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ORIGINAL ARTICLE

Effect of a Bi-herbal Formula Extract on ETS Variant-6 and Nuclear Factor Erythroid-2 Genes Expression in Phenyl-Hydrazine induced Anaemia in Albino Wistar Rats

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ABSTRACT

Background: Alterations in the expressions of ETS variant-6 (ETV6) and Nuclear Factor Erythroid 2 (NFE2) may occur as part of cellular response to phenyl-hydrazine administration and the need to replenish RBCs. Understanding the relationship between phenyl-hydrazine-induced damage, erythropoiesis, and transcriptional regulation mediated by ETV6 and NFE2 provides valuable insights into the mechanisms underlying anaemia. Therefore, this study aimed to determine the effect of bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extracts on ETV6 and NFE2 gene expressions in phenyl-hydrazine induced anaemia in albino Wistar rats.

Methods: A total of 60 adult male albino Wistar rats were divided into six groups; A, B, C, D, E and F representing control, phenyl-hydrazine group, ferrous sulphate group, phenyl-hydrazine+ 100mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus*, phenyl-hydrazine+ 200mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* and phenyl-hydrazine+ 400mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* respectively. Messenger RNA (mRNA) ETV6 and NFE2 were determined using polymerase chain reaction.

Results: mRNA expression of ETV6 of group B was higher when compared to group A ($p<0.05$). Groups C and D showed statistically significant higher expression of ETV6 when compared to group A and B ($p=0.001$). There was a significant decrease in the mRNA expression of NFE2 of group B when compared to group A ($p=0.001$). Groups E and F showed statistically significant lower expressions of NFE2 when compared to groups A and B ($p=0.001$).

Conclusion: Phenyl-hydrazine and bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* caused positive alterations in ETV6 and NFE2.

Keywords: *Picralima nitida*; *Cymbopogon citratus*; ETS variant-6; Nuclear Factor Erythroid-2; Phenyl-hydrazine.

INTRODUCTION

Plants have been utilized for their therapeutic properties for centuries, with various cultures harnessing the healing power of nature to treat ailments and promote well-being. [1] They offer a vast array of bioactive compounds such as alkaloids, flavonoids, and terpenes, which exhibit diverse pharmacological effects and have the potential to treat conditions ranging from inflammation and pain to infections and chronic diseases. [2] *Picralima nitida*, commonly known as Akuamma, is a plant native to tropical West Africa and is renowned for its medicinal properties. The plant has also been proven to have erythropoietic effect as it significantly increased packed cell volume and haemoglobin concentration of mice treated with the extract. [3] *Cymbopogon citratus*, commonly known as lemongrass, is a tropical plant prized for its culinary and medicinal uses. With its refreshing citrus flavor and aroma, lemongrass is widely used in cooking, teas, and aromatherapy. [4] It has also been demonstrated to possess erythropoietic properties. [5]

Erythropoiesis, the process by which red blood cells are produced, is a finely orchestrated biological phenomenon crucial for maintaining oxygen transport and tissue oxygenation.[6] Within the bone marrow; hematopoietic stem cells undergo a series of differentiation steps, ultimately giving rise to mature erythrocytes. [7] Transcriptional regulations plays a role in

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governing the progression of erythropoiesis, controlling the expression of genes involved in various stages of red blood cell development. This regulation ensures the timely activation and repression of specific genes required for proliferation, differentiation, and maturation of erythroid progenitor cells. Through complex signaling pathways and molecular mechanisms, transcriptional regulation tightly regulates the balance between self-renewal and differentiation of hematopoietic stem cells, ensuring the steady production of functional red blood cells to meet physiological demands. [8] Two common transcripttional regulators of erythropoiesis include the ETS variant 6 (ETV6) and Nuclear Factor Erythroid 2 (NFE2). [9] ETS variant 6 (ETV6) is a transcription factor belonging to the ETS (E26 transformation-specific) family. ETV6 plays a crucial role in various cellular processes, including haematopoiesis, development, and tumorigenesis. [10] ETV6 regulates the expression of target genes involved in cell proliferation, differentiation, and survival. In hematopoietic cells, ETV6 is essential for the maintenance of normal blood cell production and functions as a tumor suppressor by inhibiting aberrant cell growth and promoting apoptosis when necessary. [11]

However, mutations or chromosomal translocations involving ETV6 have been implicated in several hematological malignancies, such as acute lymphoblastic leukemia (ALL), myelodysplastic syndromes (MDS), and myeloproliferative disorders (MPDs), highlighting its significance in both normal haematopoiesis and leukemogenesis. [12] Nuclear Factor Erythroid 2 (NFE2) is a transcription factor primarily known for its role in regulating the expression of genes involved in erythropoiesis. NFE2 controls the expression of genes encoding globins, heme biosynthetic enzymes, and other factors critical for erythroid differentiation and maturation. [13] Dysregulation of NFE2 expression or activity can lead to defects in erythropoiesis and contribute to the pathogenesis of various hematological disorders, including polycythaemia vera. [14] Phenylhydrazine, a potent oxidizing agent, has been widely used experimentally to induce hemolytic anaemia in laboratory animals.[15] Upon administration, phenyl-hydrazine targets red blood cells (RBCs), causing oxidative damage to their membranes and disrupting their structural integrity. This leads to the premature destruction of RBCs, a condition known as haemolysis, resulting in a decrease in the total number of circulating erythrocytes and subsequent development of anaemia. [16]

Picralima nitida and *Cymbopogon citratus* have demonstrated promising medicinal properties, including anti-inflammatory, antioxidant, and haematopoietic effects, which could potentially alleviate the symptoms of anaemia. However, the molecular mechanisms underlying their therapeutic effects on anaemia remain largely unexplored. Investigating the impact of the bi-herbal formula on the expression of ETV6 and NFE2 genes is particularly relevant, as these transcription factors play crucial roles in regulating erythropoiesis. Understanding how the bi-herbal formula modulates the expression of ETV6 and NFE2 genes in the context of phenyl-hydrazine-induced anaemia could provide insights into the molecular mechanisms underlying its supposed effect.

This knowledge could facilitate the development of more targeted and effective herbal remedies for the management of anaemia and other haematological disorders. The aim of this study therefore, was to determine the effect of bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extract on ETS variant 6 (ETV6) and Nuclear Factor Erythroid 2 (NFE2) gene expressions in Phenyl Hydrazine-Induced Anaemia in Albino Wistar rats.

MATERIALS AND METHODS

Study Population

In this study, animal (rats) model was used. A total of sixty (60) of the Albino Wistar strain were purchased from the animal holdings of the Department of Anatomy, University of Benin, Benin City, Nigeria. The rats were housed at the animal housing wing of the Department of Anatomy, University of Benin. [17]

Identification of *Cymbopogon citratus and Picralima nitida* Leaves

Cymbopogon citratus and Picralima nitida leaves were obtained from Oluku community in Ovia North-East Local Government Area, Edo State. The leaves were then identified and authenticated at Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City; with ID Number: UBH-C451 and UBH-P424 for *Cymbopogon citratus and Picralima nitida* leaves respectively.

Processing of *Cymbopogon citratus and Picralima nitida* Leaves

The procedure began by removing any unhealthy leaves from the sample. Subsequently, the leaves underwent a thorough washing process followed by drainage. To facilitate proper grinding, the leaves were air-dried under shade for duration of two weeks. Further drying was then carried out using a hot air oven at 50°C for 24 hours. This ensured that the leaves were adequately dried and prepared for grinding. The grinding process itself was conducted using a high-speed grinding machine, specifically an industrial 1000A high-speed grinder. Finally, 250 grams of each leaf were precisely weighed for subsequent usage.

Preparation of Plants Extract
(Two hundred and fifty grams) 250 grams of ground powder were mixed with 2.5 liters of distilled water. Subsequently, the mixture was left to soak for duration of 24 hours under constant storage conditions. After the specified duration, the mixture underwent filtration using Whatman's (Nitro cellulose 45; 0.45µm pore size) filter paper, with the residue being discarded. Following filtration, the resulting filtrate was subjected to concentration in a Water bath maintained at 45°C until it reached a paste-like consistency. The paste obtained from this process was then accurately weighed and subsequently dissolved in distilled water to achieve the recommended concentrations for administration.

Animal Care
Animals were housed in a cross ventilated room in the animal holdings of the department of anatomy, University of Benin, Benin City. Animals were exposed to 12 hours dark and light cycles with access to feed and water *ad libitum*. The rats were acclimatized for a period of two (2) weeks before commencement of the experiment.

Ethical Consideration
Ethical approval was obtained from Research Ethics Committee on animal subjects from Edo State Ministry of Health, Benin City (Ref Number: HA/737/23/B.200600195 issued on 14th, December, 2023).

Preparation of Phenyl-hydrazine and Ferrous Sulphate Drug Solution
Phenyl-hydrazine Solution
Phenyl-hydrazine solution was prepared by combining phenyl-hydrazine (manufactured by Sigma-Aldrich, Batch Number: PHZ789001) with distilled water v/v and 2-propanol in a ratio of 1:5:5. This entailed mixing 1 part of phenyl-hydrazine with 5 parts of distilled water v/v and 5 parts of 2-propanol. Subsequently, 0.2ml of this phenyl-hydrazine solution was administered to each animal in the various test groups, with an average weight of 150g, every 48 hours for duration of 28 days.

Ferrous Sulphate Drug Solution
Ferrous Sulphate Drug Solution was made by mixing 1000mg of the powdered drug in 50ml of distilled water, and 0.3ml of this drug solution was administered orally to each animal in group C of an average weight of 150g every 48 hours for 28 days.

Research Design
Grouping of Animals: Sixty (60) Mature Wistar rats weighing 150-200g were randomly selected and divided into six groups (n = 10 per group). The Groups were the Group A, Group B, Group C, Group D, Group E and Group F.

Group A: This was the control group. Animals in this group received only standardized feed (Manufactured by KARMA AGRIC FEEDS AND FOOD LIMITED, Oyo State) and clean water *ad libitum*.
Group B: This group received only phenyl-hydrazine intraperitoneally.
Group C: Animals in this group were administered phenyl-hydrazine solution and treated with the standard drug solution (ferrous sulphate) intraperitoneally.
Group D: Animals in this group were administered phenyl-hydrazine solution intraperitoneally and treated with low dose of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally.
Group E: Animals in this group were administered phenyl-hydrazine solution intraperitoneally and treated with a higher dose of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally.
Group F: Animals in this group were administered phenyl-hydrazine solution intraperitoneally and treated with the highest dose of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally.

Administered Doses of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* Leaves Extract
Group A (control) received only standardized feed and clean water *ad libitum*. Group B (phenyl-hydrazine treated group) were administered 0.2ml of phenyl-hydrazine solution intraperitoneally every 48 hours for 28 days. Group C (ferrous sulphate drug solution treated group) were administered 0.2ml of phenyl-hydrazine solution intraperitoneally every 48 hours for 28 days and treated with 0.3ml of 6mg/ml of ferrous sulphate 48 hourly for 28 days. Group D were administered with 0.2ml of phenyl-hydrazine solution intraperiton-eally every 48 hours for 28 days and treated with 0.15ml of 100mg/kg body weight of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally using a gavage tube every 24 hours for 28 days. Group E were administered with 0.2ml of phenyl-hydrazine solution intraperitoneally every 48 hours for 28 days and treated with 0.3ml of 200mg/kg body weight of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally using a gavage tube every 24 hours for 28 days. Group F were administered with 0.2ml of phenyl-hydrazine solution intraperitoneally every 48 hours for 28 days and treated with 0.6ml of 400mg/kg body weight of bi herbal formulation of *Cymbopogon citratus* and *Picralima nitida* leaves extract orally using a gavage tube every 24 hours for 28 days.

Sacrifice of Animals and Collection of Samples

At the end of the experimental period, the animals were grossly observed for general physical characteristics. A midline incision was made through the ventral wall of the rats after anaesthetizing (using chloroform) and cervical dislocation. Bone marrow samples were also obtained from the rats by opening the femur longitudinally and exposing the marrow cavity. A sterile forceps was used to obtain the bone marrow from the cavity and placed in an Eppendorf container containing Trizol for molecular analysis.

ETS variant 6 (ETV6) and Nuclear Factor Erythroid 2 (NFE2) mRNA Assay

Isolation of Total RNA

Total RNA was isolated from whole rat samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

cDNA conversion

One (1 µg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65°C for 5 min, 42 °C for 1 h, and 80°C for 5 min. [18]

PCR amplification and Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) for the amplification of gene of interest was carried out with OneTaqR2X Master Mix (NEB) using the following primers (Inqaba Biotec, Hatfield, South Africa): PCR amplification was performed in a total of 25 µl volume reaction mixture containing cDNA, primer (forward and reverse) and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95ºC for 5 min, followed by 30 cycles of amplification (denaturation at 95ºC for 30 s, annealing for 30 s and extension at 72ºC for 60 s) and ending with final extension at 72ºC for 10 min. The amplicons were resolved on 1.0% agarose gel. The GAPDH gene was used to normalize the relative level of expression of each gene, and quantification of band intensity was done using "image J" software. [19]

Primer

ETV-6

Forward GGAAGCCTCGAATTCTCTTCTC
Reverse GCGTCCTCTGGACACAATTA

NFE-2

Forward ACAGATTGAGCTGGCCTAGA
Reverse CTGGAGAACTCAGCCTTGATTG

GAPDH

Forward: CTCCCTGGAGAAGAGCTATGA
Reverse: AGGAAGGAAGGCTGGAAGA

Statistical Analysis

Data obtained from this research was presented and analyzed using statistical package for social sciences (SPSS) version 21.0 (IBM Inc. USA). Bar chart was used to represent the mRNA gene expression patterns. A p value of ≤0.05 was considered statistically significant.

RESULTS

Figure 1 shows the expression of genes as represented by gel electrophoresis picture and internal control (Glycealdehyde-3-Phosphate Dehydrogenase {GAPDH}) of mRNA expression of ETS variant 6 (ETV6) of groups A, B, C, D, E and F, representing control, phenyl-hydrazine group, ferrous sulphate group, phenyl-hydrazine+ 100mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus*, phenyl-hydrazine+ 200mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* and phenyl-hydrazine+ 400mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* respectively, represented on different bars on the bar chart. There was a significant increase in the mRNA expression of ETV6 of group B when compared to group A ($p<0.05$). Groups C and D showed statistically significant higher expression of ETV6 when compared to groups A and B ($p<0.05$). Groups E and F showed statistically significant higher expression of ETV6 when compared to group A ($p<0.05$).

Figure 2 shows the expression of genes as represented by gel electrophoresis picture and internal control (Glycealdehyde-3-Phosphate Dehydrogenase {GAPDH}) of mRNA expression of Nuclear Factor Erythroid 2 (NFE2) of groups A, B, C, D, E and F, representing control, phenyl-hydrazine group, ferrous sulphate group, phenyl-hydrazine+ 100mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus*, phenyl-hydrazine+ 200mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* and phenyl-hydrazine+ 400mg/kg bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* respectively, represented on different bars on the bar chart. There was a significant decrease in the mRNA expression of NFE2 of group B when compared to group A ($p<0.05$). Groups C and D showed statistically significant higher expressions of NFE2 when compared to group A and B ($p<0.05$). Groups E and F showed statistically significant lower expressions of NFE2 when compared to group A and B ($p<0.05$).

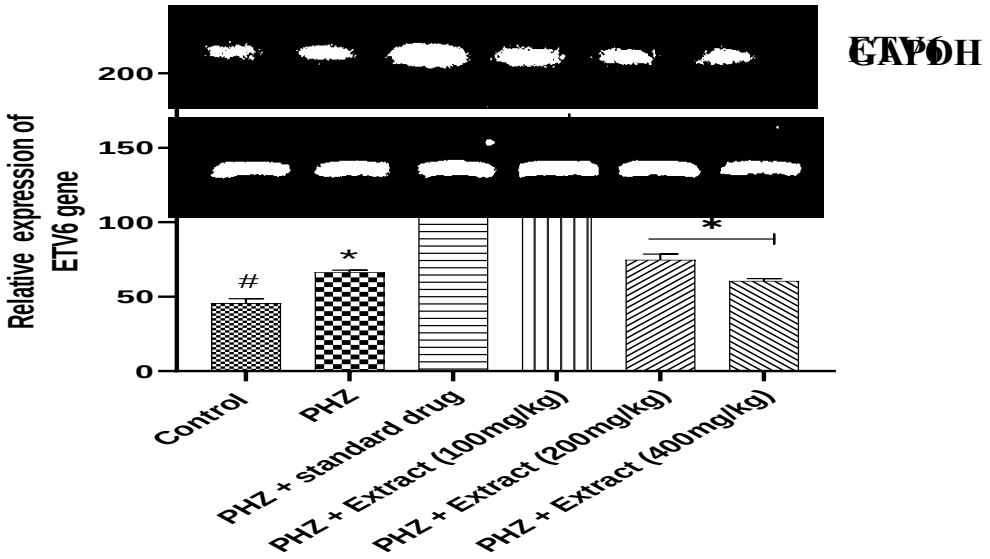

Figure 1: mRNA Expression of ETS variant-6 (ETV6) of the Studied Groups.
**Represents statistical difference to control. # Represents statistical difference to phenyl-hydrazine induced group at p<0.05.*
Key: *PHZ=Phenyl-hydrazine, GAPDH=Glyceraldehyde-3-Phosphate Dehydrogenase*

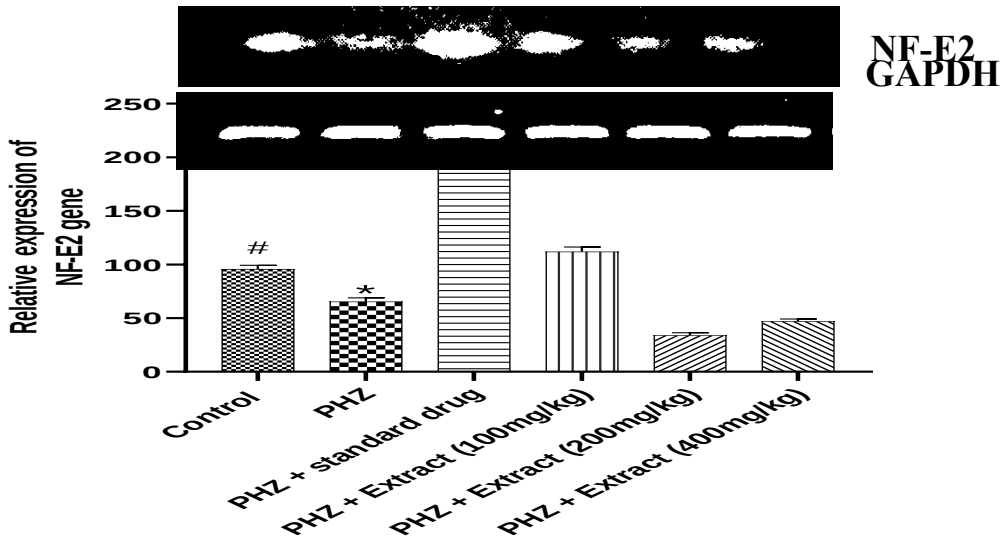

Figure 2: mRNA Expression of Nuclear Factor Erythroid 2 (NFE2) of the Studied Groups.
**Represents statistical difference to control. # Represents statistical difference to phenyl-hydrazine induced group at p<0.05.*
Key*: PHZ=Phenyl-hydrazine, GAPDH=Glyceraldehyde-3-Phosphate Dehydrogenase*

DISCUSSION

This study investigated the novel impact of the bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaves extracts on the expression of ETV6 and NF-E2 genes in Phenyl Hydrazine-induced anaemia in Albino Wistar rats. ETS Variant-6 is a transcription factor involved in haematopoiesis and the regulation of erythropoiesis, the process of red blood cell production; [20] While Nuclear Factor Erythroid-2, is another transcription factor crucial for erythropoiesis and the synthesis of haemoglobin. [21] Alterations in the expression of these genes can significantly impact erythropoiesis and contribute to the development of anaemia.

This study revealed significant alterations in ETV6 expression among the experimental groups. The group treated with phenyl-hydrazine-only, exhibited a significant increase in ETV6 mRNA expression compared to the control group, indicating a potential response to the haematotoxic effects of phenyl-hydrazine. Moreover, the groups treated with ferrous sulphate and the lowest dosage of the bi-herbal formulation (100mg/kg), showed statistically significant higher expression of ETV6 compared

to both the control group and the phenyl-hydrazine-only treated group.

This finding suggests that both ferrous sulphate supplementation and the low dosage of the bi-herbal formulation may modulate ETV6 expression, potentially influencing erythropoiesis and haemoglobin synthesis. Additionally, the groups administered higher dosages of the bi-herbal formulation (200mg/kg and 400mg/kg, respectively), exhibited statistically significant higher expression of ETV6 compared to the control group only, indicating a dose-dependent effect on ETV6 mRNA expression. A similar study [22] observed no change in ETV6 expression after treatment with a medicinal plant which is in contrast to the finding of this study.

This study also showed significant alterations in NFE2 expression among the experimental groups. The group administered phenyl-hydrazine only, exhibited a significant decrease in NFE2 mRNA expression compared to the control group, indicating a potential suppression of NFE2 expression in response to the haematotoxic effects of phenyl-hydrazine. Another study [23] also suggests that PHZ treatment may have an effect on NF-E2 gene expression, potentially influencing its timing or level during erythroid differentiation. Conversely, the groups treated with ferrous sulphate and the lowest dosage of the bi-herbal formulation (100mg/kg), respectively, showed statistically significant higher expressions of NFE2 compared to both the control group and the phenyl-hydrazine treated group. This finding suggests that both ferrous sulphate supplementation and the low dosage of the bi-herbal formulation may up-regulate NFE2 expression, potentially influencing erythropoiesis and haemoglobin synthesis. This finding agrees with another study [24] that observed erythropoietic effect by stimulation of erythropoietin expression and certain genes associated with erythropoiesis after treatment with two different plants. In contrast the groups administered higher dosages of the bi-herbal formulation (200mg/kg and 400mg/kg) exhibited statistically significant lower expressions of NFE2 compared to the control group, indicating a dose-dependent effect on NFE2 mRNA expression. The observed decrease in NFE2 expression across groups treated with higher dosages of the bi-herbal formulation suggests a potential regulatory mechanism underlying the therapeutic effects of the herbal remedy in mitigating anaemia. NFE2 is known to play a critical role in erythropoiesis and haemoglobin synthesis, and its down-regulation may impact red blood cell production and maturation.

Conclusion: Data from this study showed that phenyl-hydrazine caused an increase in ETV6 gene. Ferrous sulphate and 100mg/kg of bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* caused an increase in ETV6 compared to control and phenyl-hydrazine group. Phenyl-hydrazine caused a significant decrease in the mRNA expression of NFE2. Ferrous sulphate and 100mg/kg of bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* showed statistically significant higher expressions of NFE2 when compared to control and phenyl-hydrazine groups. Higher extract concentration led to lower expressions of NFE2 when compared to control and phenyl-hydrazine groups.

Recommendations:

Further research is recommended to elucidate the exact mechanisms involved in the regulation of ETV6 and NFE2 gene expressions by the bi-herbal extract.

It would be beneficial to explore a broader range of dosages to identify the optimal concentration that maximizes the therapeutic effects while minimizing any potential adverse effects.

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Authors' contributions: OPA: Conceptualization and manuscript writing; G-ICO: Laboratory analysis.

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