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Erythropoietic Effect of the Ethanolic Root Bark Extract of *Carissa edulis* in Phenylhydrazine-Induced Anemic Sprague-Dawley Rats

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Abstract: *Carissa edulis* has been used widely by traditional medical practitioners and herbalists to manage conditions of anemia in Ghana. This study therefore aims at investigating the erythropoietic effect of the ethanolic root bark extract of *Carissa edulis* for scientific documentation as an antianemic. Whole blood from normal Sprague-Dawley rats, phenylhydrazine-induced anemic rats and anemic rats treated with 100, 300 and 1000 mg kg^{-1} of *C. edulis* extract, 0.23 mL kg^{-1} Bioferon® or 0.23 mL kg^{-1} normal saline over a 45 days period were subjected to hematological analysis using the Cell Dyne 1800 automated analyzer®. Differences in the hematological profiles between the normal, anemic and anemic but treated rats were analyzed using GraphPad Prism 5. The 300 and 1000 mg kg^{-1} extract of *C. edulis* and Bioferon® (the reference hematinic) caused very significant increments ($p<0.01$-0.001) in red blood cell count, hemoglobin concentration and hematocrit after 23 days of treatment. Changes in mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were not significant ($p>0.05$). The extract of *C. edulis* therefore has erythropoietic activity with normocytosis and can thus be used in the management of anemic conditions. The safety for use however, needs to be ascertained in toxicity studies.

Key words: Anemia, hemoglobin concentration, red blood cells, hematological profile, *C. edulis*, Ghana

INTRODUCTION

Anemia is a hematological condition with a quantitative deficiency of circulating haemoglobin, accompanied by a reduced number of red blood cells (Oma, 1991; Rang *et al*., 1995). Red blood cells play a vital role in the body through the supply of oxygen to all the parts of the body thus, the role of erythrocytes in the body cannot be underestimated (Kumar and Clark, 2001). Anemia, a symptom of many pathologic conditions is irritating and uncomfortable and shortens the longevity (life expectancy) of the individual if the condition persists without treatment.

The World Health Organization (WHO) estimates the number of anemic people worldwide to be a staggering 2 billion (about 30% of the world's population) and that approximately 50% of all anemias can be attributed to iron deficiency and in resource-poor areas, this is frequently exacerbated by infectious diseases, malaria, worm infestation and HIV/AIDS (Tulchinsky and Varavikova, 2009; WHO, 2011). Although, anemia is associated with nutritional deficiencies, acute or chronic disease, drug usage or physiological states such as pregnancy, blood loss, impaired erythropoiesis and abnormal erythrocyte destruction are implicated (Herfindal and Gourley, 1996). In Africa, the main causes of anemia are usually due to poor nutrition and malaria. The most common causes of anemia in Ghana are inadequate dietary intake of iron, malaria and intestinal worm infestation.

The prevalence of anaemia among children has increased slightly over the past 5 years from 76% in 2003 to 78% in 2008. According to 2008, Ghana Demographic Health Survey (GDHS), about 68% of the affected children are in the urban areas while 84% are living in the rural or deprived areas. Information available indicates that a great number of Ghanaian children <5 years, representing >78% are suffering from anaemia or malnourishment while 59% of women of reproductive ages are also said to be anaemic. An earlier study has shown prevalence of anemia in Southern Ghana to be fairly common, particularly in children and women.

Even though several hematinic are available, the facts and figures reinstate the need to go into further research into new drugs that will improve the level of prognosis and compliance and help prevent the prevalence of anemia. Plants have been the major original source of

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many drugs used in the treatment of diseases today (Okolo *et al*., 1995). One such plant is *Carissa edulis*. *Carissa edulis* (Forssk.) Vahl *C. edulis* (Arabic num-num) is widespread in many parts of Africa. The plant parts are used in ethnomedicine for wide variety of illnesses such as epilepsy (Ya'u *et al*., 2008), headache, chest complaints, gonorrhea, syphilis, rheumatism, rabies as well as a diuretic (Nedi *et al*., 2004). The root bark extract has been used as anticonvulsants (Jamilu *et al*., 2007). Other folkloric uses of *Carissa edulis* include fever, sickle cell anaemia and hernia (Ibrahim, 1997). Over the years, the root bark has been used by some Traditional healers and Herbalists to treat anemia. The aim of this study, therefore is to investigate the erythropoietic properties of *Carissa edulis* on phenylhydrazine induced anaemic rats to scientifically substantiate its traditional use as an antianemic.

MATERIALS AND METHODS

Plant collection: The root bark of *Carissa edulis* was collected from the Accra plains (near Dodowa) in the Greater Accra region of Ghana and authenticated by Mr. G.H. Sam of the Department of Herbal Medicine, Faculty of Pharmacy, College of Health Sciences, KNUST where a voucher specimen with number KNUST/HM1/2001/R003 has been deposited.

Preparation of extracts: The root bark of *Carissa edulis* was chopped into small pieces and sun dried. The dry pieces were powdered using a hammer mill (Schutte Buffalo, New York, USA). A 200 g quantity of the powder was extracted with 70% ethanol with a soxhlet apparatus for 24 h.

The liquid extract was condensed under low temperature and pressure using a Buchi Rotor Evaporator (Rotavapor R-210, Switzerland) and further dried over a hot water bath (B-Tex Laboratory Engineering, Gujarat India) at 40°C. The dried extract weighing 27.8 g (percentage yield: 13.9) was labeled and stored in a desiccator. Required quantities taken and dissolved in distilled water for use in this study will be referred to as the extract or ECE.

Phytochemical screening: The ethanolic root bark extract of *Carissa edulis* was subjected to phytochemical screening in accordance with the standard procedure (Harborne, 1998).

Reference hematinic: Bioferon® (Medreich plc, England) was used as a reference hematinic. It is a preparation for treating anaemia in children and adults. Each 5 mL of the preparation contains 200 mg ferric ammonium citrate (equivalent to 41 mg elemental iron), 500 μg folic acid, 5 μg vitamin B_{12} (cyanocobalamin) and 1.75 g sorbitol. The dose of Bioferon® administered to experimental animals in this study is equivalent to the adult human dose (0.23 mL kg day^{-1}) stated by the manufacturers.

Animals and husbandry: The 5-6 weeks old Sprague-Dawley rats (200-220 g) of either sex were obtained from the Department of Pharmacology, KNUST, Animal House were made to acclimatize for a week in the laboratory prior to initiation of dosing. During this period, rats were observed (physical; in-life) daily and weighed. At initiation of treatment, animals were approximately 6-7 weeks old. Individual weights of rats placed on test were within ±30% of the mean weight for each sex. All rats were examined during the period to confirm suitability for study. Animals were housed in steel, wire mesh cages during the acclimation and the experimental periods. The rats were kept under ambient light/dark cycle, room temperature and relative humidity. The animal had free access to pelleted mice chow (GAFCO, Tema, Ghana) and water daily.

All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health Services publication No. 83-23, revised 1985). The protocols for the study were approved by the Departmental Ethics Committee.

Induction of anemia: Basal hematological profile was determined for healthy Sprague-Dawley rats using the Abbot Cell Dyn 1800 Automatic Analyzer® (Abbott Diagnostics, USA) at the Komfo Anokye Teaching Hospital, Kumasi, Ghana.

Phenylhydrazine (BDH Poole, England) was used to induce anaemia (20 mg kg^{-1}, i.p. daily for three consecutive days) as described by Yeshoda (1942) and Berger (1985) with modification. After 7 days, the hematological profile was determined again and rats with a ≥30% reduction in red blood cell count and hemoglobin concentration were considered anemic and used for this study.

Experimental procedure: Total 60 anemic rats were put into five groups of twelve and labeled A-E. A 6th group (group F) made up of 12 normal rats was also made available and put under the same laboratory conditions as the anemic rats. The groups of anemic rats were treated with 0.23 mL kg^{-1} normal saline, 0.23 mL kg^{-1} Bioferon®, or 100, 300 or 1000 mg kg^{-1} ECE. At day 23 and 45 days of treatment, blood samples were collected from experimental

animals (five from each group) into MediPlus K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) and the hematological profile performed using the Cell Dyne 1800 Automatic Analyzer®.

Statistical analysis: Differences between normal, anemic and anemic but treated rats were determined using one-way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparison test. Statistical estimates were made at a confidence limit of 95% and probability values ($p<0.05$) were considered significant. The analysis was done using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Phytochemical screening: Results of the screening revealed the presence of steroids, terpenes, flavonoids and cardiac glycosides and tannins. Anthraquinones, alkaloids, saponins were not present.

Induction of anemia: After administering phenylhydrazine to experimental animals there were very significant reductions ($p<0.001$) in RBC (Percentage reduction: 41.6%), HGB (Percentage reduction: 46.65%) and HCT (Percentage reduction: 46.54%). There were no significant differences in MCV, MCH, MCHC, RDW and PCT values between the normal and the anemic animals. WBCs increased significantly (Table 1).

Treatment of anemic rats: After 23 days of treatment, 300 and 1000 mg kg^{-1} ECE caused very significant increments ($p<0.01$-0.001) in RBC, HGB, HCT and RDW compared to the anemic rats. MCV, MCH, MCHC and PLT were not significantly different from that of the anemic rats (Table 2).

This observation was similar to the very significant effects ($p<0.001$) seen with Bioferon® treatment. There were very significant increments ($p>0.05$) in RBC, HGB, HCT while MCV, MCH, MCHC and PLT were also not significantly different in the anemic rats and in rats treated with normal saline (Table 2). After 45 days of treatment there were increments ($p<0.05$) in RBC, HGB, HCT and RDW in the 100 mg kg^{-1} ECE and the normal saline treated rats compared to the anemic rats.

The effects of 300 and 1000 mg kg^{-1} ECE and Bioferon® were very significant ($p<0.001$). The RDW was however not as elevated as in day 23. MCV, MCH, MCHC and PLT were again not significantly different from that of the anemic rats (Table 3).

Table 1: The hematological profiles of Sprague-Dawley rats prior to induction of anemia (Normal I), phenylhydrazine-induced anemic rats and normal rats kept under experimental conditions >45 days study period (Normal II)

Groups	WBC (K μL^{-1})	RBC (M μL^{-1})	HGB (g dL^{-1})	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	RDW (%)	PLT (K μL^{-1})
Normal I	7.75±0.71	6.58±1.60	16.31±1.11	46.94±2.77	71.32±3.92	23.76±3.01	34.4±2.89	15.58±1.86	318.6±46.4
Anemic	15.09±2.44†††	3.84±0.79***	8.70±1.15***	25.09±3.24***	70.58±4.19^{NS}	24.75±3.21^{NS}	33.18±3.57^{NS}	17.42±1.71†††	393.0±76.81^{NS}
Normal II	8.08±1.22^{NS}	6.53±2.31^{NS}	16.18±2.19^{NS}	45.75±2.77^{NS}	69.96±3.78^{NS}	23.04±2.45^{NS}	35.55±2.89^{NS}	16.03±1.22^{NS}	345.0±79.6^{NS}

Table 2: A comparison of the haematological profiles of phenylhydrazine-induced anemic rats and phenylhydrazine-induced anemic rats treated with 0.23 mL kg^{-1} normal saline, 0.23 mL kg^{-1} Bioferon® and 30, 100 and 300 mg kg^{-1} ECE for 23 days

Groups	WBC (K μL^{-1})	RBC (M μL^{-1})	HGB (g dL^{-1})	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	RDW (%)	PLT (K μL^{-1})
Anemic	15.09±2.44	3.84±0.79	8.70±1.15	25.09±3.24	70.58±4.19	24.75±3.21	33.18±3.57	17.42±1.71	393.0±76.81
Normal saline	7.35±1.55***	4.02±0.63^{NS}	8.93±1.83^{NS}	26.75±2.23^{NS}	67.65±3.65^{NS}	24.05±2.36^{NS}	32.60±3.53^{NS}	24.95±1.45†††	386.0±58.76^{NS}
Bioferon®	8.23±1.23***	5.92±0.72†††	11.78±1.53†††	34.69±2.67†††	66.17±2.45^{NS}	23.07±2.79^{NS}	33.49±3.72^{NS}	16.68±2.68^{NS}	418.5±62.23^{NS}
ECE (100)	7.85±1.09***	4.23±0.55^{NS}	9.14±0.96^{NS}	28.02±1.93^{NS}	65.24±2.78^{NS}	20.75±2.86^{NS}	32.60±3.93^{NS}	24.12±1.79†††	396.0±58.76^{NS}
ECE (300)	8.02±1.21***	5.08±0.87††	11.18±0.63†††	33.09±2.18†††	65.02±3.61^{NS}	21.99±2.11^{NS}	32.79±2.46^{NS}	17.32±1.18^{NS}	416.0±82.33^{NS}
ECE (1000)	8.57±1.64***	5.69±0.59†††	11.56±0.78†††	36.02±2.88†††	68.67±3.53^{NS}	22.34±2.63^{NS}	33.07±2.07^{NS}	16.72±2.84^{NS}	375.0±79.96^{NS}

Table 3: A comparison of the haematological profiles of phenylhydrazine-induced anemic rats treated with 0.23 mL kg^{-1} Normal saline, 0.23 mL kg^{-1} Bioferon®, 30, 100 and 300 mg kg^{-1} ECE for 45 days with that of phenylhydrazine-induced anemic rats (control)

Groups	WBC (K μL^{-1})	RBC (M μL^{-1})	HGB (g dL^{-1})	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	RDW (%)	PLT (K μL^{-1})
Anemic	15.09±2.44	3.84±0.79	8.70±1.15	25.09±3.24	70.58±4.19	24.75±3.21	33.18±3.57	17.42±1.71	393.0±76.81
Normalsaline	7.11±1.13***	5.21±1.76†	11.42±1.09†	31.33±3.71†	62.94±4.01^{NS}	22.67±1.58^{NS}	30.78±3.82^{NS}	21.86±2.68†	409.0±89.63^{NS}
Bioferon®	8.58±1.29***	7.37±0.74†††	16.73±1.95†††	48.89±3.33†††	65.3±3.87^{NS}	22.56±2.45^{NS}	32.19±4.52^{NS}	19.58±3.51^{NS}	387.4±39.35^{NS}
ECE (100)	7.68±0.97***	5.36±0.86†	11.82±0.98†	31.52±3.21†	65.25±1.72^{NS}	23.29±1.79^{NS}	31.08±1.60^{NS}	22.22±1.87†	379.0±75.63^{NS}
ECE (300)	7.88±1.34***	6.41±0.69†††	14.00±1.82†††	42.64±2.73†††	67.14±1.68^{NS}	22.58±2.04^{NS}	32.69±1.28^{NS}	18.87±2.59^{NS}	402.0±67.83^{NS}
ECE (1000)	8.18±1.04***	6.98±0.89†††	15.59±1.96†††	47.39±2.81†††	67.78±2.68^{NS}	22.17±0.65^{NS}	31.89±1.26^{NS}	19.71±2.18^{NS}	382.0±83.71^{NS}

Values quoted are means±SD. Levels of significant between the anemic and the treated rats were determined using one-way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparisons test. For significant decrements: ***Implies $p\leq0.001$. For significant increases: †Implies $p\leq0.05$; †††Implies $p\leq0.001$. WBC = White Blood Cell Count, RBC = Red Blood Cell Count, HGB = Hemoglobin concentration, HCT = Hematocrit, MCV = Mean Corpuscular Volume, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean Corpuscular Hemoglobin Concentration, RDW = Red Blood Cell Distribution Width, PLT = Platelet Count; NS = Non Significant

DISCUSSION

There was very severe normocytic anemia with anisocytosis in the phenylhydrazine-treated rats indicated by the very significant reduction in RBCs, HBG and HCT, non-significant change in MCV, MCH and MCHC (between the normal and the phenylhydrazine-treated rats) and the high RDW. In severe anaemia, the haematocrit may fall by ≥10% (Hall and Guyton, 1999). This was due to the haemolytic action of phenylhydrazine on red blood cells. Phenylhydrazine induces haemolysis of RBCs by inducing the formation of toxic, free radicals (peroxidation of lipids) that can attack cellular macromolecules like haemoglobin resulting in the oxidative damage within the red blood cells and oxidative degradation of spectrin in the membrane skeleton resulting in their destruction (Cary *et al*., 2000). Again, free radical attack accelerates the normal aging process of the red cells causing premature splenic sequestration (Berger, 2007). This resulted in a quantitative deficiency of circulating RBCs, HGB and HCT (Unami *et al*., 1996; Zimmermann *et al*., 1997; Nelson *et al*., 1997; Cighetti *et al*., 1999). There was no macrocytosis (as found in situations of megaloblastic, aplastic, dyserythropoietic and sideroblastic anemias) which would have been recorded as a high MCV or microcytosis (as found in microcytic anemia) which would have been recorded as a low MCV (Archer *et al*., 1982; Okhamafe *et al*., 2003). MCH and MCHC values between normal and phenylhydrazine-induced rats not being significantly different indicates that phenylhydrazine-induced anemia is not a hypochromic or spherocytic anemia (Dacie and Lewis, 1995; Bain, 1995). The elevated WBCs seen with the induction of anemia could probably be due to the body's defence mechanism of getting rid of hemolytic products of RBC hemolysis.

Carissa edulis at doses of 300 and 1000 mg kg^{-1} was able to reverse very significantly anemia caused by phenylhydrazine after 45 days of treatment without anisocytosis. This was indicated by the significant increase in RBC, HGB and HCT and the insignificant increases in RDW. A mix of both large cells and small cells will cause the RDW to be elevated (known as anisocytosis). The hematological profile values recorded for normal animals kept under experimental conditions were not significantly different from that measured prior to the study. The effects of 300 and 1000 mg kg^{-1} ECE were similar to that of Bioferon®. Similarity in effects implies that the extract could possibly have some components such as iron and the B-complex vitamins which are crucial requirements for erythropoiesis. The near constant hematological profile of normal rats prior to and under experimental conditions indicates that the experimental conditions had no detrimental effect whatsoever on the hematological profile.

Phytochemicals such as the flavonoids, tannins (polyphenols) and terpenes show antioxidant activity (Pietta, 2000; Grassmann, 2005; Zhang and Lin, 2009; Kumar *et al*., 2010) and are thus liable to protect lipids, blood and other body fluids from damage from oxidative stress.

These phytochemicals in the *Carissa edulis* extract could therefore have contributed to the faster and dose-dependent reversal of anemia during treatment compared to the normal saline group. The effect of the body's homeostatic mechanism at reversing the induced anemia (expected to be the motive behind using normal saline for treatment) could be delayed by the effect of phenylhydrazine which could still linger on awhile.

CONCLUSION

The ethanolic root bark extract of *Carissa edulis* has erythropoietic activity with normocytosis and can thus be used in the management of anemia. The safety for use however needs to be ascertained in toxicity studies.

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