as well as the circumstances of death. Nulliparous and non-pregnant female rats were used to conduct the experiment. They have been marked for identification. They were then fasted for 16 h and given only water. Two batches of five (5) rats were composed. The extract was dissolved in physiological water and then administered in a single dose to the rats by gavage with a volume relative to the weight of the rat. Batch 1 (control) received 1 mL of distilled water throughout the duration of the experiment. The second batch received the extract at a single dose of 2000 mg/Kg bw. The animals were then observed individually, at least once during the first 30 min and regularly during the first 24 h after the treatment. Four hours after access to food, the animals were again observed for possible toxicological signs. Daily during 14 days, signs such as tremor, convulsion, salivation, diarrhea, lethargy, sleep and coma were noted. The skin, hair, eyes and mucous membranes, as well as the respiratory system were also explored. During the 14 days of observation, animals were weighed daily for the first week and every two days for the second week at the same hours.

Antianemic activity of the recipe extract

The anti-anemic test of the extract was carried out according to the method of **Rafatullah (1993)** and **Leonard (1986).** After determination the hematological profile of 36 female rats, 6 batches of 6 rats, with an average weight of 175 g, were formed. The rats were all 5 months old. Five (5) batches were rendered anemic by intraperitoneal administration of 80 mg/Kg bw of 2-4 dinitrophenylhydrazine for 2 successive days. The rats with a hemoglobin lower than 12g/dL were considered as anemic and used for the experimentation. One batche was not rendered anemic. It was the non- anemic control batche. For the analyses, blood samples were taken by the method of incision or amputation of the tail. This method was quick and easily obtained 1 mL of blood in less than a minute **(Kraus, 1980; Leonard and Ruben, 1986)**. The batch of rats not treated with 2,4-Dinitrophenyl hydrazine (2.4-DNPH) was considered as batch 1 and received physiological water. Lots of rats made anemic numbered from 2 to 6. Rats in batches 2 to 6 were given saline water, vitamin B9, recipe extract doses of 200, 400 and 800 mg/Kg bw two days after induction of anemia. At the end of the treatment, the rats' blood was collected for the determination of hematological parameters: red blood cells, hemoglobin, hematocrit.

Lot 1: non-anemic control → Physiological water
Lot 2: anemic control → 2.4-DNPH + physiological water
Lot 3: → 2.4-DNPH + Vitamin B9
Lot 4: → 2.4-DNPH + extract 200 (mg/Kg bw)
Lot 5: → 2.4-DNPH + extract 400 (mg/Kg bw)
Lot 6: → 2.4-DNPH + extract 800 (mg/Kg bw)

Statistical analysis of results

The results were expressed as an average with standard deviations from the mean (mean ± SEM). The graphical representation of the data was made using GraphPad Prism 5 software. As for the two-factor analysis of variance performed by ANOVA with repeated experiments and the Dunnett test allowed us to determine significant differences at the 5% level.

RESULTS

Yield extraction and phenolic compounds composition of the recipe extract

The results of yield extraction and the determination of phenolic compounds in the recipe extract were presented in Table I. After the decoction of the recipe, a fine hygroscopic powder of brown color was obtained. The results showed that the yield of extraction was 20.7 ± 2.1 % and revealed the presence of polyphenols (821.6 mg/100 g), flavonoids (99.8 mg/100 g), tannins (108 mg/100 g).

Table 1: Yield and content of phenolic compounds in the recipe extract.

Yield and phenolic compounds	Yield (%)	Polyphenols (mg/100 g)	Flavonoids (mg/100 g)	Tannins (mg/100 g)
Recipe extract	20.7 ± 2.1	821.6±0.5	99.8±0.1	108.4±0.5

The decoction could lead to the degradation of heat-labile molecules because it requires the use of heat (**Cheaib, 2018).** These results are consistent with those (**Yao, 2020**) that showed that *Ipomoea batatas* and *Spinacia oleracea* are rich in polyphenols and flavonoids. The majority of plant pharmacological effects are attributed to phenolic compounds (**Amarowics, 2007), (Bursal & Gülçin, 2011), (Naczk & Shahidi, 2004).** These compounds play an important role against the development of chronic diseases that are influenced by oxidative stress such as sickle cell disease, cardiovascular disease and diabetes (**Wuillemin, 2017).**

Mineral's profile of the recipe extract

Table 2 presented the results of the mineral profile of the recipe extract. These results showed that the extract contained calcium (935.69 mg/100 g), potassium (907.78 mg/100 g), sodium (912.81 mg/100 g), zinc (94.16

mg/100 g), phosphorus (692.25 mg/100 g), magnesium (578.05 mg/100 g), manganese (520.03 mg/100 g), copper (70.78 mg/100 g) and iron (190.14 mg/100 g).

Table 2: **Mineral composition of the recipe extract.**

Minerals	Ca	Cu	Iron	Mg	Mn	K	P	Na	Zn
Composition (mg/100 g)	935.69	70.78	190.14	578.05	520.03	907.78	692.25	912.81	94.16

Iron incorporated in heme is the main component of hemoglobin (HB). It is therefore crucial to the transport of oxygen by erythrocytes (**Sukhbaatar et al., 2018**). It should be noted that prolonged iron deficiency leads to a reduction in iron availability and causes erythropoiesis by iron restriction in the bone marrow leading to moderate to severe anemia **(Wallace 2016; Daher et Karim 2017)**. Zinc (Zn) and copper (Cu) play a major role in the synthesis of hemoglobin. The results of **(Kaben, & Dahleb, 2017)** reported that zinc deficiency was associated with anemia and erythrocyte fragility. Zinc protects cell integrity and prevents oxidative stress **(El-Nawawy et al., 2002)**. Copper is necessary for the absorption and use of iron (Fe) in the formation of hemoglobin. In fact, enzymes containing copper catalyze the oxidation of ferrous iron to ferric iron **(Whitney et Rolfes, 2001)**. Potassium (K) is also involved in the management of sickle cell anemia (sickle cell disease) because the abnormal activation of the potassium chloride co-transport system leads to the loss of cellular potassium and dehydration observed in sickle cell anemia **(Agoreyo et Nwaeze, 2009)**. Magnesium (Mg) and calcium (Ca) are involved in blood clotting and also act in the formation of intra and extracellular blood and fluids **(Demo et al., 2007)**. In addition, calcium and phosphorus (P) play an important role in the growth and maintenance of bones, teeth and muscles **(Turan et al., 2003).** Magnesium also combines with calcium for the same purpose **(Ogbe et al., 2010).** About magnesium, note that this mineral has a preventive action against cardiomyopathy, muscle degeneration, growth retardation, alopecia, dermatitis, immunological dysfunction, gonadal atrophy, spermatogenesis disorders, malformations birth defects and bleeding disorders **(Chaturvedi et al., 2004**).

Evaluation of the acute toxicity of the recipe extract.

Table 3 presented the results of the administration of the dose of 2000 mg/Kg bw of the recipe extract of plants. Regular observation for 14 days showed no signs of toxicity (weight loss, salivation, drowsiness, coma, morbidity) and no mortality in the treated rat. During the observation, the animals having presented a slight agitation (they scratched and moved a lot) only in the first minutes compared to those of the control group. However, this is not part of the signs of toxicity. In addition, there was a slight non-significant weight gain in rats tested compared to mice in the control lot.

Table 3: Evolution of the weight of the rats after the administration of the recipe extract.

Products	**Dose administered (mg/Kg bw)**	**Average mouse weight (g)**		**Weight gain (g)**
		Day 1	**Day 14**	
Distilled water	Witness	150±11.56	172±13	22 ± 1.44
Extract from the recipe	2000	128.56± 9.41	147,93± 4,30	19.37± 5.11

The results of the acute toxicity test in showed that the recipe extract administration did not cause any death in the rats for the single dose of 2000 mg/Kg bw for 14 days of observation. The fact that the recipe extract induces no death at the single dose of 2000 mg/Kg bw means that its lethal dose 50 (LD50) is greater than 2000 mg/Kg bw.

Antianemic activity of the recipe extract

The results of hematological parameters (red blood cells, hemoglobin and hematocrit) after treatment of rats with 2.4 DNPH and the recipe extract are presented in Tables 4; 5 and 6. Day 0 corresponded to the mean value of hematological parameters in rats not yet rendered anemic. From day 2 to day 21 corresponded to the mean values of hematological parameters from the test after induction of anemia.

The results showed that rats initially treated with 2.4 DNPH were rendered anemic, and this anemia is characterized by a significant decrease in the concentration of red blood cells, hemoglobin and hematocrit. Administration of the recipe extract at different doses generated a significant increase in the concentration of red blood cells, hemoglobin and hematocrit.

Effect on red blood cell count

In Table 4, the 2,4-DNPH induced a decrease in red blood cell levels in all rats batches on day 2. The supplementation with the recipe extract at different doses (lot 4, 5, 6) resulted in recovery of this red blood cell count, and this effect was comparable to the effect of vitamin B9 (lot 3) at day 21. However, these red blood cell count after the supplementation remains much lower than those of the normal control. The dose of 800 mg/Kg bw (lot 6) was the most active, because the red blood cell count had increased from 3.4 at day 2 to $7.65x10^{6}$/µL on day 21 of the experiment.

Table 4: Effect of recipe extract on red blood cell counts during antianemic test.

RED BLOOD CELL COUNT (10^6/µL)					
Products	**Day 0**	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Lot 1	7,33 ± 2,1 x10^6/µL	7.43 ±4.1	7.54 ±1.14	7.15 ±3.12	7.56 ±2.12
Lot 2		3.2 ± 2.7	4.1 ±2.20	6.1 ± 1.63	6.1 ±1.63
Lot 3		3.7 ± 1.28	4.7± 2.11	6.5 ±5.52	7.61 ±5.52
Lot 4		3.3 ± 1.5	4.4 ±2.60	6.1 ±2.48	7.36 ±6.35
Lot 5		3.9 ±2.3	4.5 ±1.73	6.6 ±1.23	7.48 ±2.41
Lot 6		3,4 ±1,70	4,7±2,45	6,8±1,19	7,65±3,58

Effect on hemoglobin level

In Table 5, the administration of vitamin B9 and the intake of the extract of the recipe at different doses had cancelled the sharp reduction in hemoglobin, induced by 2,4-DNPH, by fully restoring this level compared to the normal control batch. On the other hand, the hemoglobin level of the negative control (Lot 2) remained much lower than that of the normal control. The dose of 800 mg/Kg bw was the most active, for the hemoglobin level had increased from 6.7 to 13.6 g/dL from day 2 to day 21.

Table 5: Effect of plant extracts on hemoglobin levels during antianemic test.

HEMOGLOBIN RATE (g/dL)					
Products	**Day 0**	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Lot 1	13,37 ± 3,2 %	13.40 ±1.07	13.31 ± 1.45	13.38 ±3.71	13.37 ±2.17
Lot 2		6.27 ±1.72	9.3 ±6.12	12.2 ±1.4	12.3 ±5.17
Lot 3		7.4 ±4.23	10.5 ±6.52	12.6 ±1.4	14.1 ±4.70
Lot 4		6.4 ±1.55	9.6 ±11.3	12.6 ±1.25	13.1 ±2.85
Lot 5		7.4 ±3.08	10.2 ±7.47	12.8 ±7.56	13.3 ±4.22
Lot 6		6.7 ±3.61	11.2 ±4.25	12.9 ±5.8	13.6 ±1.22

Effects on hematocrit level

In Table 6, The administration of vitamin B9 (Lot 3) resulted in a total suppression of the decrease caused by 2,4-DNPH while supplementation with the extract of the recipe (Lot 4, 5, 6) caused a partial suppression of this decrease, compared to the normal control (Lot 2). The hematocrit of lot 2 (negative control) remained very significantly lower than that of the normal control. The dose of 800 mg/Kg bw was the most active, because the hematocrit level had increased from 23.2 to 45.5 % from day 2 to day 21.

Table 6: Effect of recipe extract on hematocrit levels during antianemic test.

HEMATOCRIT RATE (%)					
Products	**Day 0**	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Lot 1	43,12± 2,8 %	42.66 ± 7.15	42.02 ± 11.4	42.04 ±5.17	43.03 ±5.13
Lot 2		21.2 ±8.19	31.1 ±5.94	37.3 ±4.40	40.8 ±8.2
Lot 3		22.5 ±2.55	35.8 ±3.40	42.12 ±8.14	46.2 ±7.26
Lot 4		20.1 ±6.50	32.0 ±6.13	40.6 ±5.25	41.1 ±4.8
Lot 5		22.3 ±3.84	34.2 ±2.72	41.7 ±4.11	43.6 ±3.80
Lot 6		23.2 ±2.53	35.1 ±3.35	43.2 ±6.30	45.5 ±3.09

The study on the antianemic effects of the three food plants recipe extract, at the most active dose of 800 mg/Kg bw, showed that before the administration of 2,4-DNPH, the values of the hematological parameters in the Wistar rats varied from 3.4 to 7.65 .10^6/µL for red blood cell count, from 6.7 to 13.6 g/dL for hemoglobin and from 23.2 to 45.5 % for hematocrit. These results are consistent with the reference values of hematological parameters in rats **(Okou et al., 2020)**, which means that these animals did not develop abnormalities related to these parameters before our experiment **(Gui et al., 2019).** The results of this work are consistent with those of **Zangeneh et al. (2018)** and **Gbenou et al. (2006)** who also observed a decrease in the number of red blood cells, hemoglobins and hematocrits following 2.4-DNPH administration. 2.4- DNPH, like Phenylhydrazine its derivative, is an antipyretic drug that causes hemolytic anemia in both humans and rats, by causing oxidative stress, free radical production, lipid peroxidation and degradation of the cell membrane of the spectrum and lysis of red blood cells (**Hodge & Sterner, 1943)**. Indeed, it induces destruction of the protein backbone of the erythrocyte, peroxidation and alteration of membrane phospholipids, oxidative destruction of hemoglobin, depletion of glutathione and ATP as well as a reduction in membrane deformability **(Shukla et al., 2012)**. Oral administration of the recipe extract of the three food plants at the doses of 200, 400 and 800 mg/Kg bw and the standard treatment with folic acid (vitamin B9) resulted in returning red blood cell count, hemoglobin

and hematocrit levels to normal level, compared to non-anemic control rats. This study showed light on the antianemic activity of the recipe extract. This antianemic property could be due to the presence of iron, polyphenols and flavonoids in the recipe extract.

The use of the combination of *Ceiba pentandra*, *Ipomoea batatas* and *Spinacia oleraceae* leaves in dietary nutrition in the treatment of anemia is therefore justified**.**

CONCLUSION

The objective of this study was to evaluate the antianemic activity of the recipe extract based of three food plants: *Ceiba pentandra*, *Ipomoea batatas* and *Spinacia oleracea*. The phenolic content of the extract was polyphenols, flavonoids and tannins. The mineral profile highlighted the presence of iron. Antianemic activity was evidenced by a return to normal red blood cell count, hemoglobin and hematocrit levels compared to non-anemic control rats. The different doses of extract in the recipe follow the same trend as vitamin B9. The dose of 800 mg/Kg bw of the recipe extract was similar trend than vitamin B9. This antianemic property could be due to the presence of iron, polyphenols and flavonoids in the recipe extract.

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