



Effect of Bi-Herbal Formula of *Picralima Nitida* and *Cymbopogon Citratus* Aqueous Leaf Extracts On Cyclooxygenase-2 and Interleukin-10 Gene Expressions in Phenyl-Hydrazine Induced Anaemia in Albino Wistar Rats

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ABSTRACT: *Picralima nitida* and *Cymbopogon citratus* has been associated with anti-inflammatory and hematopoietic properties, hinting at their potential effectiveness in addressing the complications of anaemia. Investigating their impact on the gene expressions of cyclooxygenase-2 (COX-2) and Interleukin 10 (IL-10) can provide insights into their modes of operation and capacity to regulate inflammatory pathways and immune responses linked to anaemia. The aim of this study was to determine the effect of bi-herbal formula of *Picralima nitida* and *Cymbopogon citratus* aqueous leaf extracts on COX-2 and IL-10 gene expressions in Phenyl Hydrazine-Induced Anaemia in Albino Wistar rats. A total of sixty 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. mRNA COX-2 and mRNA IL-10 were determined using polymerase chain reaction. Data obtained was analysed by the Statistical Package for Social Science (SPSS) software. There was a significant increase in the mRNA expression of COX-2 of groups C, D, E and F when compared to groups A and B ($p < 0.05$). There was a significant increase in the mRNA expression of IL-10 of groups C, D, E and F when compared to groups A and B ($p < 0.05$). In conclusion, the mRNA expression of COX-2 and IL-10 showed significant up-regulation in response to the bi-herbal formulation.

DOI: <https://dx.doi.org/10.4314/jasem.v28i5.15>

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Cite this Article as: OBAZELU, P. A; OGIZA, F. O. (2024). Effect of Bi-Herbal Formula of *Picralima Nitida* and *Cymbopogon Citratus* Aqueous Leaf Extracts On Cyclooxygenase-2 and Interleukin-10 Gene Expressions in Phenyl-Hydrazine Induced Anaemia in Albino Wistar Rats. *J. Appl. Sci. Environ. Manage.* 28 (6) 1765-1770

Dates: Received: 15 March 2024; Revised: 28 May 2024; Accepted: 16 June 2024 Published: 11 June 2024

Keywords: *Picralima nitida*; *Cymbopogon citratus*; Cyclooxygenase-2; Interleukin-10; Phenyl-hydrazine

Herbal medicines have been used for centuries in traditional systems of medicine for the treatment of various ailments, including anaemia. Plants such as *Picralima nitida* (Akuamma) and *Cymbopogon citratus* (Lemongrass) have been reported to possess medicinal properties, including anti-inflammatory and hematopoietic effects (Ezeamuzie *et al.*, 2004). They have also gained attention as potential therapeutic agents due to their perceived efficacy and fewer side effects compared to conventional pharmaceuticals.

Inflammation is a complex biological response triggered by the body's immune system to combat harmful stimuli such as pathogens, damaged cells, or irritants. It is a protective mechanism aimed at removing the injurious agents and initiating the healing process. During inflammation, various immune cells release signaling molecules such as cytokines, which orchestrate the immune response (Takeuchi and Akira, 2010). Anti-inflammatory cytokines are a subset of cytokines that exert

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immunosuppressive and anti-inflammatory effects, counterbalancing the pro-inflammatory signals to regulate immune responses and maintain homeostasis. These cytokines play crucial roles in resolving inflammation and preventing excessive tissue damage (Cristofori *et al.*, 2021). Among the various anti-inflammatory cytokines, Interleukin-10 (IL-10) stands out as a key mediator of immune regulation and tolerance. Produced by various immune cells, including macrophages, dendritic cells, and regulatory T cells, IL-10 acts as a potent inhibitor of pro-inflammatory cytokine production and immune cell activation. It suppresses the activity of macrophages and dendritic cells, inhibits the synthesis of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6), and promotes the differentiation of regulatory T cells (Ge *et al.*, 2020). Cyclooxygenase-2 (COX-2) is an enzyme that plays an important role in the inflammatory response by synthesizing prostaglandins from arachidonic acid. COX-2 is activated in response to inflammatory stimuli, cytokines, and growth factors. It is primarily expressed in immune cells, endothelial cells, and inflammatory tissues. COX-2-derived prostaglandins mediate inflammation-related sensations such as pain, fever, and edoema, making it a promising target for anti-inflammatory medicines (Rajakariar *et al.*, 2006). Phenyl-hydrazine is a chemical substance that is frequently used to induce haemolytic anaemia in experimental animals (Berger, 2007). Phenyl-hydrazine administration acts by promoting oxidative stress and hemoglobin destabilization, resulting in red blood cell destruction. This process triggers an inflammatory response characterized by the release of pro-inflammatory cytokines and activation of inflammatory mediators such as cyclooxygenase-2 (COX-2) (Berger, 2007). However, to counterbalance the inflammatory cascade, the body relies on anti-inflammatory mechanisms, among which interleukin-10 (IL-10) is an important agent (Lalani *et al.*, 1997). Understanding the relationship between anaemia, inflammation, and the roles of IL-10 and COX-2 is crucial for developing therapeutic strategies using plant materials aimed at modulating inflammatory pathways and ameliorating the adverse effects of haemolytic anaemia. *Picralima nitida* and *Cymbopogon citratus* have been traditionally used in folk medicine for their anti-inflammatory and hematopoietic properties, suggesting their potential efficacy in mitigating the adverse effects of anaemia. Investigating their effects on COX-2 and IL-10 gene expressions can provide insights into their mechanisms of action and their ability to modulate inflammatory pathways and immune responses associated with anaemia. Also, elucidating the effects

of the bi-herbal formula on COX-2 and IL-10 gene expressions can contribute to the development of therapeutic strategies for managing anaemia and related inflammatory conditions. 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 cyclooxygenase-2 (COX-2) and Interleukin 10 (IL-10) 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 on the 23rd of December 2023. 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 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: 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; 0.45 μ m pore size) filter paper, with the residue being discarded. Following filtration, the resulting filtrate was subjected to concentration in a Waterbath 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. 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.

Cyclooxygenase-2 (COX-2) and Interleukin-10 (IL-10) 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 (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 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 the relative level of expression of each gene, and quantification of band intensity was done using “image J” software (Olumegbon *et al.*, 2022).

Primers**COX-2**

Forward: GATTGACAGCCCACCAACTT

Reverse: CGGGATGAACTCTCTCCTCA

IL-10

Forward: GAGAGAAGCTGAAGACCCTCT G

Reverse: TCATTCATGGCCTTG TAGACAC

GAPDH

Forward: CTCCTGGAGAAGAGCTATGA

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 AND DISCUSSION

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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 Cyclooxygenase-2 (COX-2) 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 *Picalima nitida* and *Cymbopogon citratus*, phenyl-hydrazine + 200mg/kg bi-herbal formulation of *Picalima nitida* and *Cymbopogon citratus* and phenyl-hydrazine + 400mg/kg bi-herbal formulation of *Picalima nitida* and *Cymbopogon citratus* respectively, represented on different bars on the bar chart.

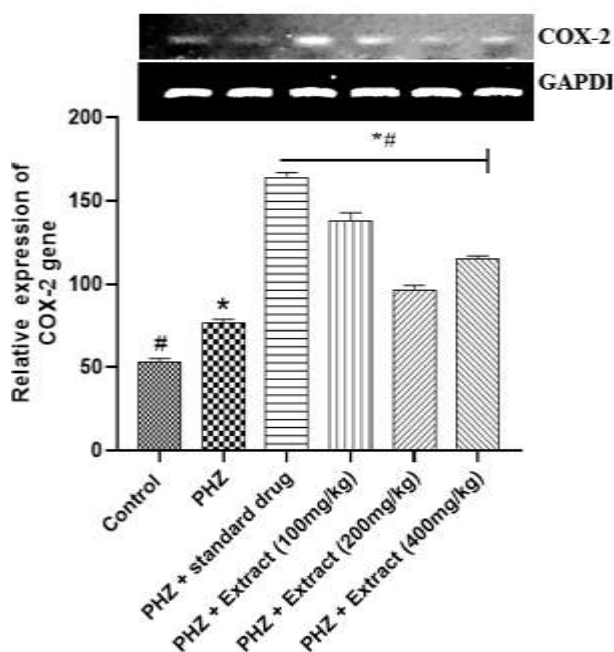


Fig 1: mRNA Expression of Cyclooxygenase-2 (COX-2) of the Studied Groups.

* 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

There was a significant increase in the mRNA expression of COX-2 of group B when compared to group A (p<0.05). There was a significant increase in the mRNA expression of COX-2 of group C, D, E and F when compared to group A and B with the expression being highest in group D (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 Interleukin-10 (IL-10) 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 *Picalima 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-10 of group B when compared to group A ($p < 0.05$). There was a significant increase in the mRNA expression of IL-10 of group C, D, E and F when compared to group A and B with the expression being highest in group D ($p < 0.05$).

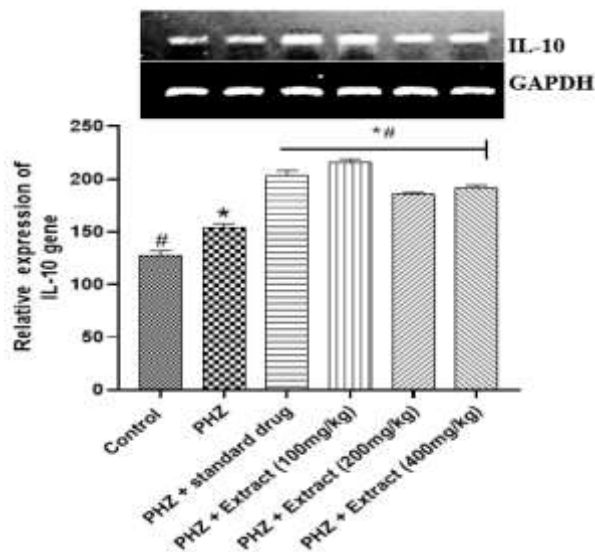


Fig 2: mRNA Expression of Interleukin-10 (IL-10) of the Studied Groups.

*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

The relationship between inflammation and anaemia has been a subject of considerable research interest, with inflammatory cytokines and mediators playing roles in the pathogenesis of anaemia (Stenvinkel, 2001). Among these mediators, cyclooxygenase-2 (COX-2) and interleukin-10 (IL-10) have emerged as key regulators of inflammation and immune response, with potential implications for erythropoiesis and red blood cell homeostasis. This study showed a notable increase in the mRNA expression of COX-2 in the phenyl-hydrazine-only treated group compared to the control group, suggesting that phenyl-hydrazine exposure induces up-regulation of COX-2 gene expression. This elevation in COX-2 mRNA expression is consistent with the known inflammatory response associated with phenyl-hydrazine induced anaemia (Shwetha *et al.*, 2019). The mRNA expression of COX-2 significantly increased in groups receiving treatment interventions, including ferrous sulphate and various doses of the bi-herbal

formulation (100mg/kg, 200mg/kg and 400mg/kg), compared to both the control and phenyl-hydrazine-only groups. However, the highest mRNA expression of COX-2 was observed in the group treated with 100mg/kg of the bi-herbal formulation, indicating a dose-dependent effect of the herbal intervention on COX-2 gene expression. These findings suggest that both ferrous sulphate and the bi-herbal formulation may potentiate the inflammatory response, as evidenced by the up-regulation of COX-2 mRNA expression (Gupta *et al.*, 2021). The results from this study also showed a significant increase in IL-10 mRNA expression in the phenyl-hydrazine-only treated group when compared to the control group, indicating an up-regulation of IL-10 gene expression in response to phenyl-hydrazine-induced anaemia. IL-10 is an anti-inflammatory cytokine known for its immuno-regulatory properties, and its up-regulation in after phenyl-hydrazine administration suggests a compensatory mechanism to counteract the inflammatory response associated with anaemia induction (Kubo and Motomura, 2012). The mRNA expression of IL-10 significantly increased in groups receiving treatment interventions, including ferrous sulphate and various doses of the bi-herbal formulation (100mg/kg, 200mg/kg and 400mg/kg), compared to both the control and phenyl-hydrazine-only groups. Interestingly, the highest mRNA expression of IL-10 was observed in the group treated with 100mg/kg of the bi-herbal formulation, indicating a potential dose-dependent effect of the herbal intervention on IL-10 gene expression. These findings suggest that both ferrous sulphate and the bi-herbal formulation may exert immuno-modulatory effects. The increased expression of IL-10 could contribute to the regulation of the inflammatory response and the maintenance of immune homeostasis in the context of phenyl-hydrazine-induced anaemia.

Conclusion: Data from this study revealed that the mRNA expression of COX-2 and IL-10 showed significant up-regulation in response to phenyl-hydrazine-induced anemia, with further increases observed following treatment with the bi-herbal formulation.

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