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Chlorogenic Acid Mitigates Phenylhydrazine-Induced Haemolytic Anaemia and Associated Oxidative Stress in Swiss Mice

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Abstract

Background and Aim: Haemolytic anaemia has been the bane of several blood borne diseases like malaria, and it appears intractable especially in endemic areas like the tropics and developing economies. It is characterized by decreased red blood cells, severe haemoglobinuria, jaundice, and high mortality rates in young subjects without prompt medical intervention. The potential ability of several nutraceuticals and plant extracts is being investigated as affordable haematinic in the management of the condition in different models of anaemia. Chlorogenic acid (CGA) is a phytonutrient with therapeutic capacities. This study is designed to investigate the haematinic property of chlorogenic acid, a plant-based polyphenol widely distributed in the diet, using phenylhydrazine-induced haemolytic anaemia model in mice.

Methods: Fifty-four adult male Swiss mice were used for the study. Forty-eight mice were administered 40 mg/kg dose of phenylhydrazine (PHZ) intraperitoneally twice at 12-hour interval to induce anaemia. Those animals that were confirmed to be anaemic were weight-matched into seven groups (n = 6/group) as follows: Group I- untreated non-anaemic animals received distilled water only, Group II- PHZ only, Group III- PHZ + ferrous sulphate (2 mg/kg), Group IV- PHZ + CGA (3.75 mg/kg), Group V- PHZ + CGA (7.5 mg/kg), Group VI- PHZ + CGA (15 mg/kg), Group VII- PHZ + CGA (30 mg/kg). The treatment with chlorogenic acid was by oral gavage for 5 consecutive days. On day 6, blood specimens were obtained for haematological parameters. The liver and spleen were also excised, weighed and processed for antioxidant assay, oxidative stress markers and liver function test.

Results: Chlorogenic acid reversed the haemolytic anaemia, reduced oxidative stress and liver damage and elevated bilirubin associated with exposure to phenylhydrazine at doses of 15 and 30 mg/kg.

Conclusion: Chlorogenic acid possesses significant haematinic, antioxidant and hepatoprotective potential at moderate doses.

Keywords: Chlorogenic acid; Haematology; Spleen; Oxidative stress; Liver function test

1. Introduction

Anaemia is a pathological condition in which the number of erythrocytes or the oxygen transporter, haemoglobin, in the blood is less than normal. Anaemia is characterized by weakness, fatigue, shortness of breath, and dizziness, among other symptoms, as a result of the reduction in the capacity of the blood to carry oxygen.

There are different types which have varying causes but the usual treatment involves diet or the use of supplements (1). For example, haemolytic anaemia, characterized by intravascular or extravascular

premature destruction of red blood cells amongst other causes, remains a significant clinical challenge associated with haemolytic diseases, poisoning and haemoglobinopathies, especially in regions with limited access to conventional therapies (2). Haemorrhagic or blood loss anaemia seen in accidents, bleeding ulcers, blood-sucking parasites is also a persistent occurrence the world over creating a need for immediate intervention and regular need for haematinics. Females, because of blood loss during menstruation and increased need during pregnancy, are also predisposed to the disease condition (1,3). Children due to their exposure to intravascular and intra-erythrocytic parasites appear more prone to the condition and the associated fatalities in cases that are not properly managed, especially in rural communities that lack blood transfusion and other medical care facilities (4,5). The World Health Organization (WHO) estimates that globally, 40% of children within the age bracket of 6-59 months, 37% of pregnant women, and 30% of women within the age range of 15-49 years are affected (1). This is a staggering number that requires the constant use of haematinic to stimulate the process of erythropoiesis in affected individuals worldwide, but more importantly in developing countries.

The use of medicinal plant extracts, food supplements, and nutraceuticals has been widely reported to gain attention as alternative treatments for anaemia due to their accessibility, traditional use, and potential therapeutic benefits. Recent studies using animal models of haemolytic and haemorrhagic anaemia, have shown that various plant extracts such as Moringa oleifera, Parquetina nigrescens, and Allium saralicum have been reportedly used in the treatment of anaemia to improve haematological parameters, restore haemoglobin levels, and reduce anaemia-related pathology (6-8).

Chlorogenic acid (CGA) is a polyphenol of the hydroxycinnamic acid family, found in coffee, tea, apples, blueberries, potatoes, carrots and tomatoes, which are widely distributed in the diet and is taken for therapeutic reasons as supplements. CGA has been documented to have anti-cancer, antilipidaemic, anti-inflammatory, hepatoprotective, cardiovascular protective, anti-diabetic, anti-obesity, antimicrobial, and anti-neurodegenerative properties (9-13). Chlorogenic acid may be a natural substitute for synthetic haematinics which are foreign to the human body.

The hypothesis is that chlorogenic acid possesses haematinic properties. This study, therefore, investigates the haematinic effect of chlorogenic acid using phenylhydrazine-induced haemolytic anaemia in experimental animal models.

2. Methods

Test and Reagents

Chlorogenic acid was obtained from AK Scientific (U.S.A.) and phenylhydrazine was a product of Sigma-Aldrich (U.S.A.). Ferrous sulphate was a product of Terrapeutic (Nigeria). All other reagents were sourced from Sigma-Aldrich (U.S.A.) or SureChem (U.K.).

Experimental Design

Fifty-four (54) Swiss mice weighing 20-24 g were obtained from the Central Animal House, College of Medicine, University of Ibadan, Oyo State, Nigeria and kept in the Experimental animal house situated there. The animals were acclimatized for one week and allowed unrestricted access to feed (Topfeed, Nigeria) and water. The cycle of 12 hours of light and 12 hours of dark was sustained. Packed cell volume (PCV) of all the animals was determined using heparinized capillary tube and micro haematocrit centrifuge. To forty-eight of the mice, 40 mg/kg dose of Phenylhydrazine (PHZ) was administered intraperitoneally twice at 12 hours interval. The PCV was then checked 12 hours later and animals with PCV less than 30% were taken to be anaemic and selected for the study. The exposure of the animals was as follows:

Group I – untreated normal animals received distilled water only (n = 6; control).

From the forty-eight animals treated with phenylhydrazine, those that had anaemia successfully induced were weight-matched evenly into six groups, and treated with ferrous sulphate and chlorogenic acid (CGA) by oral gavage for 5 consecutive days as follows:

Group II- PHZ only
Group III- PHZ + ferrous sulphate (2 mg/kg)
Group IV- PHZ + CGA (3.75 mg/kg)
Group V- PHZ + CGA (7.5 mg/kg)
Group VI- PHZ + CGA (15 mg/kg)
Group VII- PHZ + CGA (30 mg/kg)

The doses of CGA used is based on a previous study (14). On day 6, blood was collected via retro-orbital venous plexus for haematological analysis (red blood cell count, absolute neutrophils, neutrophils, platelet count, white blood cell count, packed cell volume, lymphocytes, monocytes, mean cell haemoglobin concentration, mean cell volume, and haemoglobin concentration). The liver and spleen were then excised, weighed and processed for antioxidant assay

(glutathione peroxidase (GPx) (15), superoxide dismutase (SOD) (16), glutathione-S-transferase (GST) (17) and catalase (CAT) (18) activities, ascorbic acid (19) and reduced glutathione (GSH) levels (20)); oxidative status markers (lipid peroxidation (21) and hydrogen peroxide (22) concentrations); and liver function test (bilirubin and albumin levels, alanine aminotransferase, alkaline phosphatase, and aspartate aminotransferase activities) were assayed for using Fortress Diagnostic Kits (U.K.). The Mindray BC 3000 Auto Haematology Analyzer (Mindray Bio-Medical Electronics, China) was used to assess the levels of the haematological indices. The liver and the spleen were chosen as the organs of interest because in the foetus, red blood cell formation takes place in them, as well as in certain disease conditions (23, 24).

Statistical Analysis

One-way analysis of variance (ANOVA) was employed with Tukey's post hoc test to compare the means across each group. Analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, U.S.A.). Probability value (P) less than or equal to 0.05 was considered significant at 95% confidence interval. Data were expressed as mean ± standard error of mean.

3. Results

Exposure to phenylhydrazine resulted in anaemia as confirmed in the mice before the commencement of treatment with chlorogenic acid and ferrous sulphate. As shown in Tables 1A and 1B, the anaemia persisted in the untreated group (PHZ only) as indicated by the significant ($p < 0.05$) decline in the RBC, Hb, PCV, and platelet counts while the WBC, differential neutrophil, lymphocytes, and monocytes increased significantly in the PHZ only exposure in comparison with the control. However, all the haematological parameters which were affected by PHZ were significantly ($p < 0.05$) elevated in the ferrous sulphate exposure in comparison with that of PHZ only and restored to values that were similar to the control. The significant ($p < 0.05$) increase of the haematological indices upon administration with chlorogenic acid was dose dependent in comparison with the PHZ only exposure, with the dose of 30 mg/kg of chlorogenic acid producing the most satisfactory results.

As shown in Table 2, the percentage weight of the liver and the spleen of the PHZ only exposure significantly ($p < 0.05$) reduced in comparison with the control. Similarly, the mice that were administered 3.75 mg/kg dose of CGA had significantly ($p < 0.05$) lower liver and spleen weights in comparison with the control and ferrous sulphate exposure but was higher than those of the PHZ only exposure. However, at the exposure to 30 mg/kg dose of CGA, the organs were significantly ($p < 0.05$) larger than that of PHZ only. The increases observed in the percentage organ weights (liver and spleen) in the mice treated with CGA relative to PHZ was dose dependent.

Furthermore, the activities of liver enzymes as influenced by phenylhydrazine was investigated. As shown in Table 4, the activities of the enzymes of the liver, which are alanine aminotransferase, alkaline phosphatase, and aspartate aminotransferase, were significantly ($p < 0.05$) increased in the PHZ only group in comparison with the control while it was doused in the ferrous sulphate and CGA exposures in a dose-dependent fashion, with that of the 3.75 mg/kg dose of CGA being significantly ($p < 0.05$) elevated in comparison with the control and the ferrous sulphate exposure (Table 4). Conversely, the albumin level was significantly ($p < 0.05$) reduced in the PHZ only exposure while the bilirubin concentration was significantly ($p < 0.05$) elevated in comparison with the control. However, the reversal observed by the administration of ferrous sulphate and CGA in their respective groups was dose dependent, but at the exposure of the dose of 3.75 mg/kg CGA, the concentration of bilirubin was significantly ($p < 0.05$) elevated in comparison with that of the control and ferrous sulphate (Table 4).

Figure 1 shows that mice exposed to PHZ only, had significantly ($p < 0.05$) reduced activities and concentrations of enzymatic and non-enzymatic parameters with antioxidant function in comparison with the control and ferrous sulphate exposure. Ferrous sulphate and CGA however attenuated this trend although, the groups that received CGA at the exposures of 3.75 and 7.5 mg/kg doses were significantly ($p < 0.05$) doused in the activities of GPx, GST, CAT, and SOD as well as the levels of GSH and ascorbic acid in comparison with the control and the ferrous sulphate exposure (Figure 1).

Table 3 shows that hydrogen peroxide concentration and the level of lipid peroxidation significantly ($p < 0.05$) appreciated in the PHZ only exposure in comparison with the control in the liver. This was attenuated in the group administered with ferrous sulphate. It was observed that upon administration of CGA, the reduced levels of hydrogen peroxide and lipid peroxidation in the anaemic mice were dose-dependent, with the exposure to CGA at 3.75 and 7.5 mg/kg doses being significantly ($p < 0.05$) elevated in comparison with the control and the ferrous sulphate groups (Table 3).

Figure 2 shows that in the spleen, the enzymatic antioxidant parameters; glutathione peroxidase, superoxide dismutase and, glutathione S-transferase, were significantly ($p < 0.05$) reduced in the PHZ only group in comparison with the control while the ferrous

sulphate and CGA exposures were significantly ($p < 0.05$) elevated compared to the PHZ only. The elevation in the CGA groups was in a dose-dependent presentation with the 3.75 mg/kg dose being significantly ($p < 0.05$) reduced in comparison with the control and the ferrous sulphate groups (Figure 2). Furthermore, the non-enzymatic indices with antioxidant function - reduced glutathione and ascorbic acid- were also significantly ($p < 0.05$) reduced in the PHZ only exposure in comparison with the control while that of the ferrous sulphate and the CGA exposures significantly ($p < 0.05$) appreciated in comparison with PHZ only. The reversal that was observed was dose dependent in the CGA exposures compared to the PHZ only, with that at the 3.75 and 7.5 mg/kg doses being significantly ($p < 0.05$) doused in comparison with that of ferrous sulphate. While the values of enzymatic and non-enzymatic parameters with antioxidant function obtained in the mice administered 15 and 30 mg/kg doses of CGA, were similar to the data obtained in the control and ferrous sulphate exposure (Figure 2).

According to Table 5, the oxidative stress indicators- H_2O_2 and lipid peroxidation (malondialdehyde (MDA)) - in the spleen followed a similar trajectory observed in the liver. For example, the values of H_2O_2 and MDA were significantly ($p < 0.05$) elevated in the PHZ only exposure in comparison with the control. That of the ferrous sulphate group was significantly doused ($p < 0.05$) in comparison with the PHZ only exposure. The groups administered CGA were also significantly reduced in a dose-dependent fashion in comparison with the PHZ only, but that of the mice with the 3.75 and 7.5 mg/kg doses of CGA significantly ($p < 0.05$) appreciated in comparison with the control and ferrous sulphate groups (Table 5).

Table 1A. Influence Of Chlorogenic Acid Administration On Erythrocyte Parameters In Anaemic Swiss Mice

GROUP	PCV %	HB g/dL	RBC $x10^6/\mu L$	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	44.10±0.15	10.75 ± 0.05	8.30±0.15	70.33±1.76	12.50±0.50	28.0±0.57
PHZ only (40 mg/kg)	23.50±1.20[a]	3.75 ± 0.05[a]	1.00±0.08[a]	38.66±0.88[a]	25.33±1.08[a]	16.00±2.00[a]
PHZ + FS (2 mg/kg)	42.80±0.15[b]	9.70±0.50[a,b]	7.63±0.20[b]	64.50±3.67[b]	15.50±0.50[b]	26.50±0.50[b]
PHZ + CGA (3.75 mg/kg)	33.60±0.70[a,b,c]	6.10±0.10[a,b,c]	3.46±0.13[a,b,c]	51.30±0.88[a,b,c]	19.00±0.57[a,b]	22.50±0.50[a,b]
PHZ + CGA (7.5 mg/kg)	35.70±0.73[a,b,c]	6.83±0.03[a,b,c]	4.65±0.15[a,b,c]	53.30±1.67[a,b,c]	17.00±0.57[a,b]	23.50±0.50[a,b]
PHZ + CGA (15 mg/kg)	40.70±0.62[a,b]	7.85±0.04[a,b,c]	5.85±0.05[a,b,c]	62.00±1.73[a,b,c]	15.50±1.50[b]	25.50±0.31[b]
PHZ + CGA (30 mg/kg)	41.80±0.15[b]	8.83±0.41[a,b]	7.06±0.20[a,b]	64.30±2.33[b]	15.00±1.00[b]	26.33±0.33[b]

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA -chlorogenic acid; FS- ferrous sulphate; PHZ- Phenylhydrazine; PCV- packed cell volume; HB- haemoglobin concentration; RBC- red blood cell; MCHC- mean corpuscular haemoglobin concentration; MCV- mean corpuscular volume; MCH- mean corpuscular haemoglobin.

Table 1B. Influence Of Chlorogenic Acid Administration On Leucocytes And Platelet Parameters In Anaemic Swiss Mice

GROUP	WBC $x10^3$/µL	PLATEL ETS $x10^3$/ µL	NEUT $x10^3$/ µL	LYMP $x10^3$/ µL	EOSIN $x10^3$/ µL	MONO $x10^3$/ µL	BASO $x10^3$/ µL
Control	11.08±4.24	1436.67±31.79	1.82 ± 1.11 (17.20±3.75%)	6.78±3.58 (59.80±5.67%)	0.23±0.07 (2.75±0.55%)	1.80±0.78 (15.80±2.08%)	0.51±0.19 (5.00±1.00%)
PHZ only (40 mg/kg)	18.27±7.07[a]	316.66±12.02[a]	7.42±1.02 (24.56±5.34%)	15.32±4.19[a] (58.80±14.08%)	0	5.59±4.46[a] (15.40±3.92%)	1.14±2.17 (1.40±1.10%)
PHZ + FS (2 mg/kg)	10.55±2.42[b]	1350.00±24.49[b]	1.10±0.88[b] (12.67±1.76%)	6.97±1.31[b] (66.75±3.43%)	0	2.14±0.47[b] (20.50±1.19%)	0.22±0.20 (2.50±0.50%)
PHZ + CGA (3.75 mg/kg)	13.40±2.62[a,b,c]	603.33±8.82[a,b,c]	2.81±1.43[a,b,c] (33.00±4.36%)	6.03±3.03[a,b,c] (51.4±9.20%)	0.009±0.02 (0.20±0.23%)	1.00±0.95[a,b,c] (10.60±2.36%)	0.29±0.26 (3.00±0.71%)
PHZ + CGA (7.5 mg/kg)	11.23±2.42[a,b,c]	680.00±11.55[a,b,c]	1.93±0.79[a,b,c] (17.75±1.25%)	6.55±1.10[a,b,c] (63.80±4.98%)	0	1.97±1.25[a,b,c] (17.8±4.59%)	0.29±0.24 (2.60±0.76%)
PHZ + CGA (15 mg/kg)	8.28±2.40[a,b,c]	1000.00±57.74[a,b,c]	1.59±0.61[a] (25.50±6.02%)	4.56±2.86[a,b,c] (56.40±10.56%)	0	1.29±0.51[a,b,c] (19.00±5.01%)	0.20±0.18 (2.40 ±0.76%)
PHZ + CGA (30 mg/kg)	12.58±3.15[b]	1250.00±50.00[b]	2.41±0.51[b] (21.0±2.45%)	7.34±3.18[b] (55.80±6.88%)	0	2.62±0.85[a,b] (21.20±2.59%)	0.60±0.32 (2.35±0.53%)

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA -chlorogenic acid; FS-ferrous sulphate; PHZ- Phenylhydrazine; NEUT- neutrophils; MONO- monocytes; LYMP-lymphocytes; WBC- white blood cell; Percentage differential leucocytes counts in parenthesis; BASO-basophils; EOSIN-eosinophils

Table 2. Effect Of Chlorogenic Acid Administration On The Weight Of The Liver And Spleen In Anaemic Swiss Mice

GROUP	LIVER (%)	SPLEEN (%)
Control	6.61 ± 0.23	7.95 ± 0.18
PHZ (40 mg/ kg)	4.26 ± 0.23[a]	3.63 ± 0.29[a]
PHZ + FS (2 mg/ kg)	5.16 ± 0.29[b]	7.21 ± 0.19[b]
PHZ + CGA (3.75 mg/kg)	4.96 ± 0.24[a,b,c]	5.47 ± 0.25[a,b,c]
PHZ + CGA (7.5 mg/kg)	5.17 ± 0.25[a,b]	5.65 ± 0.18[a,b,c]
PHZ + CGA (15 mg/ kg)	5.41 ± 0.29[a,b]	6.41 ± 0.15[b]
PHZ + CGA (30 mg/ kg)	5.62 ± 0.24[b]	7.25 ± 0.18[b]

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate

Table 3. Effect Of Chlorogenic Acid Administration On The Oxidative Stress Indicators In The Liver Of Anaemic Swiss Mice

GROUP	HYDROGEN PEROXIDE	LIPID PEROXIDATION
Control	10.87 ± 1.45	1.29 E-06 ± 0.06 E-06
PHZ (40 mg/kg)	46.67 ± 1.20[a]	6.26 E-06 ± 0.20 E-06[a]
PHZ + FS (2 mg/kg)	15.67 ± 1.22[b]	1.32 E-06 ± 0.03 E-06[b]
PHZ + CGA (3.75 mg/kg)	39.75 ± 2.29[a,c]	4.32 E-06 ± 0.13 E-06[a,c]
PHZ + CGA (7.5 mg/kg)	35.37 ± 1.90[a,c]	3.13 E-06 ± 0.05 E-06[a,c]
PHZ + CGA (15 mg/kg)	31.60 ± 0.47[b]	2.25 E-06 ± 0.11 E-06[b]
PHZ + CGA (30 mg/kg)	28.70 ± 1.18[b]	1.90 E-06 ± 0.04 E-06[b]

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate. Parameter (unit): - Lipid peroxidation (µmol malondialdehyde formed/ mg protein), Hydrogen Peroxide (µM)

Table 4. Influence Of Chlorogenic Acid Administration On The Function Of The Liver In Anaemic Swiss Mice

GROUP	AST (U/L)	ALT (U/L)	ALP (U/L)	ALBUMIN (mg/dL)	BILIRUBIN (mg/dL)
Control	2.00 ± 0.13	0.68 ± 0.02	23.55 ± 1.98	64.25 ± 1.77	0.16 ± 0.02
PHZ (40 mg/kg)	4.12 ± 0.10[a]	1.63 ± 0.05[a]	42.60 ± 1.79[a]	33.72 ± 2.85[a]	0.33 ± 0.01[a]
PHZ + FS (2 mg/ kg)	2.37 ± 0.06[b]	0.81 ± 0.05[b]	19.70 ± 6.36[b]	61.06 ± 5.53[b]	0.17 ± 0.03[b]
PHZ + CGA (3.75 mg/kg)	3.09 ± 0.10[a,b,c]	1.34 ± 0.04[a,b,c]	33.32 ± 2.99[a,b,c]	44.66 ± 7.53[a,b,c]	0.30 ± 0.02[a,c]
PHZ + CGA (7.5 mg/kg)	2.72 ± 0.14[a,b]	1.22 ± 0.04[a,b,c]	31.70 ± 2.66[b]	48.53 ± 3.60[a,b]	0.26 ± 0.02[a,b]
PHZ + CGA (15 mg/kg)	2.35 ± 0.09[b]	1.11 ± 0.02[a,b]	30.18 ± 2.15[b]	56.05 ± 1.77[b]	0.20 ± 0.02[b]
PHZ + CGA (30 mg/kg)	2.17 ± 0.14[b]	0.85 ± 0.04[b]	28.67 ± 2.23[b]	58.78 ± 3.62[b]	0.18 ± 0.03[b]

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate; AST- Aspartate aminotransferase; ALT- Alanine aminotransferase; ALP- Alkaline pho sphatase

Table 5. Influence Of Chlorogenic Acid Administration On The Oxidative Stress Indicators In The Spleen Of Anaemic Swiss Mice

GROUP	HYDROGEN PEROXIDE	LIPID PEROXIDATION
Control	18.40 ± 1.21	0.002 ± 0.00
PHZ (40 mg/kg)	47.04 ± 0.81[a]	0.008 ± 0.00[a]
PHZ + FS (2mg/kg)	23.83 ± 2.43[b]	0.002 ± 0.00[b]
PHZ + CGA (3.75 mg/kg)	43.09 ± 1.06[a,c]	0.005 ± 0.00[a,c]
PHZ + CGA (7.5 mg/kg)	35.83 ± 1.65[a,c]	0.004 ± 0.00[a,c]
PHZ + CGA (15 mg/kg)	30.99 ± 0.68[b]	0.003 ± 0.00[b]
PHZ + CGA (30 mg/kg)	25.68 ± 0.83[b]	0.002 ± 0.00[b]

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate. Parameter (unit): - Lipid peroxidation (nmol malondialdehyde formed/mg protein), Hydrogen Peroxide (µM)

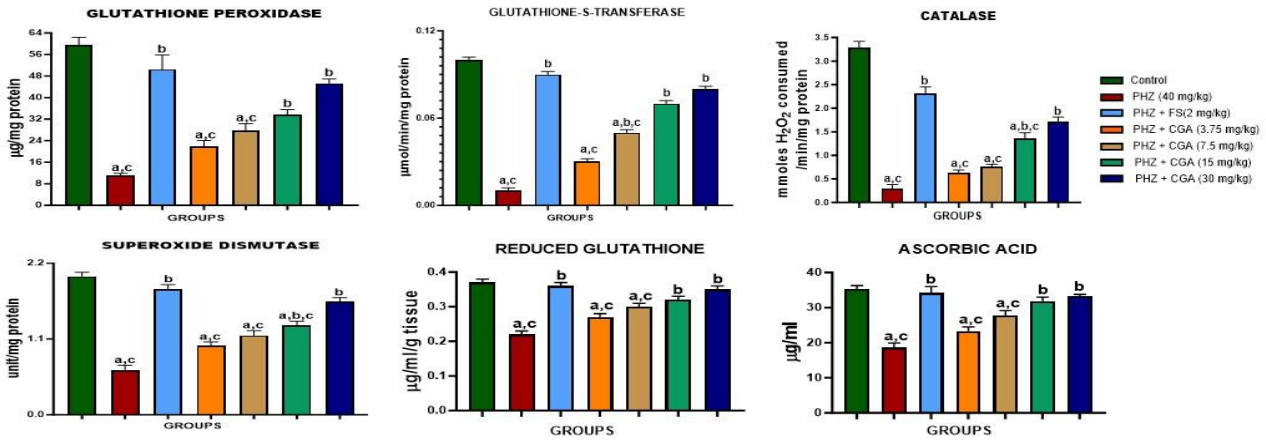

Figure 1. Influence of Chlorogenic Acid Administration on the Antioxidant Parameters in the Liver of Anaemic Swiss Mice

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate

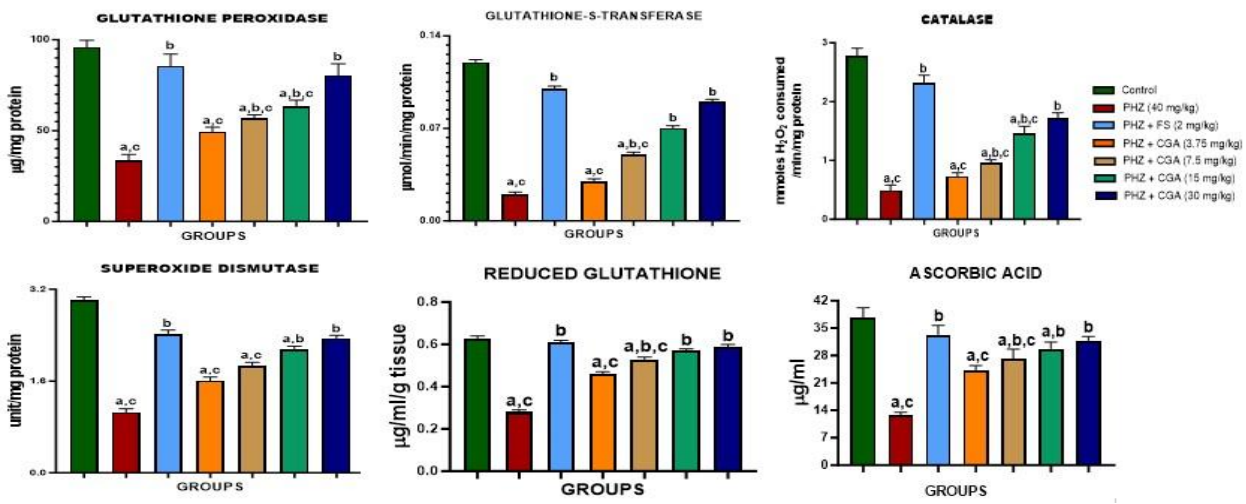

Figure 2. Effect of Chlorogenic Acid Administration on the Antioxidant Parameters in the Spleen of Anaemic Swiss Mice

Note: n = 6; Values with superscript alphabet a – are significantly different from the control at $p < 0.05$; b – are significantly different from PHZ only at $p < 0.05$; while c – are significantly different from PHZ + FS at $p < 0.05$; CGA-chlorogenic acid; PHZ-Phenylhydrazine; FS-ferrous sulphate

4. Discussion

Haemolytic anaemia is a very common type of anaemia, and it appears difficult to manage, especially in developing nations and the tropics because of its association with poisoning and several haemoprotozoan diseases such as malaria, viral diseases such as Lassa and yellow fever, as well as bacterial haemolytic diseases which are endemic or common in the tropics and in most developing countries (25-27). Therefore, the need for available and affordable antioxidants and nutraceuticals that can mitigate the condition is of high priority. This study shows that administration of chlorogenic acid improved the haematological and antioxidant parameters, as well as the hepatocellular disruptions and oxidative stress associated with phenylhydrazine induced anaemia at moderate doses.

The packed cell volume, platelet, neutrophil, white blood cell count, lymphocyte, absolute lymphocyte, red blood cell count, haemoglobin concentration as well as mean cell volume, were the haematological indices significantly affected by phenylhydrazine. A microcytic hypochromic anaemia and leukocytosis with associated neutrophilia, basophilia, lymphocytosis and monocytosis were observed in the mice that were not treated for anaemia. Following treatment with chlorogenic acid however, the observation of a significant improvement and restoration of those haematological parameters was dose dependent. At the exposure of 3.75 and 7.5 mg/kg of chlorogenic acid, the values of the haematological parameters were not as high as that of the control and the group treated with ferrous sulphate, but were higher than the phenylhydrazine only group. Whereas, chlorogenic acid at 15 and 30 mg/kg considerably ameliorated the microcytic hypochromic anaemia in the mice. It also restored the thrombocytopenia and the white blood indices to results that were similar to those of the control. This is akin to the observation of Ekweogu *et al*. (28) who documented that *Solanum aethiopicum* at a dose of 400 mg/kg increased platelet count, packed cell volume, lymphocytes, red blood cell count, mean cell haemoglobin, white blood cell count, neutrophils, haemoglobin concentration, and mean cell volume in Wistar rats. Similar study by Azeez *et al*. (8), reported an amelioration and stabilization of erythrocyte membrane in haemorrhagic anaemia and phenylhydrazine induced haemolytic anaemia by the leaf extract of *Parquetina nigrescens*. Ajibade *et al*. (29) and Oladele *et al*. (30) also reported haemolytic anaemia induced by phenylhydrazine which was reversed by extracts of *Telfairia occidentalis* in separate studies. These studies reinforce the role of phenylhydrazine as the drug of choice for experimental model of anaemia. This is because

administration of phenylhydrazine can cause haematotoxicity (toxicity in blood and haematopoietic tissues), culminating in haemolytic anaemia which results in lowering of red blood cell count with subsequent decrease in overall blood volume, which eventually leads to a relative decrease in the concentration of white blood cells. This it does by the generation of oxidants and subsequent lipid peroxidation, culminating in the disruption of the red blood cell membrane and haemoglobin (31,32). This also explains why the other haematological parameters are affected in the phenylhydrazine only group, as shown in this investigation. But the effects can be easily reversed by a strong haematinic and antioxidant compound and thus indicate the potential of chlorogenic acid as a good haematinic agent, especially at the exposures of 15 and 30 mg/kg.

The observed change in the relative organ weight of the liver and the spleen in the phenylhydrazine only group may be as a result of the toxic effect of phenylhydrazine. The reversal of this observation in the other treatment groups may buttress the ameliorative effect of ferrous sulphate and chlorogenic acid at moderate doses in these organs.

The role of enzymes that have antioxidant function like catalase, glutathione peroxidase, superoxide dismutase, and glutathione-S-transferase in protecting the body against reactive oxygen species generated during oxidative phosphorylation cannot be over emphasized. Catalase has been found to be a vital, ubiquitous enzyme located in cells which have access to oxygen. It catalyzes the hydrolysis of hydrogen peroxide to water and oxygen (33). Superoxide dismutase is an enzyme which contains manganese and alternately catalyzes the partitioning of superoxide anion into hydrogen peroxide or molecular oxygen (34). Glutathione-S-transferase represents a family of enzymes which function in catalyzing electrophilic and hydrophobic compounds being conjugated to reduced glutathione and detoxifying them (35). Glutathione peroxidase is the most important antioxidant enzyme which contains selenium and uses reduced glutathione to reduce lipid peroxides and hydrogen peroxide, thus defending cells from damage as a result of oxidation (36). Ascorbic acid is an antioxidant which is water soluble and removes reactive oxygen species and free radicals (37). Reduced glutathione is a tripeptide which functions in donating an electron to oxidants (38). Being antioxidants, catalase, glutathione-S-transferase, reduced glutathione, glutathione peroxidase, ascorbic acid, and superoxide dismutase are all involved in the protection of cells from oxidative damage. In both the liver and spleen, at the 3.75, 7.5 and 15 mg/kg exposures of chlorogenic acid, the above antioxidant enzymes were reduced considerably as a result of exposure to phenylhydrazine. But the treatment with chlorogenic acid led to a dose dependent restoration of the endogenous antioxidant status in an increasing stepwise manner as seen in Figures 1 and 2, with the levels of these enzymes fully restored to values similar to the non-anaemic control as well as those treated with ferrous sulphate at the dosage of 30mg/kg of chlorogenic acid. This shows that at the lower exposures of chlorogenic acid, the antioxidant effect on these parameters was not prominent hence chlorogenic acid was not effective in dousing the perturbations in these parameters as a result of exposure to Phenylhydrazine. But at the higher doses (15 and 30 mg/kg), the antioxidant effect of chlorogenic acid was observed. This is similar to the report of Aloke *et al*. (32) and Ajibade *et al*. (29) using *Copaifera salikounda* and *Telfairia occidentalis*, respectively, which expressed that the leaf extracts increased the activities of the antioxidant enzymes in phenylhydrazine induced haemolytic anaemia and oxidative stress in Wistar rats.

Apart from direct free radical scavenging capacity of chlorogenic acid, it affects the Keap1-Nrf2/HO-1 pathway where it modifies reactive cysteine residues, and thus results in the disruption of the Keap1-Nrf2 complex. Furthermore, it inhibits the expression of pro-inflammatory cytokines such as Tumor Necrosis Factor-α and Interleukin-6 as well as enzymes that produce free radicals such as NADPH oxidase. Therefore, chlorogenic acid protects against cell death, especially in the eye and nervous tissues. This it does by enhancing the Phosphatidylinositol 3-kinase/ Protein kinase B (P13K/AKT) signaling pathway (a pathway that regulates processes like growth, proliferation, metabolism, and survival in the cell) (39-41).

Aspartate aminotransferase is an enzyme involved in amino acid metabolism which catalyzes the transfer of α-amino group between glutamate and aspartate (42). Alanine aminotransferase is also an enzyme involved in amino acid metabolism which catalyzes the transfer of α-amino group between alanine and α-ketoglutarate (43). Alkaline phosphatase constitutes a group of isozymes found on the exterior part of the cell membrane and facilitate the catalysis of organic phosphates situated in the extracellular space (44). Impairment in the activities of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase is an indication of hepatic damage. Phenylhydrazine resulted in significant hepatocellular damages as evidenced by the elevation of these enzymes in phenylhydrazine only group when compared with the non-anaemic control. The evidence of liver perturbations was, however, reduced in those anaemic rats that were treated with chlorogenic acid, especially at 15 and 30 mg/kg doses, thus showing the

ameliorative potential of chlorogenic acid against hepatocellular damage associated with xenobiotic and other oxidants. This tends to support the findings of Chaphekar *et al*. (45) with copper sulphate and ferrous sulphate in Wistar rats where combination of the two reversed the observed elevated activities of aspartate aminotransferase and alanine aminotransferase in the phenylhydrazine only exposure. Furthermore, akin to this was the report by Allahmoradi *et al*. (46) with hydroethanol leaf extract of *Cynara scolymus* in male Wistar rats in a dose-dependent fashion as well as in the observations of Ezeigwe *et al*. (47).

Biologically, hydrogen peroxide is a major reactive oxygen species with the potential of damaging cells and tissues when in excessive amounts (48). Lipid peroxidation takes place as a result of the attack by free radical on carbon-carbon double bonds found in certain lipids, especially those of the cell membrane. A chain reaction ensues and this results in the manifestation of various diseases as a result of damage to the cell membrane (49). In a manner consistent with previous observation on the antioxidant enzymes, we observed that the elevated hydrogen peroxide generation and lipid peroxidation was only corrected at the exposures of 15 and 30 mg/kg of chlorogenic acid. These levels were reduced compared to the phenylhydrazine exposure. This again depicts the ameliorative potential of chlorogenic acid and aligns with the documentation of the *in vitro* investigation by Banerjee *et al*. (31) who used goat erythrocytes to assess the protective outcome of oleic acid on the disruption of the erythrocyte membrane by phenylhydrazine. They showed that phenylhydrazine resulted in increased lipid peroxidation but this was corrected by exposure to oleic acid, especially at the highest concentration (31). It also supports the fact that phenylhydrazine causes lipid peroxidation as evidenced in the reports of Madhikarmi and Murthy (50). These results confirm the hypothesis that chlorogenic acid possesses haematinic capacity.

5. Conclusion

Deducibly, chlorogenic acid demonstrated significant haematinic effect, which is comparable to ferrous sulphate, especially at moderate doses of 15 and 30mg/kg because it corrected the anaemia by restoring all the haematological parameters. It also demonstrated considerable hepatoprotection and antioxidant activities against the perturbations and toxicity associated with exposure to phenylhydrazine.

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Ethical Considerations and Compliance with Ethical Guidelines

This study was approved by the Animal Care Use and Research Ethics Committee of the University of Ibadan, Nigeria and the number UI-ACUREC/074-0524/17 was assigned.

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Conflict of interest

The authors declare no conflict of interest.

AI Using Declaration

The authors declare no artificial intelligence chatbot use.

Author's contributions

All authors equally contributed to preparing this article.

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