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Pharmacological Assessment of Anti-anaemic property of chloroform fraction of *Cnidoscolus aconitifolius* leaves in phenylhydrazine-induced anaemic rats.

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Abstract

Cnidoscolus aconitifolius is a globally consumed vegetable that has been utilized for its nutritive and medicinal relevance, especially for the treatment of anaemia in some parts of Nigeria. Since the crude extract has been proven to ameliorate anaemic symptoms, this study then aims to ascertain if some of the anti-anaemic properties of C. aconitifolius reside within the chloroform fraction. The crude ethanol extract of C. aconitifolius was fractionated using n-hexane, chloroform, ethyl acetate, n-buthanol, and water. Acute toxicity study was carried out using Lorke's method, before proceeding to test the extract's potency in vivo. Twenty-five male Wistar rats were randomized into five groups: A, B, C, D and E, of five rats each. Groups A, and B were the Normal and the anaemic untreated control groups respectively; Group C was administered the standard drug (Emzoron), while Groups D and E received 100 and 200mg/kg bodyweight of the chloroform fraction. Anaemia was induced for four consecutive days using phenylhydrazine, followed by fourteen days of treatment with the fraction. The haematological and biochemical analysis were done using standard methods. The result of the acute toxicity study showed that the lethal dose of the chloroform fraction is above 5000 mg/kg bodyweight. The results showed a significant ($p<0.05$) increase in haemoglobin, packed cell volume and red blood cell count in the test groups treated with 100 and 200mg/kg doses of the fraction; while the white blood cell count, neutrophils and platelets showed significant ($p<0.05$) decrease when compared to the anaemic untreated group. There was a significant decrease ($p<0.05$) in the triglyceride, total cholesterol, low-density lipoprotein, alanine transaminase, aspartate transaminase, alkaline phosphatase, urea and creatinine concentrations of the test groups compared with the anaemic untreated control group. These results suggest that the chloroform fraction of the ethanol extract of C. aconitifolius does indeed harbor anti-anaemic properties without altering the biochemical parameters, among other medicinal attributes. Therefore, its use as a blood supplement in herbal medicine should be encouraged.

Keywords: *Cnidoscolus aconitifolius, anaemia, fractionation, phenylhydrazine, chloroform, Emzoron.*

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I. INTRODUCTION

Among the numerous maladies that nature has inflicted upon man, anaemia remains one of the most prevalent and dangerous. Anaemia is described as the overall reduction in the amount of red blood cells, or hemoglobin levels in the blood; which inadvertently reduces the ability of the blood to carry oxygen (Williams and Wilkins, 2006). When one is accosted with anaemia, the symptoms are often vague and may include fatigue, and shortness of breath; whereas if acute, the symptoms manifest as lightheadedness, loss of consciousness, and increased thirst (Janz *et al.,* 2013; Enemchukwu *et al*., 2022). Although it has similar symptoms, anaemia is encumbered with different types which have unique causes and statistics. There are the; Pernicious, Iron-deficiency, Haemolytic, Sickle-cell, Thalassaemia, and Aplastic anaemia (Soundarya and Sudanthi, 2015), whose causes are as different as their names imply.

Annually, anaemia affects over a billion people globally, with the highest prevalence found among preschool-age children (Benoist *et al*, 2008). In 2020, there were over nine million diagnosed cases of anaemia in USA, and over eleven million cases in Japan (Merrik, 2021), depicting its trajectory and relevance as a major disease afflicting the world. Nature has provided man with a wealth of herbs which are both immensely nutritive, as well as medicinal, and since antiquity, humans have always depended on these herbs, either as natural remedy, or as a precursor for the production of synthetic drugs. Among many of such herbs is the tree spinach, *Cnidoscolus aconitifolius*, cultivated mostly for its medicinal relevance and potency (Abdala and Parra, 2005).

Cnidoscolus aconitifolius, is a progeny of the Euphorbiaceae which is a family of evergreen shrubs that usually occupy rocky outcropping, disturbed places and forests (Lucena and Alves, 2010). *C. aconitifolius* is commonly referred to as either "obarandu" (Ezeigwe *et al,* 2020), or *ogwu obala* (blood tonic) (Iwuji *et al.,* 2005); and it is utilized by all and sundry, especially those in the southern part of Nigeria to treat numerous diseases like; anemia, alcoholism, diabetes, insomnia, gout, scorpion stings and venereal diseases (Chittendon, 1956, Ezeigwe *et al*., 2022). It is also renowned for its ability to strengthen fingernails, darken greying hair, and improve cerebral potency (Chittendon, 1956).

The leaves of *C. aconitifolius* are consumed as a protein-rich vegetable which is abundant in calcium, iron, carotene, riboflavin, niacin, and ascorbic acid (Dave and Eric*,* 2015), albeit with a toxically high hydrocyanate content (Dave and Eric*,* 2015). Proximate analysis of *C. aconitifolius* reveals that the nutrients within *C. aconitifolius* is two to threefolds greater than there are in any other land based green vegetable (Ganiyu, 2005, Ezeigwe *et al*., 2020). These characteristics make it a very peculiar and interesting plant to study in depth. Decoctions of the leaves of *C. aconitifolius* have been employed in multiple rural anemic therapies; and also has the crude ethanol extract been proven to possess anti-anaemic properties (Ezeigwe *et al.,* 2020, Onuabuchi *et al*., 2022). So, the current *in vitro* cum *in vivo* study was undertaken to affirm whether some of the anti-anaemic properties of *C. aconitifolius* are resident within the chloroform fraction. Also, some specific biochemical parameters were assayed, to appropriately define the effects of administering any dose of this extract's fraction on the vital organs of Wistar rats.

II. METHODS

Sample Collection and Identification

The leaves of *C. aconitifolius* were collected from Adazi-Ani, Anaocha Local Government Area, Anambra State, Nigeria. The sample was identified by a taxonomist in the Department of Botany, Nnamdi Azikiwe University, Awka. The voucher number as deposited in the herbarium of Nnamdi Azikiwe University, Awka is NAU/168.

Preparation of Ethanol Extract of C. aconitifolius Leaf

The leaves were properly washed and air dried at room temperature for two weeks. The dried leaves were pulverized into powder using corona manual grinding machine. Exactly 1.5 kg of the pulverized leaf powder of *C. aconitifolius* was soaked in 6 litres of 70% ethanol for 24 hrs for ethanol extraction. The ethanol mixture was sieved using muslin cloth and filtered using Whatman no 1 filter paper. The filtrate was concentrated using waterbath at 50^0 C. The biological yield of the extract after extraction was 173.6g. The ethanol extract was stoppered in universal bottles and preserved in the refrigerator for use.

Fractionation of C. aconitifolius Leaf Extract

The crude ethanol leaf extract (173.6g) was fractionated by the method of Wu *et al*. 2005. The method involved successive extraction by increasing polarity with n-hexane, chloroform, ethyl acetate, n-butanol and water. Twenty-five grams (25g) of the ethanol extract was dissolved in 250ml of Methanol/Water ($MeOH/H_2O$) (1:1) mixture and shaken with n-hexane (2x250ml). Combined extract was left to dry on the bench to yield n-hexane fraction. Methanol (MeOH) was further fractionated by successive solvent extraction with chloroform (1x250ml), ethyl acetate (2 x 200ml) and n-butanol (1x250ml). Each fraction was left to evaporate to dryness on the bench to yield n-hexane fraction (3.23g), chloroform fraction (9.75g), ethyl acetate fraction (5.63g), n butanol fraction (2.50g) and water fraction (3.89g).

Test Animals

A total of 38 male Wistar albino rats weighing between 120–150g were purchased from Chris Experimental Animal Farm and Research Laboratory, Awka, Anambra State and used for the experiment. They were maintained and housed in cages under standard environmental conditions (27°C±3°C, 12-hour light/dark cycle) in the Department of Applied Biochemistry Laboratory, Nnamdi Azikiwe University, Awka. They were

allowed to acclimatize with the environment for one week before use. The animals were fed Vital grower's mash pellets purchased from Vital Feed Distributor at Awka, Anambra state and fed *ad libitum*. At the end of the one-week acclimatization period, the animals were weighed, grouped and labeled.

Acute toxicity (LD_{50}) evaluation

The median lethal dose (LD_{50}) for each of the extracts were determined using Lorke's method (1983). Thirteen (13) male rats were used for the determination of the median lethal dose for the chloroform fraction. The thirteen (13) rats were randomized into six groups; three rats each for the first phase which was given 10, 100 and 1000mg/kg bw and one rat each for the second phase which was given 1600, 2900 and 5000mg/kg bw. The animals were monitored for changes in behaviour and mortality within 2 hrs, 24 hrs and 14 days after a single administration of the extracts.

Study design for Anti-anaemic Properties

A total of twenty-five (25) male Wistar rats were randomized into 5 groups of 5 rats each. After the induction of anaemia with phenylhydrazine, the animals were treated for 14 days after which blood was collected by cardiac puncture under chloroform anesthesia and used for haematological and biochemical analysis. They were grouped as follows:

Group A: Normal Control
Group B: Negative Control (Anaemic untreated)
Group C: Positive Control (Std Drug-Emzoron)
Group D: Anaemia + 100 mg/kg bw. of chloroform fraction of *C. aconitifolius* leaf
Group E: Anaemia + 200 mg/kg bw. of chloroform fraction of *C. aconitifolius* leaf

Induction of Anaemia

Anaemia was induced intraperitoneally in the rats using 20mg/kg b.w. of phenylhydrazine for four consecutive days. The animals were confirmed to be anaemic on the 5th day before the commencement of treatment. Blood was collected by *retro orbital sinus* for hematological analysis before and after the induction of anaemia to monitor the animals for the symptoms of anaemia before the commencement of treatment.

Determination of Weight

The weight of the experimental subjects was checked using an electronic weighing scale. The weight of the rats was monitored before, during, and after the experiment to know whether the chloroform fraction has an effect on the body weight of the experimental rats.

Random Blood Glucose Concentration

The blood glucose levels of the rats were checked before the induction of anaemia, during, and after treatment using One Touch Glucometer (Life Scan, USA) and test strips based on the method of Trinder 1972.

Haematological Analysis

Haematological parameters were determined using automated haematology analyzer (Mindray-BC-5300). The haematological parameters that were analysed include Haemoglobin (HGB), Packed Cell Volume (PCV), Red Blood Cells (RBC), Platelets (PLT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Cells (WBC), Neutrophils (NEUT), Lymphocytes (LYMPH), Monocytes (MON), Eosinophils (EOS), Basophils (BAS).

Lipid Profile

The lipid profile (Total Cholesterol, Triglycerides, High-Density Lipoprotein-cholesterol, Low-Density Lipoprotein-cholesterol and Very Low-Density Lipoprotein-cholesterol) were determined using Randox test kits (Trinder, 1969 and Tietze *et al*., 1990). Low-density Lipoprotein-cholesterol (LDL-c) was calculated using a standard formula (Friedwald *et al.,* 1972). The procedure used was according to the manufacturer's instructions provided in the manual.

Liver Function Test

Serum biochemical indices routinely estimated for liver functions were analysed. They include: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), direct and total bilirubin. The parameters were determined using Randox diagnostic test kits. The procedures used were according to the manufacturer's instruction.

Kidney Function Test

Urea and creatinine were analysed using Randox test kits. The procedures were carried out according to the manufacturer's instructions.

Electrolyte Concentration

The serum electrolyte concentration was analysed using AFT-300 electrolyte analyzer. The whole blood sample of the wistar rat was centrifuged at 4000 rpm for 10 mins. The serum was separated and used for the analysis. The probe of the electrolyte analyser aspirates the serum of the Wistar rat which passes through the electrodes, aspiration pump and the electronic circuits which measure and process the electromotive force to give the test ion concentration. The electrolytes that were analyzed include Potassium ion (K^+), Sodium ion (Na^+), Chloride ion (Cl^-), Bicarbonate ion (BCO_3^-), Total Calcium (T^{cal}) and Ionized Calcium (n^{cal}).

Lipid Peroxidation

Lipid peroxidation was determined by the thiobarbituric acid-reacting substances (TBARS) assay method of Buege and Aust (1978). The reaction depends on the formation of complex between malondialehyde and theobarbituric acid (TBA). 0.4ml of serum was collected into the test tubes; 1.6mlof 0.25N HCl was added together with 0.5ml of 15% trichloroacetic acid and 0.5ml of 0.375% of thiobarbituricacid and then mixed thoroughly.

The reaction mixture was then placed in 100^{o}C boiling water for 15 minutes, allowed to cool and centrifuged at 3000 rpm for 10 minutes.The supernatant was collected and the optical density recorded at 532nm against reagent blank containing distilled water.

The lipid peroxidation activity was calculated using the formula:

$$\frac{\text{Optical density}}{\text{Time}} \times \frac{\text{extinction co-efficient}}{\text{amount of sample}}$$

Where the extinction coefficient value is $1.56 \times 10^{-5}M^{-1}CM^{-1}$

The unit is expressed as umol/MDA/mg of protein.

Lactate Dehydrogenase

Serum lactate dehydrogenase enzyme was determined using Randox diagnostic test kits. The procedures used were according to the manufacturer's instruction.

Statistical Analysis

Data obtained from the experiments were analyzed using the Statistical Package for Social Sciences software for windows version 23 (SPSS Inc., Chicago, Illinois, USA). All the data collected were expressed as Mean ± SEM. Statistical analysis of the results obtained were performed by using ANOVA Tests to determine if significant difference exists between the mean of the test and control groups. The limit of significance was set at $p<0.05$.

III. RESULTS

Results of the Acute Toxicity (LD_{50}) Test

The oral administration of *C. aconitifolius* produced signs of distress; albeit no mortality, at a dose of 5000 mg/kg, therefore the LD_{50} was calculated to be above 5000 mg/kg (table 1).

Table 1: Acute toxicity studies of chloroform fraction of crude ethanol extract of *C. aconitifolius* leaf

Phase	Dose mg/kg	Death recorded in rats	Observations
First	10	0/3	-
	100	0/3	-
	1000	0/3	-
Second	1600	0/1	-
	2900	0/1	-
	5000	0/1	very weak, breathing fast

Result of Bodyweight

Administration of 100 and 200mg/kg of the chloroform fraction of the crude ethanol extract of *C. aconitifolius,* decreased the mean weights of the rats when compared to the anaemic untreated after the 7th and 14th day of treatment although the decrease was not statistically significant (table 2).

Table 2: Bodyweight of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius*.

Groups	Weight (g)			
	Before induction Day 0	**After 4th day of ind. of anaemia Day 5**	**After 7th day of treatment Day 12**	**After 14th day of treatment Day 19**
Normal Control	133.20±2.54	143.60±3.33	150.00±4.83	161.20±4.68
Anaemic Untreated	126.00±3.11	133.00±2.89	156.50±7.10	162.80±4.55
Anaemia + Standard drug (Emzoron)	125.60±5.14	129.80±3.98	144.80±4.77	155.00±4.11
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	137.00±2.02	139.40±5.28	146.00±4.81	159.60±3.78
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	132.00±4.85	136.40±2.94	143.00±3.58	148.80±2.76

Result of Random Blood Glucose Concentration

Chloroform fraction of the ethanol extract of *C. aconitifolius* at doses 100 and 200mg/kg did not cause any noticeable change in the random blood glucose levels of the test group when compared to the normal control. The random blood glucose concentrations remained normal in the course of the experiment (table 3).

Table 3: Random blood glucose concentrations of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius*.

Groups	Glucose Level (g/dl)			
	Before induction Day 0	**After 4th day of ind. of anaemia Day 5**	**After 7th day of treatment Day 12**	**After 14th day of treatment Day 19**
Normal Control	94.80±11.20	81.40±4.03	102.00±2.98	107.80±11.00
Anaemic Untreated	100.40±8.77	82.40±5.26	104.0±6.36	99.50±2.78
Anaemia + Standard drug (Emzoron)	96.00±6.45	78.60±3.01	114.60±7.81	105.60±5.91
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	102.20±10.21	83.20±3.15	101.2±5.34	104.80±3.60
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	74.60±4.21	82.60±6.58	98.20±6.37	102.00±7.94

Result of Haematological Analysis

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Haemoglobin Concentration (HGB) of Phenylhydrrazine-induced anaemic rats

Following a prior reduction in the concentrations of hemoglobin across the test group as a consequence of the induction of anaemia, a subsequent significant (p<0.05) increase in the HGB levels was recorded after 14 days of treatment. At doses 100 and 200mg/kg, results show a significant (p<0.05) decrease in the concentration of HGB as recorded on the fifth day after the forth day induction; then consequently a significant (p<0.05) increase in the concentration of HGB was recorded after 14 days of treatment when compared to the anaemic untreated. The Emzoron group followed the same trajectory of significantly (p<0.05) falling first, then rising much later (table 4).

Table 4: Haemoglobin (HGB) concentration of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius*.

Groups	HGB (g/dl)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	12.43±0.50	12.13±0.19	11.70±1.40
Anaemic Untreated	12.20±0.34	8.73±0.50[b]	8.30±1.55
Anaemia + Standard drug (Emzoron)	13.17±0.27	10.60±0.60[b]	14.10±0.10[c]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	12.47±0.54	9.70±0.32[b]	14.00±0.96[c]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	12.17±0.13	8.77±0.52[b]	13.33±0.38[c]

[b]Significant decrease with respect to day 0; [c]Significant increase with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Packed Cell Volune (PCV) of Phenylhydrazine-induced Anaemic Rats

Induction of anaemia for four consecutive days in groups B to E caused a significant (p<0.05) decrease in PCV compared to the normal control which was not induced. A subsequent significant (p<0.05) rise in the PCV at doses 100 and 200mg/kg; in tandem with the positive control, was recorded (>15%); after 14 days of treatment with the chloroform fraction of the ethanol extract, when compared to the anaemic untreated (table 5).

Table 5: Packed Cell Volume (PCV) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	PCV (%)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	46.87±1.72	46.73±2.66	45.83±2.13
Anaemic Untreated	37.43±0.46	30.57±1.77[b]	23.73±2.87[bd]
Anaemia + Standard drug (Emzoron)	40.47±0.99	33.43±2.97[b]	43.87±0.74[c]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	43.83±1.28	27.63±2.06[b]	43.57±3.99[c]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	37.60±0.46	24.47±1.30[b]	40.93±1.77[c]

[b]Significant decrease with respect to day 0; [c]Significant increase with respect to day 5; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Red Blood Cell (RBC) of Phenylhydrazine-induced Anaemic Rats

Induction of anaemia for four consecutive days in groups B to E caused a significant (p<0.05) decrease in RBC count compared to the normal control which was not induced. However, a significant (p<0.05) increase was recorded on Day 19 in the test groups administered 100 and 200mg/kg when compared to the anaemic untreated after 14 days of treatment (table 6).

Table 6: Red Blood Cells (RBC) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	RBC (g/dl)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	7.91±0.06	7.85±0.32	6.38±0.74
Anaemic Untreated	8.26±0.18	3.15±0.25[b]	3.54±0.38[b]
Anaemia + Standard drug (Emzoron)	8.37±0.36	6.27±0.64[b]	5.86±0.15[b]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	7.45±0.27	4.66±0.35[b]	5.80±0.36[b]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	7.76±0.16	4.28±0.33[b]	5.76±0.20[bc]

[b]Significant decrease with respect to day 0; [c]Significant increase with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Platelets (PLT) Count of Phenylhydrazine-induced Anaemic Rats

Induction of anaemia for four consecutive days in groups B to E caused a significant (p<0.05) decrease in platelet count compared to the normal control which was not induced. Doses 100 and 200mg/kg alongside the positive control produced a significant (p<0.05) increase in the PLT count when compared to the anaemic untreated after 14 days of treatment (table 7).

Table 7: Platelets (PLT) count of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	PLT (10^9/L)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	798.70±36.29	794.00±52.62	780.70±81.77
Anaemic Untreated	751.30±81.88	445.70±65.03[b]	488.70±168.90[b]
Anaemia + Standard drug (Emzoron)	713.00±4.62	756.30±10.17	572.00±115.9
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	772.30±4.33	858.30±92.34	739.70±32.00
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	753.00±36.17	908.70±32.21	513.00±198.70[bd]

[b]Significant decrease with respect to day 0; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Mean Corpuscular Volume (MCV) of Phenylhydrazine-induced Anaemic Rtas

Induction of anaemia for four consecutive days in groups B to E caused a significant (p<0.05) decrease in the MCV levels compared to the normal control which was not induced. The MCV levels at doses 100 and 200mg/kg were also found to be significantly (p<0.05) increased when compared to the anaemic untreated (Table 8).

Table 8: Mean Corpuscular Volume (MCV) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius.*

Groups	MCV (fL)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	57.40±1.23	59.50±1.30	55.77±0.55
Anaemic Untreated	52.67±1.09	97.47±2.80	66.83±1.10[ad]
Anaemia + Standard drug (Emzoron)	51.17±1.10	53.47±1.18	75.00±2.69[ac]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	55.73±2.57	59.37±2.30	74.87±4.62[ac]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	50.97±4.14	53.23±1.11	71.07±2.34[ac]

[a]Significant increase with respect to day 0; [c]Significant increase with respect to day 5; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Mean Corpuscular Hemiglobin (MCH) of Phenylhydrazine-induced Anaemic Rats

Single administration of the chloroform fraction of the ethanol extract of *C. aconitifolius* at doses 100 and 200mg/kg produced significant (p<0.05) increase in the MCH when compared to Day 5 after 14 days of treatment (table 9).

Table 9: Mean Corpuscular Hemoglobin (MCH) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	MCH (pg)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	16.40±1.29	15.50±0.66	18.33±0.20
Anaemic Untreated	15.96±0.84	27.80±0.90[a]	23.40±0.06[ad]
Anaemia + Standard drug (Emzoron)	16.53±0.52	17.10±1.51	24.10±0.45
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	15.57±0.32	20.90±1.12[a]	24.07±0.17[ac]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	16.67±0.67	19.37±0.98[a]	23.20±0.82[ac]

[a]Significant increase with respect to day 0; [c]Significant increase with respect to day 5; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Mean Corpuscular Haemoglobin Concentration (MCHC) of Phenylhydrazine-induced Anaemic Rats

Groups C, D and E, showed increase in MCHC on Day 5; after the 4 days of induction, when compared to Day 0. The test group administered 100 and 200mg/kg showed some decrease in the MCHC when compared to Day 5. Also, doses 100 and 200mg/kg of the extract as well as those treated with Emzoron produced a reduction in the MCHC when compared with the negative control (table 10).

Table 10: Mean Corpuscular Hemoglobin Concentration (MCHC) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius.*

Groups	MCHC (g/dl)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	26.23±2.33	26.13±1.57	32.90±0.26[ac]
Anaemic Untreated	27.70±0.75	28.50±0.10	35.10±0.56[ac]
Anaemia + Standard drug (Emzoron)	28.10±0.38	31.97±1.18[a]	32.17±0.67[a]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	30.43±1.55	35.27±1.60[a]	32.23±1.02

Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	30.50±1.14	37.20±0.30[a]	32.67±0.55

[a]Significant increase with respect to day 0; [c]Significant increase with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the White Blood Cell (WBC) Count of Phenylhydrazine-induced Anaemic Rats

The WBC count significantly (p<0.05) increased in the induced groups after four days of induction when compared with the normal control. However, with continuous administration of the extract and the standard drug to their respective groups significantly (p<0.05) decreased the WBC volume measured after 14 days of treatment when compared to both results from Day 5 and the anaemic untreated. The WBC count of the negative control however remained on the ascendency and showed a potential of even exceeding the $32.28*10^9$/L had the *in vivo* study continued beyond 20 days (table 11).

Table 11: White Blood Cells (WBC) count of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	WBC (10^9/L)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	11.17±0.92	11.43±1.86	14.45±0.93
Anaemic Untreated	9.14±1.85	21.51±5.94[a]	32.28±4.82[ac]
Anaemia + Standard drug (Emzoron)	7.73±0.73	31.65±4.75[a]	14.96±2.31[ad]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	8.73±1.96	39.69±5.68[a]	14.38±1.63[ad]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	8.74±1.78	29.54±9.01[a]	9.18±2.25[d]

[a]Significant increase with respect to day 0; [c]Significant increase with respect to day 5; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Neutrophilis (NEUT) of Phenylhydrazine-induced Anaemic Rats

Following induction with phenylhydrazine, a significant (p<0.05) increase in the NEUT levels was recorded in the test groups when compared to the normal control. However, a significant (p<0.05) decrease in the NUET levels was recorded at 100 and 200mg/kg bw of the extract when compared with the anaemic untreated after 14 days of treatment (table 12).

Table 12: Neutrophils (NEUT) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	Neut (%)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	27.77±0.35	27.53±1.67	24.83±6.67
Anaemic Untreated	29.53±0.66	32.13±1.08	30.70±0.96
Anaemia + Standard drug (Emzoron)	27.40±1.04	30.57±1.08	17.80±1.59[bd]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	28.03±0.26	31.60±0.72	22.07±4.62[d]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	27.93±0.87	35.60±1.08	18.87±5.67[bd]

[b]Significant decrease with respect to day 0; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Lymphocytes (LYMPH) of Phenylhydrazine-induced Anaemic Rats

Phenylhydrazine produced significant (p<0.05) decreases in the lymphocyte levels across the test groups after four days of induction when compared to the normal control. Doses 100 and 200mg/kg bw of extract alongside Emzoron significantly (p<0.05) increased LYMPH concentration when compared to the anaemic untreated after 14 days of treatment (table 13).

Table 13: Lymphocytes (LYMPH) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	Lymph (%)		
	Before induction Day 0	**After 4th day of induction of anaemia Day 5**	**After 14th day of treatment Day 19**
Normal Control	70.10±0.25	70.70±0.80	74.67±6.87
Anaemic Untreated	66.60±1.57	52.97±1.80[b]	69.10±1.08[c]
Anaemia + Standard drug (Emzoron)	65.33±2.72	52.53±2.13[b]	82.13±1.63[ac]
Anaemia + 100 mg/kg chloroform	65.47±2.71	53.53±1.82[b]	77.57±4.66[ac]

fraction of *C. aconitifolius*			
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	71.20±0.81	62.27±1.07[b]	83.97±7.61[ac]

[a]Significant increase with respect to day 0; [b]Significant decrease with respect to day 0 [c]Significant increase with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Monocytes (MON) of Phenylhydrazine-induced Anaemic Rats

Significant (p<0.05) increase in the monocyte levels in all the induced groups were recorded on Day 5 when compared to either the normal control or Day 0. After the 14th day, there was a significant general reduction in the monocyte percentage in all the groups when compared to the percentages recorded on Day 5. Doses 100 and 200mg/kg produced a significantly (p<0.05) high monocyte percentage on the 19th day when compared to all and sundry (table 14).

Table 14: Monocytes (MON) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	Mon (%)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	0.33±0.03	0.37±0.33	0.03±0.03[bd]
Anaemic Untreated	0.43±0.03	1.47±0.03	0.03±0.03[bd]
Anaemia + Standard drug (Emzoron)	0.37±0.03	1.40±0.06	0.00±0.00[bd]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	0.40±0.00	1.40±0.06	0.20±0.15[d]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	0.43±0.09	1.47±0.03	0.10±0.06[d]

[b]Significant decrease with respect to day 0; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Eosinophils (EOS) of Phenylhydrazine-induced Anaemic Rats

EOS percentage recorded a significantly (p<0.05) lowered levels across the induced groups on Day 5 when compared to these same levels on Day 0. Doses 100 and 200mg/kg maintained these levels throughout the duration of the study which was significantly (p<0.05) decreased when compared to the normal control (table 15).

Table 15: Eosinophils (EOS) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	Eos (%)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	1.13±0.03	1.10±0.06	0.27±0.17
Anaemic Untreated	0.23±0.07	0.13±0.03	0.17±0.09
Anaemia + Standard drug (Emzoron)	0.23±0.03	0.17±0.03	0.07±0.03[bd]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	1.03±0.12	0.17±0.03	0.17±0.12
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	1.13±0.03	0.17±0.03	0.17±0.17

[b]Significant decrease with respect to day 0; [d]Significant decrease with respect to day 5.

The Effect of Chloroform Fraction of the Ethanol Extract of *C. aconitifolius* on the Basophyls (BAS) of Phenylhydrazine-induced Anaemic Rats

The Basophyl percentage was significantly (p<0.05) increased within the induced groups as recorded on Day 5 when compared to the normal control and Day 0. However, there was a subsequent unanimous zeroing of the basophyl levels as was recorded after the 14th day across the groups (table 16).

Table 16: Basophyls (BAS) of phenylhydrazine-induced anaemic rats treated with chloroform fraction of crude ethanol extract of *C. aconitifolius* leaves.

Groups	Bas (%)		
	Before induction Day 0	After 4th day of induction of anaemia Day 5	After 14th day of treatment Day 19
Normal Control	0.10±0.00	0.10±0.00	0.00±0.00[bd]
Anaemic Untreated	0.10±0.00	0.33±0.03	0.00±0.00[bd]
Anaemia + Standard drug (Emzoron)	0.17±0.03	0.37±0.03	0.00±0.00[bd]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	0.20±0.06	0.33±0.03	0.00±0.00[bd]

Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	0.23±0.03	0.33±0.03	0.00±0.00[bd]

[b]Significant decrease with respect to day 0; [d]Significant decrease with respect to day 5.

Result of Biochemical Analysis

Effect of Extract on Lipid Profile

Significant (p<0.05) reductions were noticed in the TCHOL, TRIG, LDL-C and vLDL levels of both groups treated with Emzoron; and those treated with varying doses of the chloroform fraction of the ethanol extract when compared to anaemic untreated control. The HDL-C of the test groups was also significantly (p<0.05) increased when compared to the HDL-C of the negative control (table 17).

Table 17: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on lipid profile of phenylhydrazine-induced anaemic rats.

Groups	TCHOL (mg/dl)	HDL-C (mg/dl)	TRIG (mg/dl)	LDL-C (mg/dl)	VLDL (mg/dl)
Normal Control	92.67±5.46	45.33±2.85	27.33±1.45	41.87±5.88	5.47±0.29
Anaemic Untreated	139.67±3.76[e]	35.33±4.70	38.67±3.84[e]	95.27±8.73[e]	9.07±0.93[e]
Anaemia + Standard drug (Emzoron)	113.60±3.18[h]	42.00±5.03	32.67±2.03	65.13±8.23[h]	6.53±0.41[h]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	102.67±7.13[h]	45.67±8.88	32.33±3.53	50.53±14.00[h]	6.47±0.71[h]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	116.00±8.50	40.67±5.61	38.67±3.84[e]	67.20±9.93	7.73±0.77[e]

[e]Significant increase with respect to normal control; [h]Significant decrease with respect to anaemic untreated.

Effect of Extract on Liver Function Parameters

Results show a significant (p<0.05) decrease in the AST, ALP, ALT, T.BIL, D.BIL levels of the test groups treated with the chloroform fraction when compared to the negative control/anaemic untreated. The levels of all these parameters within the negative control models show significant (p<0.05) increase when compared to the normal control (table 18)

Table 18: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on liver function parameters of phenylhydrazine-induced anaemic rats.

Groups	AST (U/L)	ALP (U/L)	ALT (U/L)	T. BIL (mg/dl)	D. BIL (mg/dl)
Normal Control	17.67±0.88	30.57±3.58	10.33±1.20	1.14±0.16	0.22±0.04
Anaemic Untreated	29.00±1.15[e]	62.50±9.97[e]	30.00±2.08[e]	2.10±0.32[e]	0.85±0.17[e]
Anaemia + Standard drug (Emzoron)	21.67±1.33[h]	42.73±14.10[h]	16.67±2.60[h]	1.48±0.16[h]	0.34±0.12[h]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	22.00±1.00[h]	41.17±6.85[h]	13.33±1.85[h]	1.67±0.08[h]	0.31±0.07[h]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	20.67±1.45[h]	37.90±6.37[h]	14.67±3.76[h]	1.66±0.08[h]	0.31±0.07[h]

[e]Significant increase with respect to normal control; [h]Significant decrease with respect to anaemic untreated.

Effect of Extract on Kidney Function Parameters

Doses 100 and 200 of the extract showed significant (p<0.05) decrease in both the urea and the creatinine levels of the teat group when compared to the negative control, so also does the standard drug. However, the anaemic untreated group showed significant increase (p<0.05) in the urea and creatinine concentrations as compared to the normal control and the other groups (table 19).

Table 19: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on kidney parameters of phenylhydrazine-induced anaemic rats.

Groups	Urea (mg/dl)	Creatinine (mg/dl)
Normal Control	9.27±1.28	1.73±0.03
Anaemic Untreated	15.17±1.18[e]	2.10±0.40
Anaemia + Standard drug (Emzoron)	10.70±0.45[h]	1.57±0.20
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	12.23±0.32[h]	1.70±0.06
Anaemia + 200 mg/kg chloroform fraction	11.43±0.67[h]	1.77±0.32

of *C. aconitifolius*		

[e]Significant increase with respect to normal control; [h]Significant decrease with respect to anaemic untreated.

Effect of Extract on Electrolyte Levels

Results show a significant (p<0.05) decrease in the concentrations of K^+ of the models treated with varying doses of the chloroform fraction of the ethanol extract of *C. aconitifolius* when compared to the negative and the normal control. Also, doses 100 and 200mg/kg showed significant (p<0.05) increase in the concentrations of Na^+ and Cl^- when compared to the either the negative or the normal control. Significant (p<0.05) increase was also recorded in the BCO_3^- when compared to the normal control (table 20).

Table 20: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on electrolyte levels of phenylhydrazine-induced anaemic rats.

Groups	K^+ (mmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	BCO_3^- (mmol/L)	T^{cal} (mmol/L)	n^{cal} (mmol/L)
Normal Control	8.20±1.40	127.33±1.86	97.00±0.00	18.67±1.20	1.27±0.03	0.60±0.00
Anaemic Untreated	8.20±1.52	130.00±3.61	96.67±2.03	20.67±1.20	1.27±0.07	0.63±0.03
Anaemia + Standard drug (Emzoron)	5.47±0.52[fh]	130.66±1.33	106.7±0.67[eg]	21.33±0.88	1.07±0.18	0.53±0.09
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	6.30±1.08	134.00±0.58[e]	102.30±1.86[egj]	22.67±1.20[e]	1.23±0.33	0.60±0.00
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	4.80±0.35[fh]	134.0±1.00[e]	100.3±1.33[j]	19.67±0.88	1.40±0.12[i]	0.70±0.58[i]

[e]Significant increase with respect to normal control; [f]Significant decrease with respect to normal control; [g]Significant increase with respect to anaemic untreated; [h]Significant decrease with respect to anaemic untreated; [i]Significant increase with respect to standard drug; [j]Significant decrease with respect to standard drug.

Effect of Extract on Lipid Peroxidation (Malondialdehyde)

Peroxidation result shows a significant (p<0.05) decrease in the MDA concentration in groups treated with either Emzoron or extract, when compared to the negative control/anaemic untreated. However, the negative control showed significant (p<0.05) increase in the MDA concentration when compared to either normal control or test groups (table 21).

Table 21: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on malondialdehyde (MDA) concentration of phenylhydrazine-induced anaemic rats.

Groups	MDA (µmol/L) x 10^{-9}
Normal Control	3.15±0.97
Anaemic Untreated	4.51±0.26
Anaemia + Standard drug (Emzoron)	2.44±0.58[h]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	2.50±0.95[h]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	2.57±0.65[h]

[h]Significant decrease with respect to anaemic untreated.

3.4.6 Effect of Extract on Lactate Dehydrogenase Activity

Induction of anaemia for four consecutive days in groups B to E caused a significant (p<0.05) decrease in LDH activity compared to the normal control which was not induced. However, the standard drug was most effective at lowering the activity of LDH to almost match those of the normal control. The activity of LDH in the negative control group showed a significant (p<0.05) increase in the activity of LDH when compared to the test groups (table 22).

Table 22: Effect of treatment with chloroform fraction of crude ethanol extract of *C. aconitifolius* on lactate dehydrogenase (LDH) activity of phenylhydrazine-induced anaemic rats.

Groups	LDH (U/L)
Normal Control	294.67±12.41
Anaemic Untreated	427.00±24.02[e]
Anaemia + Standard drug (Emzoron)	249.30±22.28[h]
Anaemia + 100 mg/kg chloroform fraction of *C. aconitifolius*	320.67±36.84[h]
Anaemia + 200 mg/kg chloroform fraction of *C. aconitifolius*	305.67±36.84[h]

[e]Significant increase with respect to normal control; [h]Significant decrease with respect to anaemic untreated.

IV. DISCUSSION

The oral median lethal dose (LD_{50}) of the chloroform fraction of the ethanol extract of *Cnidoscolus aconitifolius* was found to be above 5000mg/kg bw which designates the extract as non-toxic since no death was recorded at a high dose of 5000 mg/kg. The Organization for Economic Cooperation and Development (OECD), Paris, France has a recommended chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values. Values below 50mg/kg are toxic; and those below 5 mg/kg are termed very toxic. However, samples with an LD_{50} value between 50 and 500mg/kg are designated as harmful; while those whose value is above 500mg/kg are nontoxic. The doses selected and used in this study were lower than 30% of the LD_{50} which is labeled, 'safe', for ethnopharmacological research (Voungtao *et al,* 2004, Onochie *et al*., 2020).

This study revealed the antianaemic effect of the chloroform fraction of the ethanol extract of *C. aconitifolius* as evidenced in tables 4, 5, 6, 7, 11, 13 and 14. The extract showed significant alterations in the levels of the parameters assayed when compared to anaemic untreated. The extract significantly (p<0.05) increased the Haemoglobin (HGB) concentration; the Packed Cell Volume (PCV); the Red Blood Cell (RBC) count; and the Lymphocyte percentage of the induced test models, as much as the standard drug after 14 days of treatment. The increase in HBG, PCV, RBC and Lymphocyte levels indicate the antianaemic property of the chloroform fraction of the extract. These significant increases recorded could be attributed to an iron-rich ethanol extract, alongside other nutrients. This agrees with the findings of Upasana *et al* (2018) that the abundance of iron in the leaves of *Moringa oleifera* is responsible for the antianaemic property inherent within.

The chloroform fraction of the ethanol extract, in tandem with the standard drug significantly decreased the White Blood Cell (WBC) count; Blood Platelet volume (PLT); and the Neutrophile concentration after 14 days of treatment. This supports the claim that chloroform fraction has antianaemic properties since immune response is quite related to health status as evidenced by Olusoye *et al.,* (2021), who figured out that a dietary deficiency in iron is directly proportional to an increased susceptibility to infections (Olusoye *et al.,* 2021).

In the present study, chloroform fraction of the ethanol extract of *C. aconotifolius* did not significantly alter the blood glucose levels (BGL). The insignificant change in BGL indicates that the extract's fraction has little; or no antihyperglycaemic activity which can be attributed to the little presence of phytochemical constituents like; alkaloids, flavonoids, and saponins (Ezeigwe *et al.,* 2020). This is in agreement with earlier report by (Roy *et al.,* 2004), that the presence of secondary metabolites such as saponins, alkaloids and flavonoids in *Mangifera indica* are responsible for the antihyperglycaemic effect.

The ethanol extract also had a profound effect on the lipid profile of the test models, significantly (p<0.05) altering those with respect to the anaemic untreated. Levels of Total cholesterol (TCHOL), Triglyceride (TRIG), Low density lipoprotein-cholesterol (LDL-C) and very Low-density lipoprotein (VLDL) were found to be significantly (p<0.05) higher in the negative control group than it was in the test groups after 14 days of treatment with the extract. A decreased plasma LDL-Cholesterol concentration has been associated with a reduced risk for cardiovascular diseases like myocardial infarction, and arteriolosclerosis (Ugochukwu and Babady, 2002). This is in line with previous studies by Oluwatosin *et al* (2011) where the ethanol extract of *Cnidoscolius aconitifolius* helped restore the lipid levels of ethanol-treated rats to near normal, thereby reducing the incidence of a fatty liver associated with alcohol consumption. However, the High-density lipoprotein-cholesterol level was significantly (p<0.05) lowered after 14 days of treatment. The significant decrease in plasma LDL-cholesterol level after administration of extract may be due to alterations in the small intestine, which might increase conversion of VLDL to LDL (Workman, 1992, Ezeigwe *et al*., 2019).

The chloroform fraction of the ethanol extract significantly (p<0.05) decreased the Malondialdehyde and Lactate Dehydrogenase levels when compared to the untreated group. This goes to show that the fraction coupled with a potential anti-anaemic property, also remedies cellular damage (Aisha and Sarah, 2021).

V. CONCLUSION

The chloroform fraction of the ethanol extract of *Cnidoscolus aconitifolius* does indeed possess antianaemic properties as evidenced by this study. It has the capability of boosting haematological indices favourably without significantly upstaging the essential biochemical parameters in Wistar rats. This therefore gives more credence to its use as a blood tonic for the treatment of anaemia and paves a path for further pharmacological studies.

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