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ORIGINAL ARTICLE

Effect of a Bi-Herbal Formula of Picralima Nitida and Cymbopogon Citratus aqueous leaf extracts on Glucose-6-Phosphate Dehydrogenase and Krüppel-Like Factor- 1 Genes In Phenyl-Hydrazine induced Anaemia in Albino Wistar Rats

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Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) is crucial in protecting red blood cells from oxidative stress, while Krüppel-like factor-1 (KLF-1) regulates erythropoiesis and haemoglobin. Understanding how the bi-herbal formulation modulates these genes could provide valuable insights into its potential as a therapeutic intervention for anaemia.

Aim: The aim of this study was to determine the effect of the bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extracts on glucose-6-phosphate dehydrogenase (G6PD) and Krüppel like factor-1 (KLF-1) gene expressions in phenyl-hydrazine induced anaemia in albino Wistar rats.

Methods: A total of thirty (30) adult male albino Wistar rats were divided into six (6) groups; A, B, C, D, E and F representing control, a 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. mRNA of G6PD and KLF-1 were determined using a polymerase chain reaction. Data obtained was analysed by the Statistical Package for Social Science (SPSS) Software.

Results: There was a significant increase in group B's mRNA expression of G6PD compared to group A ($p<0.05$). Groups C, D, E and F had a higher mRNA expression when compared to groups A and B ($p<0.05$). There was a significant increase in the mRNA expression of KLF1 in groups C, D and E compared to groups A and B ($p<0.05$).

Conclusion: This study concludes that administering a bi-herbal formulation altered the gene expression of G6PD and KLF-1.

Key words: *Picralima nitida*, *Cymbopogon citratus*, Glucose-6-phosphate dehydrogenase, Krüppel-like factor-1, Phenyl hydrazine.

Introduction

Medicinal plants, also known as medicinal herbs, are any plant in which one or more of its organs contain substances that can be used for therapeutic purposes or which are precursors for synthesising useful drugs (1). All plants synthesise hundreds of chemical compounds which serve various functions. The compounds found in these plants are diverse, most in four biochemical classes, namely alkaloids, glycosides, polyphenols, and terpenes. These phytochemicals have the potential for use as drugs, and the known pharmacological activity of these substances forms the scientific basis for their use in modern medicine if scientifically confirmed (2). *Picralima nitida* is a medicinal plant from the genus *Picralima* and the plant family *Apocynaceae*. Several studies have previously shown that extracts of this plant are good sources of phytochemicals (3). *Cymbopogon citratus*, commonly known as lemon grass, is a tropical perennial and aromatic plant that belongs to the family *Gramineae* (Poaceae) and the genus *Cymbopogon*. Scientific studies have shown that C. *citratus* oil possesses various pharmacological properties such as antibacterial, anti-amoebic, anti-filarial, antidiarrheal, anti-inflammatory, antifungal, antimalarial, anti-mycobacterial, anti-mutagenicity, hypoglycaemic, antioxidants and neurobehavioral. It also possesses antiprotozoal, anti-carcinogenic, antioxidant, anti-rheumatic and cardio-protective properties (4). Glucose-6-phosphate dehydrogenase is an enzyme found in the cytoplasm of all cells in the body. It is a housekeeping enzyme that is vital in preventing cellular damage from reactive oxygen species. It does this by providing substrates to prevent oxidative damage (5). Erythroid Krüppel-like factor (EKLF) or KLF1, an erythroid-specific transcription factor, plays a critical role in erythropoiesis (6). It is a master erythroid regulator, which plays a pivotal role during erythroid lineage development by regulating the expression of many erythroid genes (7). Phenyl hydrazine (PHZ) is a potent chemical that causes various tissue toxicity at various levels. Administration of phenyl hydrazine mainly causes haematotoxicity, which leads to haemolytic anaemia (8).
The scientific community recognises the need for alternative therapeutic approaches, and herbal formulations have shown great promise in various health conditions. The medicinal plants Picralima nitida and Cymbopogon citratus have been used traditionally for their medicinal benefits, including antioxidant, anti-inflammatory, and anti-anaemic properties. Anaemia is a prevalent health condition characterized by a reduction in red blood cells or haemoglobin levels, and phenyl hydrazine is a compound known to induce haemolytic anaemia in experimental animal models. G6PD is an enzyme that protects red blood cells from oxidative stress, while KLF1 is a transcription factor that regulates gene expression in erythropoiesis. Alterations in the expression or activity of these genes can contribute to the development of anaemia, making them potential targets for therapeutic interventions. Many traditional medicines have been used for centuries to manage various health conditions, including anaemia, but scientific validation of their efficacy and mechanisms of action is often lacking, which necessitates this study. By exploring the effects of this bi-herbal formula on G6PD and KLF1 gene expressions in an anaemic model, this research intends to provide valuable information that could advance our understanding of anaemia management and contribute to developing effective and

safe therapeutic strategies. The aim of this study, therefore, is to determine the effect of the bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extract on glucose-6-phosphate dehydrogenase (G6PD) and Krüppel-like factor 1 (KLF1) gene expressions in phenyl-hydrazine induced anaemia in albino Wistar rats.

Materials and Methods

Study Population

In this study, an animal model (rats) was used. Thirty (30) albino Wistar strain rats were purchased from the animal holdings of the Department of Anatomy, University of Benin, Benin City, Nigeria. The rats were housed at the Department of Anatomy, University of Benin's animal housing wing.

Identification of *Cymbopogon citratus and Picralima nitida* Leaves

Cymbopogon citratus and Picralima nitida leaves were collected at the Oluku community in Ovia North-East Local Government Area of Edo State, Nigeria, on December 23, 2023. Dr. A. O Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, then identified and authenticated the leaves.

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 a duration of two weeks. Further drying was then carried out using a hot air oven at 50^{o}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 Plant Extract

Two hundred and fifty (250) grams of ground powder were mixed with 2.5 litres of distilled water. Subsequently, the mixture was left to soak for a duration of 24 hours under constant storage conditions. After the specified duration, the mixture underwent filtration using Whatman's (Nitrocellulose 45; 0.45µm pore size) filter paper, which discarded the residue. 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. They were exposed to 12-hour dark and light cycles and had access to feed and water ad libitum. The rats were acclimatized for two (2) weeks before the experiment commenced.

Ethical Consideration

Ethical approval was obtained from the 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 phenylhydrazine with 5 parts of distilled water v/v and 5 parts of 2-propanol. Subse-

quently, 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 a duration of 28 days.

Ferrous Sulphate Drug Solution

A ferrous sulphate drug solution was made by mixing 1000mg of the powdered drug in 50 ml of distilled water. 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: Thirty (30) mature Wistar rats weighing 150-200g were randomly selected and divided into six groups (n = 5 per group): 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 was administered with 0.2ml of phenyl hydrazine solution intraperitoneally 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 was 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 was 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

The animals were grossly observed for general physical characteristics at the end of the experimental period. After anaesthetizing (using chloroform) and cervical dislocation, a midline incision was made through the ventral wall of the rats. 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, which was placed in an Eppendorf container containing Trizol for molecular analysis.

Glucose-6-Phosphate Dehydrogenase (G6PD) and Krüppel-like Factor-1 (KLF-1) mRNA Assay

Isolation of Total RNA

Total RNA was isolated from whole rat samples with the Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following the treatment of DNAse I (NEB, Cat: M0303S). The RNA was quantified at 260 nm, and its purity was confirmed at 260 nm and 280 nm using an 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 [9].

PCR Amplification and Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) for the amplification of the 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 conditions: Initial denaturation at 95°C for 5 min, followed by 30 cycles of amplification (denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C for 60 s) and ending with final extension at 72°C for 10 min. The amplicons were resolved on 1.0% agarose gel. The GAPDH gene was used to normalize each gene's relative expression level, and band intensity was quantified using "image J" software [10].

Primers
KLF1
Forward: CCGGAAGGCCATACAAAGAA
Reverse: TTTACTTGGCGGCCTGTATC

G6PD
Forward: GCTCCGTGCCTCTGATAAA
Reverse: CCACGAAAGATAGCGAGAG-TAG

GAPDH
Forward: CTCCCTGGAGAAGAGCTAT-GA
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). A 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 Glucose-6-Phosphate dehydrogenase (G6PD) 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 G6PD of group B when compared to group A ($p<0.05$). Groups C, D, E and F had a higher mRNA expression when compared to group A and B ($p<0.05$).

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 Krüppel-like Factor 1 (KLF1) 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 KLF1 of groups C, D and E when compared to groups A and B ($p<0.05$).

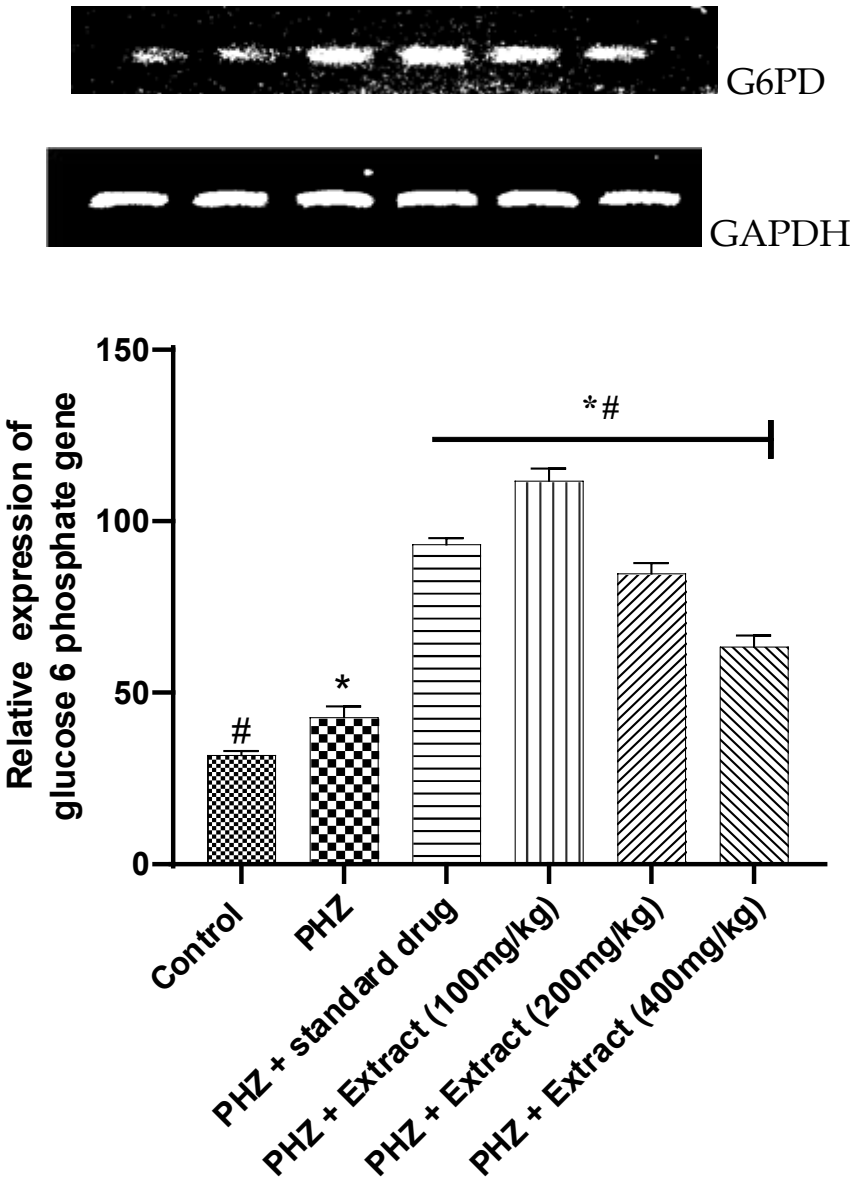

Figure 1: mRNA Expression of Glucose-6-Phosphate Dehydrogenase (G6PD) of the Studied Groups.

* 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

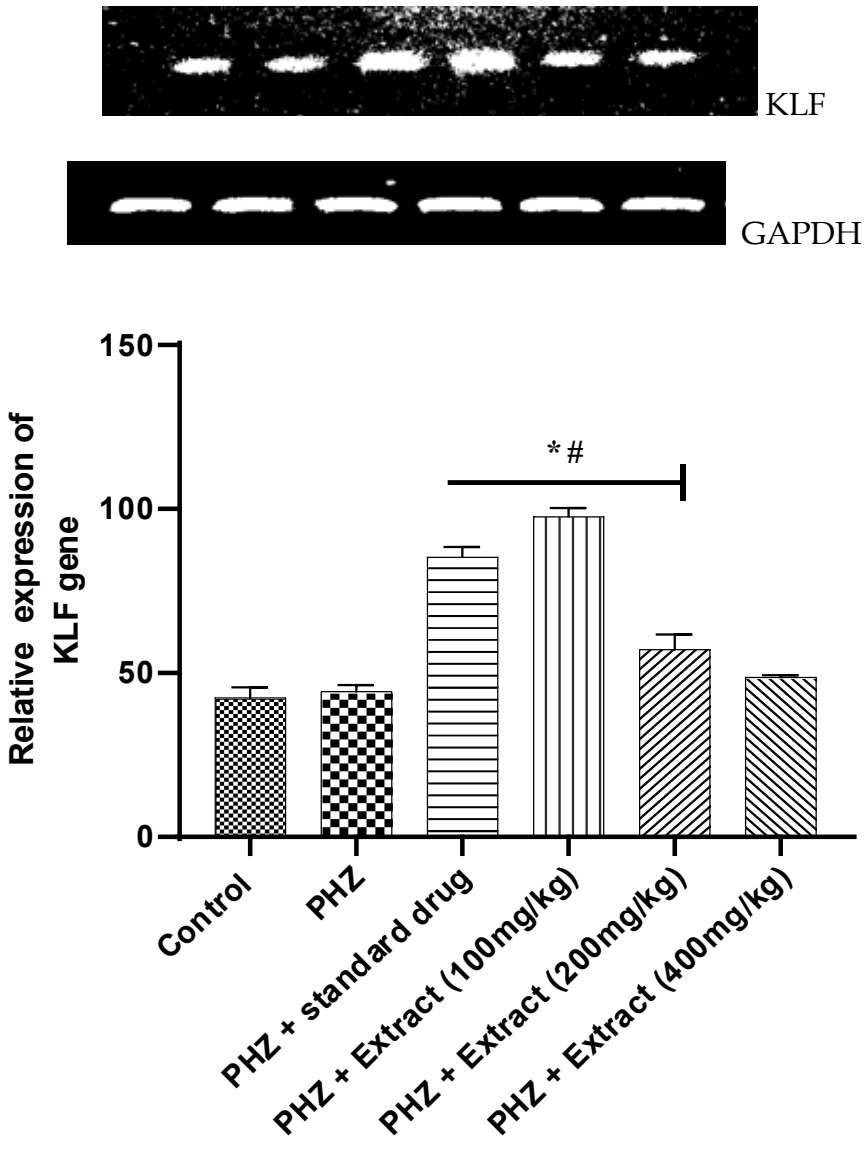

Figure 2: mRNA Expression of Krüppel-like Factor 1 (KLF1) of the Studied Groups.

* 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

The exploration of natural remedies derived from medicinal plants has gained significant interest in recent years, offering promising avenues for therapeutic interventions against various health conditions (11). The genetic factors underlying physiological processes, particularly those associated with haematopoiesis and erythropoiesis, have been of great research interest. Glucose-6-phosphate dehydrogenase (G6PD) and Krüppel-like Factor-1 (KLF-1) genes regulate erythrocyte function and development. Alterations in their expression or activity can profoundly affect red blood cell production and function, potentially contributing to haematological disorders. Amidst the exploration of botanical interventions and genetic mechanisms, the detrimental effects of certain chemical agents on haematopoiesis cannot be overlooked.

This study investigated the potential effects of a bi-herbal formula comprising *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extracts on phenyl hydrazine-induced anaemia in albino Wistar rats while also seeking to show the impact of the bi-herbal formulation on the expression of G6PD and KLF-1 genes. This study observed the mRNA expression of Glucose-6-Phosphate Dehydrogenase (G6PD) among different experimental groups; the initial observation reveals a significant increase in G6PD mRNA expression in the group administered PHZ compared to the control, indicating a pronounced effect of phenyl hydrazine exposure on the expression of this gene. Moreover, the groups administered ferrous sulphate, 100mg/kg, 200mg/kg and 400mg/kg of the bi-herbal formulation exhibited even higher mRNA expression levels of G6PD compared to both the control and the phenyl hydrazine-exposed group, signifying the potential impact of ferrous sulphate treatment and the bi-herbal formulation on enhancing G6PD expression. Notably, higher doses of the bi-herbal formulation (200mg/kg and 400mg/kg) demonstrated lower mRNA expression levels of G6PD compared to the group administered 100mg/kg, suggesting a possible dose-dependent response to the bi-herbal formulation.

There was a significant increase in KLF-1 mRNA expression in the groups administered ferrous sulphate, 100mg/kg and 200mg/kg when compared to both the control and the phenyl hydrazine-exposed group, also indicating a notable effect of ferrous sulphate treatment and the bi-herbal formulation on enhancing KLF-1 expression. The lower expression of KLF-1 in the group administered 2000mg/kg of the bi-herbal formulation compared to the groups treated with ferrous sulphate and 100mh/kg of the bi-herbal formulation suggests a potential dose-dependent response to the bi-herbal formulation. These findings are particularly relevant when considering the role of KLF-1 in erythropoiesis. Krüppel-like Factor-1 is a critical transcription factor involved in regulating erythropoiesis, playing a pivotal role in the differentiation and maturation of red blood cells (12). Increased expression of KLF-1 is associated with enhanced erythropoiesis, leading to the production of mature and functional red blood cells (13). Therefore, the significant increase in KLF-1 expression observed in the groups administered ferrous sulphate, 100mg/kg and 200mg/kg suggests a potential mechanism by which ferrous sulphate and the bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus* stimulate erythropoiesis and promote red blood cell production.

Conclusion

Data from this study showed that administering phenylhydrazine, ferrous sulphate, and various concentrations of the bi-herbal formulation increased the Glucose-6-Phosphate dehydrogenase (G6PD) gene. An increase in the mRNA expression of the KLF-1 gene was observed in the group administered PHZ and ferrous sulphate, 100mg/kg, and 200mg/kg of the bi-herbal formulation of *Picralima nitida* and *Cymbopogon citratus.*

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Conflict of Interest

There is no conflict of interest as declared by the Authors.

Contribution of the authors

Obazelu, Progress Arhenrhen: Corresponding Author, conceptualization and manuscript writing.

Evwaire, Oghenetejiri Peace: Analysis of samples

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