PROINFLAMMATORY EFFECT OF BI-HERBAL FORMULA OF *PICRALIMA NITIDA* AND *CYMBOPOGON CITRATUS* AQUEOUS LEAF EXTRACT IN PHENYL HYDRAZINE-INDUCED ANAEMIA IN ALBINO WISTAR RATS

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ABSTRACT
Background: *Picralima nitida* and *Cymbopogon citratus* are known for their medicinal use possessing bioactive compounds with anti-inflammatory and hematopoietic properties, offer promises for novel treatments. By examining the impact of the bi-herbal formula on IL-1β and TGF-β gene expressions, the study aims to investigate the molecular mechanisms underlying its therapeutic effects.
Aim: This aim of this study was to determine the effect of bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extract on interleukin 1β (IL-1β) and Transforming growth factor β (TGF-β) gene expressions in Phenyl Hydrazine-Induced Anaemia in Albino Wistar rats.
Methods: A total of sixty (60) adult male albino Wistar rats were divided into six (6) groups; A, B, C, D, E and F representing control, phenylhydrazine group, ferrous sulphate group, phenylhydrazine + 100mg/kg Biherbal formulation of *Picralima nitida* and *Cymbopogon citratus*, phenylhydrazine + 200mg/kg Biherbal formulation of *Picralima nitida* and *Cymbopogon citratus* and phenylhydrazine + 400mg/kg Biherbal formulation of *Picralima nitida* and *Cymbopogon citratus* respectively. mRNA IL-1β and TGF-β were determined using polymerase chain reaction. Data obtained was analysed by the Statistical Package for Social Science (SPSS) software.
Results: There was a significant increase in the mRNA expression of IL-1β of group C and D when compared to group A and B ($p<0.05$). There was a significant decrease in the mRNA expression of TGF-β of group B when compared to group A ($p<0.05$). Groups C showed statistically significant higher expressions of TGF-β when compared to group B ($p<0.05$). Groups D and E showed statistically significant lower expressions of TGF-β when compared to group A and B ($p<0.05$). Groups F showed statistically significant lower expressions of TGF-β when compared to group A ($p<0.05$).
Conclusion: This study concludes that treatment with the bi-herbal formulation resulted in a variable effects on IL-1β and TGF-β mRNA expression.
Key words: *Picralima nitida, Cymbopogon citratus*, Interleukin 1β, Transforming growth factor β, Phenyl hydrazine.

INTRODUCTION
The use of plants for their medicinal effects dates back thousands of years, with various cultures harnessing the healing potential of botanicals to treat ailments and promote well-being. Today, modern scientific research continues to explore the pharmacological properties of plants, identifying bioactive compounds that can be utilized in pharmaceuticals and nutraceuticals Patwardhan *et al*. (2004).

Medicinal plants contain a number of bioactive compounds which includes alkaloids, flavonoids, terpenes, and phenols, among other constituents Saxena *et al*. (2013). These bioactive constituents exhibit a diverse range of pharmacological properties, including inflammatory activities Bernhoft (2010). *Picralima nitida*, commonly known as Akuamma, is a plant native to West Africa that has gained attention for its potential medicinal properties. Recent studies have identified alkaloids present in *Picralima nitida* seeds, such as akuammine and akuammidine, which exhibit analgesic and anti-inflammatory effects Erharuyi *et al*. (2014). *Cymbopogon citratus*, or lemongrass, is a tropical plant widely cultivated for its culinary and medicinal uses Majewska *et al*. (2019). Research has shown that lemongrass contains bioactive compounds like citral, which exhibit antioxidant, antimicrobial, and anti-inflammatory activities Promila and Madan (2018). Inflammation is a complex biological response triggered by the immune system in response to harmful stimuli such as pathogens, tissue injury, or toxins Kumar *et al*. (2013). During inflammation, cytokines which are signaling molecules are secreted by immune cells and play an important role in orchestrating the inflammatory response. Cytokines may either be pro-inflammatory of anti-inflammatory depending on their function O'Shea and Murray (2008).

Interleukin-1β is a pro-inflammatory cytokine released by activated macrophages and immune cells in response to infection, damage, or stress. IL-1β initiates and amplifies the inflammatory response by regulating adhesion molecules, activating immune cells, and producing pro-inflammatory cytokines Awad *et al*. (2017). Transforming growth factor beta (TGF-β) is a multifunctional cytokine involved in the regulation of cell growth, differentiation, and immune response Li *et al*. (2006); it plays a role in promoting tissue repair and fibrosis. Dysregulation of TGF-β signaling is associated with numerous diseases, including cancer, fibrosis, autoimmune disorders, and developmental abnormalities Massagué and Sheppard (2023). Phenyl-hydrazine is a strong chemical molecule that has received a lot of interest in laboratory research due to its tendency to cause hemolytic anaemia in experimental animal models. This substance works by inducing oxidative damage to red blood cells, eventually affecting their function and triggering a cascade of events similar to the pathophysiology of hemolytic anaemia in humans.

Picralima nitida and *Cymbopogon citratus* are known for their traditional medicinal use and bioactive compounds with anti-inflammatory and hematopoietic properties, offer promises for novel treatments. By examining the impact of the bi-herbal formula on IL-1β and TGF-β gene expressions, the study aims to elucidate specific molecular mechanisms underlying its therapeutic effects, thereby contributing to a deeper understanding of inflammation regulation and tissue homeostasis. The aim of this study therefore, is to determine the effect of bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extract on interleukin 1β (IL-1β) and Transforming growth factor β (TGF-β) 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 Obazelu and Faluyi (2023).

Identification of *Cymbopogon citratus and Picralima nitida* Leaves

Cymbopogon citratus and Picralima nitida leaves were collected at Oluku community in Ovia North-East Local Government Area, Edo State.

The leaves were then identified and authenticated by Dr. A. O Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City.

Processing of *Cymbopogon citratus and Picralima nitida* Leaves

The leaves of *Cymbopogon citratus and Picralima nitida* were thoroughly washed, drained and air-dried under shade for duration of two weeks. Further drying was then carried out using a hot air oven at 50ºC for 24 hours. This ensured that the leaves were adequately dried and prepared for grinding. The grinding process was done using an industrial 1000A high-speed grinder. Finally, 250 grams of each leaf were precisely weighed for subsequent usage Ezeonwu and Dahiru (2013).

Preparation of Plants Extract

Two hundred and fifty (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 Ezeonwu and Dahiru (2013).

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 Obazelu and Faluyi (2023).

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. 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 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 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 Obazelu and Faluyi (2023). .

Interleukin 1β (IL-1β) and Transforming growth factor β (TGF-β) mRNA Assay

Extraction of Total RNA

Total RNA was extracted 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) Elekofehinti *et al.* (2020).

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 Elekofehinti *et al.* (2020).

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 Olumegbon *et al*. (2022).

Synthesized Primers Sequences

IL-1 Beta

Forward: TCTGACAGGCAACCACTTAC
Reverse: TTGTCCGTGTGTATGGGATG

TGF-β

Forward: AGAGCCCTGGATACCAACTA
Reverse: CAACCCAGGTCCTTCCTAAAG

GAPDH

Forward:
CTCCCTGGAGAAGAGCTATGA
Reverse: AGGAAGGAAGGCTGGAAGA

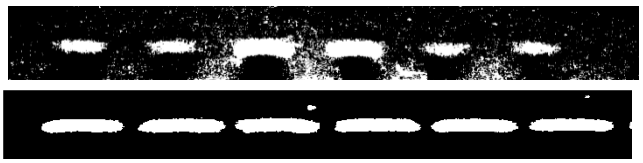
Statistical Analysis

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 Interleukin 1β (IL-1β) 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 IL-1β of group C and D 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 Transforming growth factor β (TGF-β) 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 TGF-β of group B when compared to group A ($p<0.05$). Groups C showed statistically significant higher expressions of TGF-β when compared to group B ($p<0.05$). Groups D and E showed statistically significant lower expressions of TGF-β when compared to group A and B ($p<0.05$). Groups F showed statistically significant lower expressions of TGF-β when compared to group A ($p<0.05$).



IL-1β

GAPDH

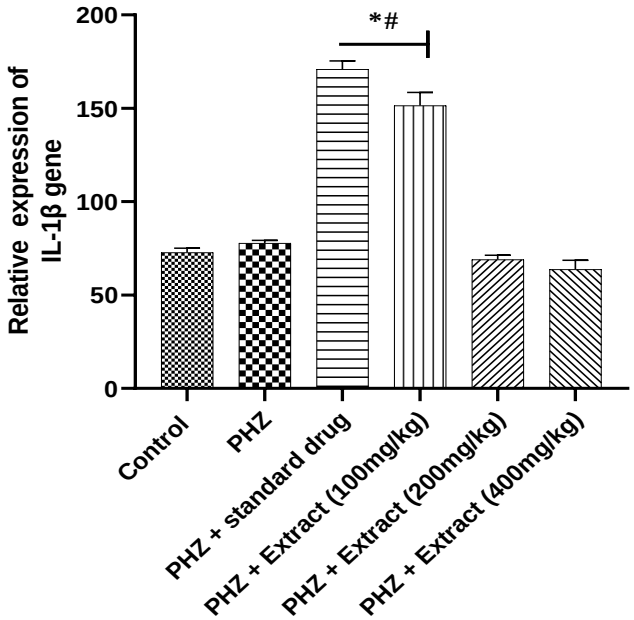


Figure 4.1: mRNA Expression of Interleukin 1β (IL-1β) of the Studied Groups.
* Represents statistical difference to control. # Represents statistical difference to benzene induced group at p<0.05.
Key: PHZ= Phenyl-hydrazine, GAPDH=Glyceraldehyde-3-Phosphate Dehydrogenase

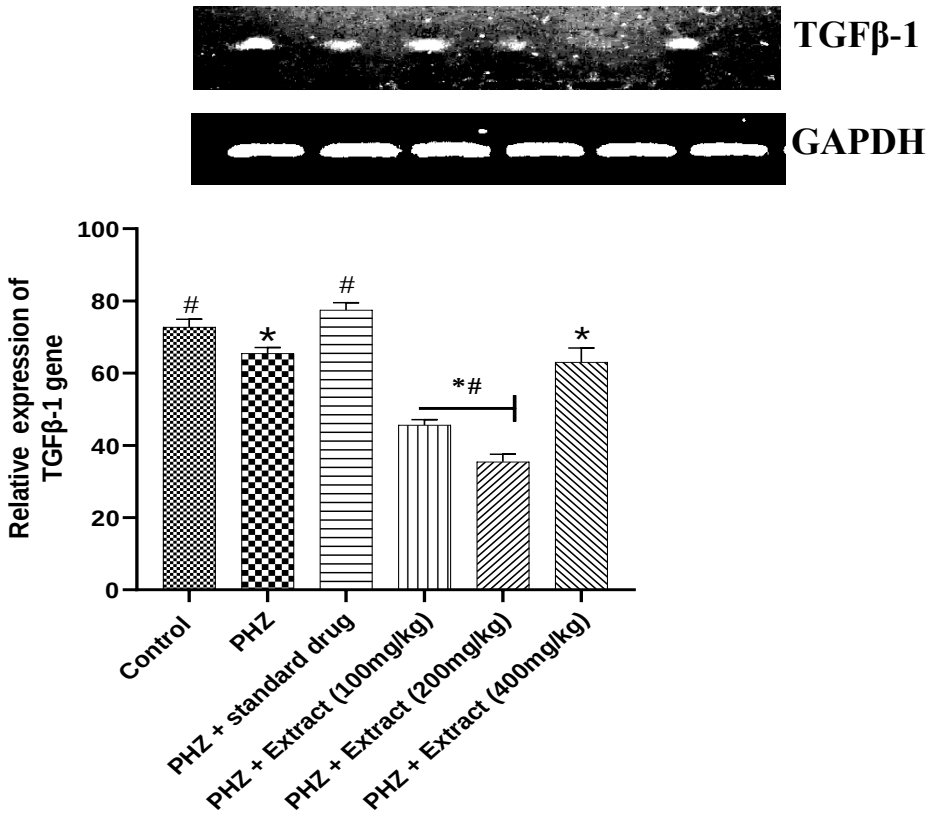


Figure 4.2: mRNA Expression of Transforming growth factor β (TGF-β) of the Studied Group
* Represents statistical difference to control. # Represents statistical difference to benzene induced group at p<0.05.
Key: PHZ= Phenyl-hydrazine, GAPDH=Glyceraldehyde-3-Phosphate Dehydrogenase

DISCUSSION

Interleukin-1 beta (IL-1β) serves as a pro-inflammatory cytokine implicated in the regulation of immune responses and haematopoiesis Mantovani *et al*. (2019), while transforming growth factor beta (TGF-β) plays a role in erythropoiesis and tissue repair mechanisms Fortunel *et al*. (2000). Understanding the impact of the bi-herbal formula on these cytokines can provide valuable insights into its therapeutic potential in phenyl hydrazine-induced anaemia. The findings from this study revealed a significant increase in IL-1β mRNA expression in the groups administered ferrous sulphate and 100mg/kg of bi-herbal formulation compared to the control group and phenyl-hydrazine treated group. This up-regulation of IL-1β gene expression in response to phenyl-hydrazine and treatment interventions (ferrous sulphate and lower doses of the bi-herbal formulation) suggests a potential role of ferrous sulphate and low dose bi-herbal formulation in modulating IL-1β expression. Interestingly, the mRNA expression of IL-1β in the groups treated with higher doses of the bi-herbal formulation (200mg/kg and 400mg/kg), appears to be comparable to that of the control group, indicating a potential dose-dependent modulation of IL-1β expression by the bi-herbal formulation. There were significant variations in TGF-β mRNA expression among the experimental groups. The phenyl-hydrazine-induced anaemic group exhibited a significant decrease in TGF-β expression compared to the control group, suggesting a potential down-regulation of TGF-β in response to anaemia induction. Conversely, the group treated with ferrous sulphate, demonstrated a statistically significant increase in TGF-β expression compared to the phenyl-hydrazine treated group, implying a potential compensatory mechanism or therapeutic effect of ferrous sulphate on TGF-β levels. The groups treated with lower doses of the bi-herbal formulation (100mg/kg and 200mg/kg), exhibited significantly lowered TGF-β expression compared to the control and anaemia groups, indicating a dose-dependent modulation of TGF-β by the bi-herbal formulation while the group treated with the highest dose of the bi-herbal formulation (400mg/kg), displays significantly lower TGF-β expression compared to the control group, suggesting a potential suppressive effect of the higher dose on TGF-β levels.

CONCLUSION

Data from this study revealed that in response to phenyl-hydrazine-induced anaemia, interleukin-1β (IL-1β) mRNA expression showed no change, while transforming growth factor beta (TGF-β) mRNA expression increased. Ferrous sulphate treatment led to an increase in IL-1β mRNA expression. Treatment with the bi-herbal formulation resulted in a variable effects on IL-1β and TGF-β mRNA expression**.**

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