

Erythropoetic and Bone Marrow Stimulating Activity of *Terminalia Catappa* Extract: Possible Role of Nitric Oxide Signaling

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ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aims: We assessed the capacity and mechanism of *Terminalia catappa* (TC) to induce erythropoiesis *in vivo* in phenylhydrazine induced anemic mice.

Place and Duration of Study: Sample: This study was carried out at Department of Biochemistry and Center for Biotechnology Research and Training Ahmadu Bello University Zaria, and National Research Institute for Chemical Technology, Zaria. The duration spanned between Jan 2011 and Feb 2012.

Methodology: solvent fractions of *Terminalia catappa* aqueous extract was used to treat phenylhydrazine-induced anemic mice. Treatment was done for four days, erythropoietic activity of each fraction was assayed by determining the effect of these fractions on intracellular hemoglobin and reticulocyte level from the blood, arginase was also assayed. Bone marrow carbonic anhydrase was assayed to monitor bone marrow erythropoietic stimulation.

Results: *Terminalia Catappa* was able to up-regulate the synthesis of intracellular hemoglobin ($0.135 \pm 0.004 \mu\text{mol}/0.1\text{ml}$) significantly comparable to hydroxyurea (HU) ($0.158 \pm 0.006 \mu\text{mol}/0.1\text{ml}$), and normalize the peripheral blood reticulocyte index significantly at $P=0.05$ $0.94 \pm 0.25\%$ close to the non anemic mice $0.97 \pm 0.25\%$ and bone marrow carbonic anhydrase activity. TC induced nitric oxide synthase activity significantly ($P=0.05$) comparable to hydroxyurea.

Conclusion: The results demonstrate *Terminalia Catappa* extract as an erythropoietic agent that supports normal erythroid differentiation *in vivo* in phenylhydrazine-induced anemic mice in a synergistic fashion.

Keywords: *Terminalia catappa*, Nitric oxide, arginase, phenylhydrazine, sickle cell anemia, erythropoiesis and hemolytic anemia.

1. INTRODUCTION

Terminalia catappa (almond) from the family Combrataceae is known to originate from sub-tropical zones of India and Indonesia. Almond is known to have a barrage of medicinal uses: anti-inflammatory uses,

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hepatoprotective, antioxidant, antiparasitic and antimicrobial among other uses (Chu et al., 2007; Kinoshita et al., 2007; Babayi et al., 2004; Fan et al., 2004). In Nigeria, the ethanol extracts of almond leaf have shown potential in treating sickle cell anemia (SCA) (Ameh et al., 2010; Moody et al., 2004).

Anemia could result from rapid loss of red blood cells (RBC) which is a common feature of SCA and the β -thalassemia or ineffective RBC synthesis from the bone marrow (Esbach et al., 1987), usually a balance in synthesis and depletion of RBC is the key at maintaining a healthy hemoglobin level. Nitric oxide (NO) serves as a potent signaling molecule causing vasodilation and increased local blood flow [Kim et al., 2007; Etten et al., 2002; Spieker et al., 2002]. It has been shown previously that its level is decreased in chronic hemolytic anemia like SCA and β -thalassemia cases [Perrine, 2011; Eberhardt et al., 2003; King, 2003; Morris et al., 2003]. In addition, NO has been implicated in hydroxyurea (HU) and hydroxybutyrate induced gamma globin gene activation [Bianchi et al., 2007; King, 2003; Cokic et al., 2003].

Erythropoiesis involves the proliferation and differentiation of pluripotent stem cells into immature erythroid progenitors and finally into reticulocytes which usually takes place in the bone marrow in adult primates [Maria et al., 1999; Kenedy et al., 1997]. Studies into the mechanisms of proliferation of the erythroid cells has implicated NO as a major candidate inducer and high NO levels have been shown to be associated with heightened erythropoietic activity, [Hsu et al., 2006; Beleslin-cokic et al., 2004]. The availability of NO in the blood is modulated by the activity of arginase which depletes plasma arginine, the substrate for NO production by NO synthase [Little et al., 2009; Kato et al., 2007; Chang et al., 1998].

Pharmacologic mediation of improved fetal hemoglobin synthesis through the induction of NO could be of interest in the chemotherapy of hemolytic anemia like sickle cell anemia and β -thalasemias [Fibach et al., 2003]. We have shown previously that TC methanolic extract enhances intracellular hemoglobin content in Balb C mice [Aimola et al., 2011], but the precise mechanism is not known. To investigate this we evaluated TC extract potency in stimulating bone marrow erythropoietic activity in phenylhydrazine- induced anemic Balb C mice.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

TC was obtained from the Botanical Garden of Ahmadu Bello University, Zaria (Longitude 7.72518 and latitude 11.11324). It was identified at the Department of Biological Sciences Herbarium by U.S. Gallah and deposited with voucher number 3940-TC.

2.1 Fractionation of *Terminalia cattapa* L.

The fresh leaves of TC were oven dried to a constant weight at 27°C in a Gallenkamp OGS60 oven for 72hrs and the dried leaves were then pulverized. 50g of the pulverized sample was extracted exhaustively with 200ml methanol and distilled water and subsequently filtered using a Whatman No.1 filter paper and the filtrate was concentrated using a water bath at 40°C. The extract was reconstituted in 10ml of methanol and loaded on a pre-equilibrated silica gel column. The extract was then partitioned according to solubility in different solvents as previously described (Akiyama et al., 2005; Newton et al., 2002). The active fractions were eluted using 20ml of analytical grade petroleum ether, methanol, ethanol, acetone (Pharmacia). The eluate was air dried and stored in amber bottles.

2.2 Animals

All experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

Thirty-six apparently healthy Balb C mice weighing 20±5g/mice were housed in well- ventilated cages in the Animal House of the Department of Pharmacology, Ahmadu Bello University, Zaria, allowed to acclimatize for three weeks and maintained on conventional rat chow (Reaven et al., 2003) and water. The mice were divided into 3 control groups (A, B and C) and groups (D, E, F, G, H, I). Hemolytic anemia was induced by intraperitoneal administration of phenylhydrazine (20mg/kg bodyweight) to all the groups except group A. Group B was not treated for the duration of the experiment, group C was treated with hydroxyurea 15mg/kg bodyweight orally [Grant et al., 1997]. Groups D, E, F, G, H and I were treated with 43mg/kg bodyweight of the TC solvent fractions in water orally corresponding to one-fifth of the documented LD₅₀ of TC (Ahmed et al., 2005). Group A received phosphate buffered saline only orally. The treatment regime was for 5 days and blood was collected in EDTA for further analysis. The packed cell volume (PCV) of the mice was assayed using standard procedures as previously described (Hanson et al., 1997). Bone marrow was also extracted from the femur of the mice as previously described (Zhang et al., 2007).

2.3 Assay for Intracellular Hemoglobin and Reticulocytes

The number of reticulocytes in 10µl of blood from tail vein was determined using the methylene blue assay (Riley et al., 2001). Intracellular hemoglobin was assayed by lysing RBCs using repeated freezing and thawing in distilled water and the lysate subjected to benzidine assay in 10mM phosphate buffer using hemoglobin as standard (Riley et al., 2001).

2.4 Arginase Assay

The arginase assay was carried out spectrophotometrically by measuring the formation of L-ornithine at 515 nm with ninhydrin as previously described (Hafid-Medheb et al., 2003) with slight modifications. 50µl serum samples were diluted to 1ml with 0.01mM carbonate bicarbonate buffer containing 0.13% v/v sodium azide and 0.43% w/v L-arginine. The mixtures were incubated at 37°C for 20min. The reaction was stopped by cooling on ice and the addition of 2ml 0.71% ninhydrin containing 91% acetic acid v/v and 1.1% v/v phosphoric acid. Color formation proceeded for 15min at 95°C. The concentration of L-ornithine was measured spectrophotometrically at 515 nm in 1.5 ml cuvettes. Representative data are presented as activity in µmol L-ornithine/min

2.5 Carbonic Anhydrase Assay

Bone marrow Carbonic anhydrase (CA) was assayed using CO₂ saturated water as substrate as previously described (Zhang et al., 2007). Briefly 100µl bone marrow extracted from the femur of the mice was reconstituted in Tris-HCl buffer pH 8.3 and maintained on ice, then 4ml of the substrate was then introduced, the reaction was monitored and time recorded for pH drop to 6.3. A similar setup (excluding the bone marrow) was carried out for the blank. The activity was expressed as units/ml of enzyme.

Statistical Analysis

For each treatment and control, data are expressed as means±SD. An unpaired two tailed t-test was employed to detect significant differences between treatment and control groups..

3. RESULTS AND DISCUSSION

3.1 Terminalia catappa supports normal erythroid differentiation in phenylhydrazine-induced anemic mice

We assessed the ability of the different fractions of TC to stimulate normal erythroid differentiation by determining its effect on bone marrow erythroid cell carbonic anhydrase activity. Bone marrow carbonic anhydrase activity was significantly suppressed ($P=0.05$) in groups administered with methanolic TC methanolic fraction (1.503 ±0.09 unit/ml), methanolic TC petroleum ether fraction (1.218 ±0.1unit/ml), methanolic TC acetone fraction(0.666 ±0.005unit/ml), Aqueous TC Ethanol fraction (1.019 ±0.209unit/ml) aqueous TC petroleum ether fraction (0.966±0.02unit/ml) and aqueous TC methanolic fraction (1.503 ±0.123unit/ml) when compared to the negative control (1.974 ±0.06unit/ml) as shown in Figure 3. While no significant difference was observed when compared to the normal control (1.075 ±0.08unit/ml) also there was no significant difference in group treated with hydroxyurea (1.589±0.005unit/ml) when compared to aqueous TC methanolic fraction (1.503 ±0.09unit/ml). TC appeared to normalize bone marrow carbonic anhydrase activity comparable to hydroxyurea treated mice.

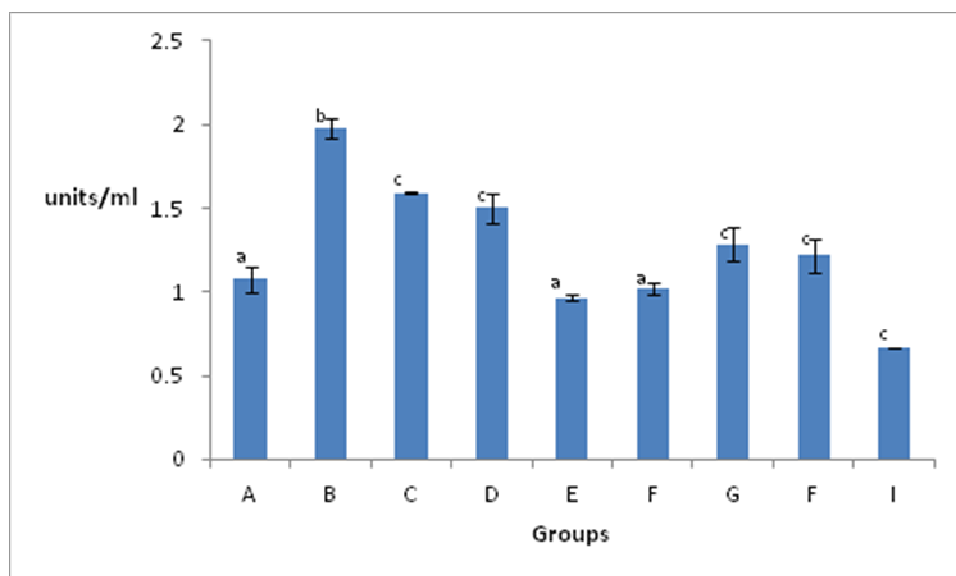


Figure 1: Carbonic anhydrase activity in phenylhydrazine- induced anemic mice

Carbonic anhydrase activity was assayed in bone marrow tissues using CO₂ saturated water as substrate in the different groups of Phenylhydrazine induced anemic mice (n=3) which were treated for four days orally with different solvent fractions of TC aqueous and methanolic extracts: The results are presented as mean $\pm$ SD of four independent experiments. Activity of carbonic anhydrase was represented units/ml of bone marrow cells (6×10^7 cells). All the groups were induced with hemolytic anemia using 20mg/kg phenyl hydrazine except group A. Group B was not treated in the course of the experiment, while mice were subsequently treated orally with 0.15mg/kg bodyweight hydroxyurea group C. 0.43mg/kg bodyweight of different solvent fractions of aqueous and methanolic extracts of TC was used for treatment. Group D is aqueous extract methanolic fraction of TC, group E is aqueous extract ethanolic fraction of TC, group F is aqueous extract petroleum ether fraction of TC, group G is methanolic fraction of methanolic extract of TC group H is petroleum ether fraction of methanolic extract of TC and group I is acetone fraction of methanolic extract of TC. The different alphabets a, b and c represent significant differences between treatment groups and the positive control A or negative control B ($P = .05$).

3.2 Terminalia catappa Improves bone marrow activity in Balb C mice

We assayed the ability of TC to restore normal bone marrow function in phenyl hydrazine induced anemic mice by assaying for its effect on number of circulating reticulocytes and total formed elements in the blood. We observed that TC equalized the number of circulating erythrocytes significantly comparable to that of the normal (non anemic) mice and hydroxyurea treated mice. The proportion of reticulocytes in the peripheral circulation was significantly elevated $1.53 \pm 0.11\%$ ($P=.05$) in the phenyl hydrazine induced anemic mice compared to the treated groups as shown in Figure 2. The PCV of the anemic treated and untreated mice was significantly elevated above the normal control as shown in Figure 3.

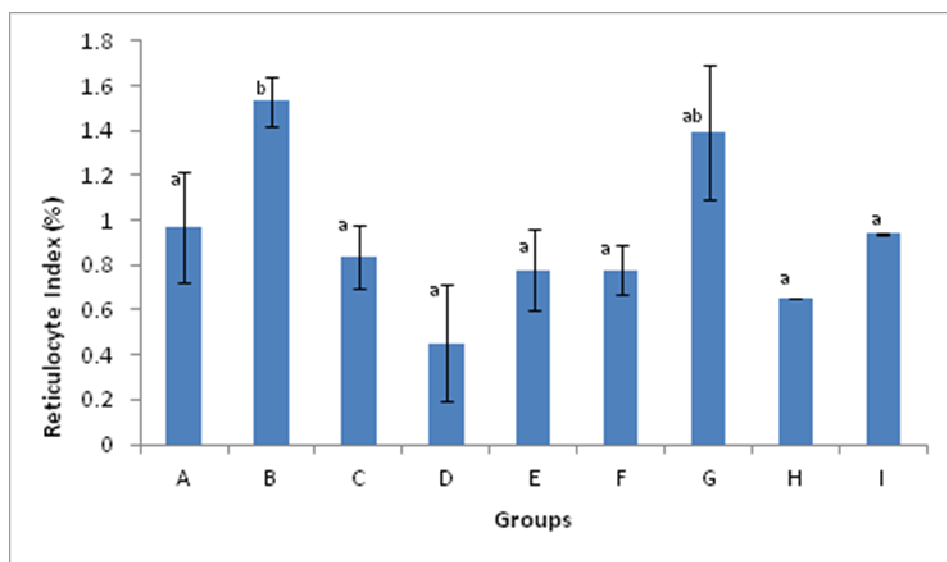


Figure 1 Reticulocyte index of phenyl hydrazine induced hemolytic mice:

Phenylhydrazine induced anemic mice (n = 3) were treated for four days orally with different solvent fractions of TC aqueous and methanolic extracts, reticulocyte index was represented as percentage of circulating erythrocytes to total blood formed elements. The results are presented as mean $\pm$ SD of four independent experiments. All the groups were induced with hemolytic anemia using 20mg/kg phenyl hydrazine except group A. Group B was not treated in the course of the experiment, while mice were subsequently treated orally with 0.15mg/kg bodyweight hydroxyurea group C. 0.43mg/kg bodyweight of different solvent fractions of aqueous and methanolic extracts of TC was used for treatment. Group D is aqueous extract methanolic fraction of TC, group E is aqueous extract ethanolic fraction of TC, group F is aqueous extract petroleum ether fraction of TC, group G is methanolic fraction of methanolic extract of TC, group H is petroleum ether fraction of methanolic extract of TC and group I is acetone fraction of metanolic extract of TC. The alphabet a differ significantly in the percentage of circulating erythrocytes from b the negative control ($P = .05$).

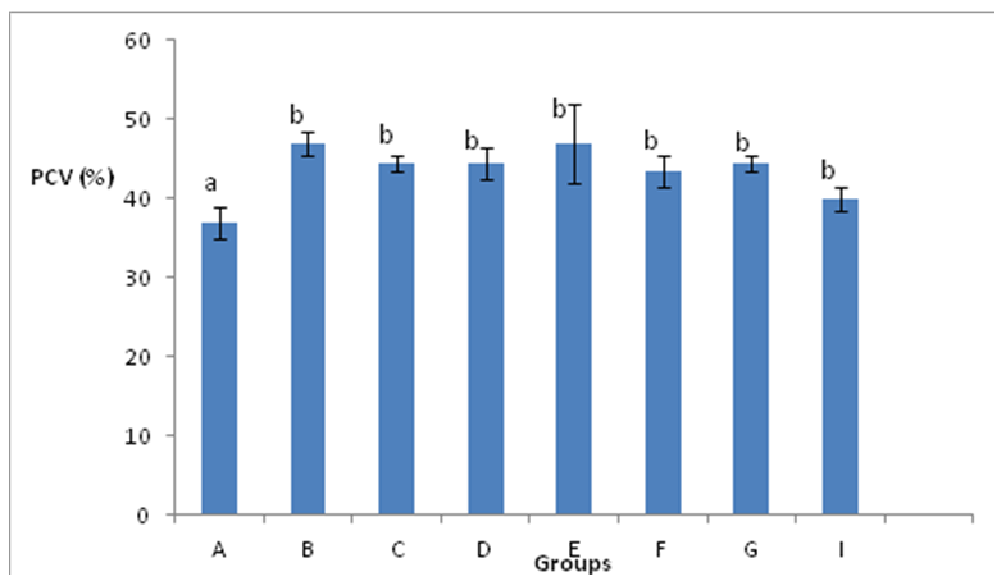


Figure 3: Packed cell volume in phenylhydrazine induced anemic mice

Packed cell volume was assessed using standard procedures to assess the potential of TC extract solvent fractions to support production of total formed elements in the blood. Phenyl hydrazine induced anemic mice (n=3) were treated for four days orally with different solvent fractions of TC aqueous and methanolic extracts. The results are presented as mean $\pm$ SD of four independent experiments. All the groups were induced with hemolytic anemia using 20mg/kg

phenyl hydrazine except group A. Group B was not treated in the course of the experiment, while mice were subsequently treated orally with 0.15mg/kg bodyweight hydroxyurea group C. 0.43mg/kg bodyweight of different solvent fractions of aqueous and methanolic extracts of TC was used for treatment. Group D is aqueous extract methanolic fraction of TC, group E is aqueous extract ethanolic fraction of TC, group F is aqueous extract petroleum ether fraction of TC, group G is methanolic fraction of methanolic extract of TC, group H is petroleum ether fraction of methanolic extract of TC and group I is acetone fraction of methanolic extract of TC. The alphabet a differ significantly from b ($P= .05$) No significant difference was observed between the treated groups and untreated phnylhydrazine-induced anemic mice was observed.

3.3 Terminalia catappa improves hemoglobin synthesis

TC increased the content of intracellular hemoglobin significantly higher ($P =.05$) 0.135 ± 0.001 than the phenyl hydrazine induced anemic untreated mice 0.00995 ± 0.003 and the normal untreated control mice 0.078 ± 0.002 as shown in Figure 4.

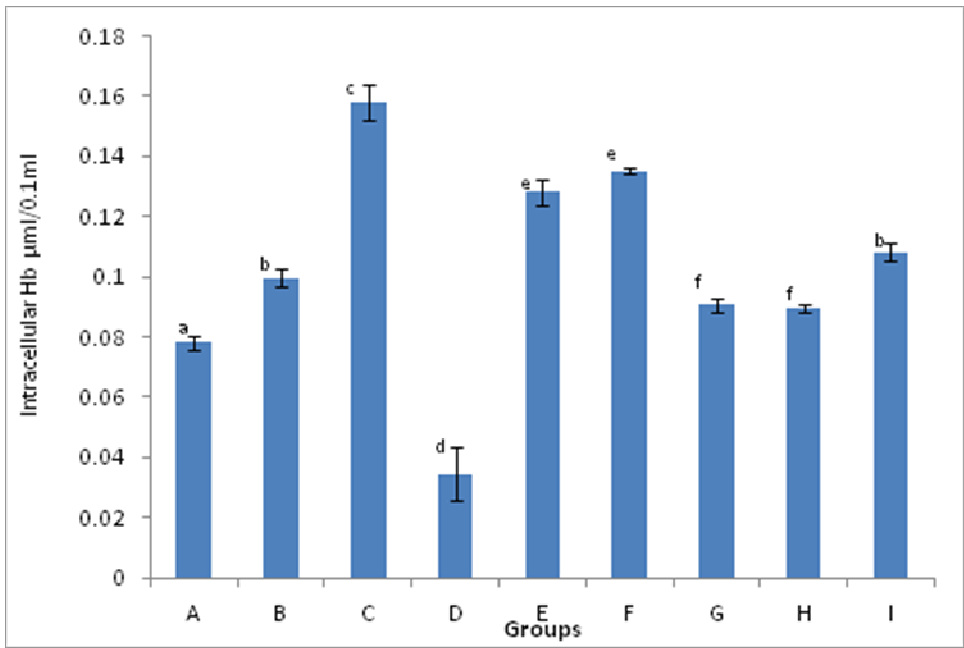


Figure 4: Intracellular hemoglobin in anemic mice:

Phenyl hydrazine induced anemic mice (n=3) were treated for four days orally with different solvent fractions of TC aqueous and methanolic extracts: The results are presented as mean $\pm$ SD of four independent experiments. All the groups were induced with hemolytic anemia using 20mg/kg phenyl hydrazine except group A. Group B was not treated in the course of the experiment, while mice subsequently treated orally with 0.15mg/kg bodyweight hydroxyurea group C. 0.43mg/kg bodyweight of different solvent fractions of aqueous and methanolic extracts of TC was used for treatment. Group D is aqueous extract methanolic fraction of TC, group E is aqueous extract ethanolic fraction of TC, group F is aqueous extract petroleum ether fraction of TC, group G is methanolic fraction of methanolic extract of TC group H is petroleum ether fraction of metanolic extract of TC and group I is acetone fraction of metanolic extract of TC. The alphabets a, b, c, d, e and f differ significantly from each other ($P = .05$). All the treatment groups significantly ($P=.05$) increased intracellular hemoglobin content compared to the untreated groups A and B.

3.4 Terminalia catappa increases production of Nitric Oxide *in vivo*

NO has been implicated in increased fetal hemoglobin synthesis. NO production has been shown to be modulated by the activity of arginase. We studied the ability of TC to function as a stimulant for production of NO by assaying for its effect

on serum arginase activity. The results revealed a serum arginase suppressive activity of the solvent fractions of TC, comparable to the control erythropoietic drug; hydroxyurea as shown in Figure 4.

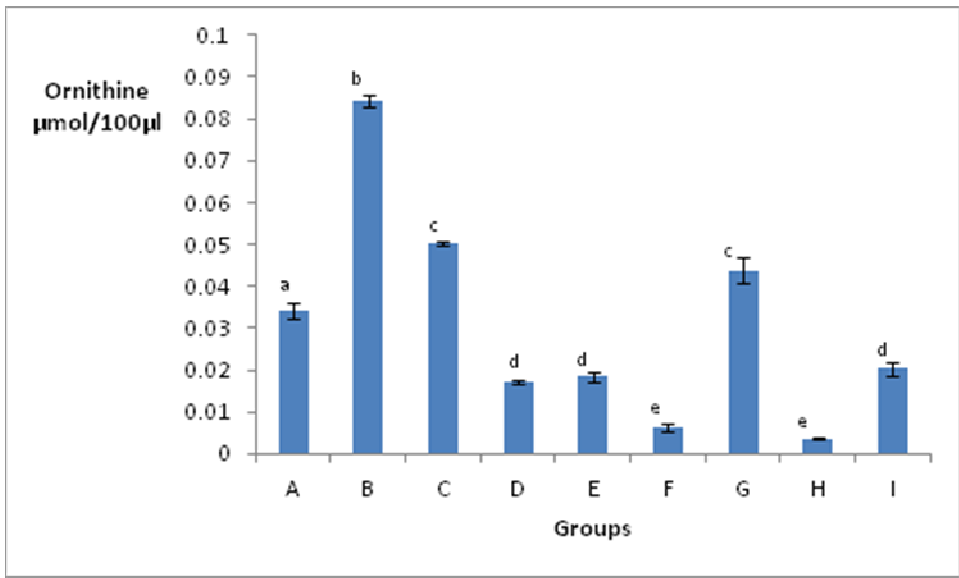


Figure 5: Serum arginase activity in phenylhydrazine induced anemic mice

The activity of serum arginase was monitored to check if the solvent fractions of TC can sufficiently suppress arginase activity thereby allowing for an increased activity of nitric oxide synthase by assessing the amount of ornithine liberated by the enzyme in the serum of treated and untreated phenyl hydrazine induced anemic mice (n=3) and expressed as μmol/100μl of serum Phenylhydrazine induced anemic mice were treated for four days orally with different solvent fractions of TC aqueous and methanolic extracts: The results are presented as mean ± SD of four independent experiments. All the groups were induced with hemolytic anemia using 20mg/kg phenyl hydrazine except group A. Group B was not treated in the course of the experiment, while mice were subsequently treated orally with 0.15mg/kg bodyweight hydroxyurea group C. 0.43mg/kg bodyweight of different solvent fractions of aqueous and methanolic extracts of TC was used for treatment. Group D is aqueous extract methanolic fraction of TC, group E is aqueous extract ethanolic fraction of TC, group F is aqueous extract petroleum ether fraction of TC, group G is methanolic fraction of methanolic extract of TC, group H is petroleum ether fraction of methanolic extract of TC and group I is acetone fraction of methanolic extract of TC. The alphabets a, b, c, d and e differ significantly from each other (P = .05). All of the solvent fractions of TC suppressed aerum arginase activity significantly (P = .05) when compared to the untreated control.

Discussion

Hemolytic anemia is a condition in which there are not enough RBCs in the blood due to premature destruction of the RBCs or blood loss from the body. Anemia ensues when the bone marrow cannot effectively make up for the lost RBCs, similarly as is found in sickle cell hemolytic anemia there exists an insufficiency in the synthesis of RBC from the bone marrow (Mcintosh, 1968). Herein, we demonstrate the capacity for active fractions of TC to induce erythropoiesis in bone marrow through a NO signaling pathway.

We found out that the active fractions of TC increased intracellular hemoglobin significantly (P<0.05) above the untreated mice and non hemolytic mice in a similar fashion with HU. This is consistent with our previous finding that the crude extract of TC induces hemoglobin synthesis (Aimola et al., 2011).

Active hemoglobin synthesis occurs in committed erythroid cells, and is accompanied with increased synthesis of carbonic anhydrase in normal erythroid development. Consequently, carbonic anhydrase expression is linked to the fetal hemoglobin (HbF) to adult hemoglobin (HbA) switch at the late phase of erythroid development (Hebbel and Miller, 1984).

We observed that the active fractions appeared to lower the expression of carbonic anhydrase significantly comparable to the normal non anemic, non treated mice as measured by the appearance of CA activity as opposed to phenylhydrazine-induced hemolytic anemic mice treated with HU. HU an established fetal hemoglobin inducer, increased the expression of CA significantly above the phenylhydrazine- induced hemolytic untreated mice. This demonstrates that HU and the plant extracts induce normal erythroid development in Balb C mice through different mechanism which requires further studies.

We investigated the potential of TC active plant fractions to induce NO synthesis in murine anemic mice models to ascertain its potential to induce fetal hemoglobin synthesis in SCA patients in northern Nigeria who commonly use this plant as a treatment modality. The effect of TC on NO production was carried out using an indirect assay of NO by assaying its activity on blood arginase activity. NO has been demonstrated previously to be involved in the activation of λ -globin activation through the activation of soluble guanyl cyclase (Cokic et al., 2003). In addition, Nitric oxide synthase activity has been shown to be modulated by arginase which competes with the enzyme for L-arginine, the physiologic precursor of NO (Laminkra et al., 2009; Durante et al., 2007; Chang et al., 1998; Hey et al., 1997; Brady et al., 1990). The study revealed that the active fractions of TC suppressed serum arginase activity significantly higher than HU which is indicative of a potential of TC as a possible potentiating agent for HbF synthesis (Cokic et al., 2003).

We analyzed for the possibility of an *in vivo* dyserythropietic response to TC by the mice in comparism to HU which have been shown to stimulate normal erythropoietic response in bone marrow of mice [Hey et al., 1997]. A significantly improved reticulocyte response was observed with administration of TC similar to HU as shown in Figure 3

HU stimulates the synthesis of fetal hemoglobin through an induced bone marrow erythroid development with an increased erythropoietic response and has been demonstrated to be therapeutic and highly useful in hemolytic blood disorders like sickle cell anemia and β -thalassemia [Cokic et al., 2003; Hey et al., 1997]. The improved erythropoietic response in mice with hemolytic anemia due to TC that we describe here underscores a huge potential of TC active fractions activity in hemolytic blood disorders. TC appeared to suppress the expression of CA in the later phase of erythroid development suggesting the presence of components that masks the expression of CA in TC. The experimental model described herein revealed little or no side effects associated with administration of TC leaf extracts. Further trials are required, though, to establish a dose dependent response to TC and to establish the structure and chemical nature of the components of the active fractions of TC.

4. CONCLUSION

TC solvent extracts showed varied erythropoietic activities comparable to HU and the extracts were able to enhance erythropoiesis in phenylhydrazine-induced anemic mice models in a mechanism similar to HU. We also observed that the extract supports normal erythroid development and suppressed serum arginase activity thus enhancing NO synthase activity during the course of treatment.

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COMPETING INTERESTS

The authors declare that no competing interest exists

AUTHORS' CONTRIBUTIONS

Aimola I.A., Inuwa H.M., Nok A.J., Mamman A.I designed the study, and also read and approved the final manuscript.
Habila1 N., Muhammad1 A. designed the animal experiments section also read and approved the final manuscript.
Igoche1 A., Ogungbemi1 K., Tighil1 M Executed the laboratory aspects of the work
Agbaji A.S., contributed with plant selection and collection Usman Y. worked on the fractionation of TC extract.

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