

Possible Inflammatory Responses in the Traditional Application of Raw Liquid Extract of *Cnidoscolus aconitifolius* Leaf (Iyana Ipaja-Chaya) In the Treatment of Anaemia

Mathew Folaranmi OLANIYAN*, Temitayo AFOLABI

Department of Medical Laboratory Science, Achievers University, Owo, Nigeria

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*Corresponding author

Mathew Folaranmi
OLANIYAN

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Abstract: Inflammatory response is a form of immune response which could be caused by infectious agents and toxic chemicals. *Cnidoscolus aconitifolius* leaf extract contains phytonutrients such as protein, vitamins, calcium, and iron; and is also a rich source of antioxidants which could be attributed to the traditional and scientific claims in the treatment of anaemia. This work was designed to determine possible pro and anti-inflammatory responses in the traditional application of raw liquid extract of *Cnidoscolus aconitifolius* leaf (iyana ipaja-chaya) in the treatment of anaemia. Twenty three (23) out of Thirty one (31) anaemic patients aged 12-32 years (including 2 females aged 12/15 years and 21 males aged 17 – 32 years) with PCV \leq 20% receiving treatment at 5 traditional homes in Saki-west Local Government of Oyo State-Nigeria. Age matched 50 apparently healthy volunteers (Female-25; Male-25) with a PCV of $42 \pm 3.0\%$ were recruited as control subjects. Thirty one anaemic patients initially volunteered themselves for the work but only 23 were successfully monitored. The patients were recruited before the commencement of the treatment. Each of the test and control subjects was subjected to stool microscopy for intestinal parasite, Giemsa thick staining procedure for plasmodium and Serological test for anti HIV, anti-HCV and HBsAg. All subjects who were negative to the aforementioned laboratory procedures were selected as subjects. Freshly prepared sixty milliliters (60ml) of the liquid was administered to each of the 23 anaemic patients and the 50 normal control volunteers 3 times on daily basis for 14 days when the PCV was found to have increased appreciably. Plasma ALT, IL-4, IL-6, TNF- α (before and after the administration of the extract), HIV-1 p24 Antigen ELISA, anti-HCV, HBsAg were determined in all subjects by ELISA technique while whole blood was used to determine PCV by microhaematocrit and the identification of Plasmodium infection using Geimsa thick staining techniques. The result obtained showed a significantly higher plasma value of TNF- α and ALT in Aneamic Patients After they were given Raw liquid Extract of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf (Supplement) compared with the result obtained in the Control (before extract supplementation) and also with the same aneamic patients when they were not given *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf (Supplement) with $p < 0.05$. There was also a significantly higher PCV in both the anemic patients and normal controls following the supplementation with raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja) leaf than when they were not supplemented with the raw liquid extract with $p < 0.05$. This work revealed increase in Packed Cell Volume, plasma ALT and TNF- α . Following the administration raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf which a possible indication of proinflammatory response and a potential natural supplement in the treatment of anaemia.

Keywords: *Cnidoscolus aconitifolius*, anaemia, Serological test.

INTRODUCTION

Pro and inflammatory cytokines interplay in immune responses as a result of inflammation especially of the liver. Cytokines are indices of inflammation which are classified as pro-inflammatory cytokines which include interleukin-1 (IL-1), tumor

necrosis factor (TNF), gamma-interferon (IFN-gamma), IL-12, IL-18, granulocyte-macrophage colony stimulating factor while major anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13 and transforming growth factor-beta are recognized as anti-inflammatory

cytokines. Cytokines are also a bridge between innate and acquired immunity [1].

Interleukin 6 (IL-6) can act as both a pro-inflammatory cytokine and an anti-inflammatory cytokine [2]. Pro-inflammatory cytokines (inflammatory cytokines) are excreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote inflammation. Pro-inflammatory cytokines like IL-1 β , IL-6, and TNF- α trigger pathological pain. Proinflammatory action of proinflammatory cytokines could make the disease or the symptoms of a disease worse by causing fever, inflammation, tissue destruction, and in some cases, even shock and death [3,4].

Anti-inflammatory cytokines act in anti-inflammatory or antiinflammatory process to reduce inflammation or swelling. Anti-inflammatory cytokines are immunoregulatory molecules that control the proinflammatory cytokine response [5]

Alanine and Aspartate transaminases are found in the liver. Their plasma level increase in inflammation of the liver or in liver damage [6,7].

Cnidoscolus aconitifolius (iyana ipaja) is a common vegetable cooked as sauce to supplement food like pounded yam, amala produced from yam powder and rice in Nigeria. It has some traditional and scientific claim in the treatment of anemia and leucopenia. Some traditional homes in Saki-West Local Government squeeze *Cnidoscolus aconitifolius* (iyana ipaja) leaf to extract the liquid content to treat anemia owing to its phytochemical constituents such as Pro-vitamin A, Ascorbic acid (Vitamin C), Riboflavin, Vitamin B₆, Vitamin B₁₂, Iron, zinc and copper [8].

Onuoha *et al.*, [8] also reported a significant increase in haemoglobin, packed cell volume, red blood cell and white blood cell in rats induced with anaemia and treated with raw and boiled *Cnidoscolus aconitifolius* leaf juices which reveals that the raw and boiled *Cnidoscolus aconitifolius* leaf juices possess haematinic property. This haematinic property of *Cnidoscolus aconitifolius* leaf was also demonstrated by Azeez *et al.*, [9] in their report on Ameliorative effects of *Cnidoscolus aconitifolius* on alloxan toxicity in Wistar rats where the toxicity of alloxan induced anemia and leucopenia as Azeez *et al.*, [9] reported that "Following treatment with alloxan, there was anaemia, thrombocytopenia and leucopenia, while the sperm count, motility and live/dead ratio were significantly reduced and that there were significant increases in the PCV, RBC, Hb, WBC, MCV and the platelet values. Erythrocyte osmotic fragility, sperm count, motility and live/dead ratio also improved significantly when these rats were treated with *Cnidoscolus aconitifolius* leaf

extract. *Cnidoscolus aconitifolius* leaf (Chaya) is a good source of protein, vitamins, calcium, and iron; and is also a rich source of antioxidants [10].

This work was therefore designed to determine the possible pro and anti-inflammatory responses in the traditional application of raw liquid extract of *Cnidoscolus aconitifolius* leaf (iyana ipaja-Chaya) in the treatment of anaemia in traditional homes at Saki-west Local Government Area of Oyo-State Nigeria.

MATERIALS AND METHOD

Study Area

Five traditional homes in Saki-west Local Government Area of Oyo State Nigeria. Saki West is a Local Government Area in Oyo State, Nigeria. Its headquarters are in the town of Saki. Shaki, Nigeria is located at the extreme end of Oyo state. It has a resettlement center of 2nd Mechanized division of Nigerian Army, The Oke-Ogun Polytechnic, and a Technical college. Shaki, Nigeria is also one of the largest cities in Oyo state. It has an area of 2,014 km² and a population of 278,002 at the 2006 census. The postal code of the area is 203 [11]

Study Population

Twenty three (23) out of Thirty one (31) anaemic patients aged 12-32 years (including 2 females aged 12/15 years and 21 males aged 17 – 32 years) with PCV \leq 20% receiving treatment at 5 traditional homes in Saki-west Local Government of Oyo State-Nigeria. Age matched 50 apparently healthy volunteers (Female-25; Male-25) with a PCV of 42 \pm 3.0% were recruited as control subjects

Thirty one anaemic patients initially volunteered themselves for the work but only 23 were successfully monitored. The patients were recruited before the commencement of the treatment.

Each of the test and control subjects was subjected to Urine and stool microscopy for intestinal parasite, Giemsa thick staining procedure for plasmodium and Serological test for anti HIV, anti-HCV and HBsAg. All subjects who were negative to the aforementioned laboratory procedures were selected as subjects.

Inclusion and Exclusion Criteria

1. Only anaemic subjects who were negative to Urine and stool microscopy for intestinal parasite, Giemsa thick staining procedure for plasmodium and Serological test for anti HIV, anti-HCV and HBsAg were recruited for the study.
2. Subjects with any episode of Jaundice were excluded.
3. Subjects on Hematinic or on any other blood chemicals were excluded from the study

4. Only individuals who volunteered and consented as test or control subject were included in the study.
5. Only anemic patients who volunteered to receive treatment from herbal homes and yet to commence any form of treatment were included in this study.

Ethical Consideration

The proposal for this study was reviewed and approved by Ethical and Research Committee of Baptist Medical Centre Saki-Nigeria.

Preparation of *Cnidioscolus aconitifolius* leaf (iyana ipaja-Chaya) Extract

The liquid extract was prepared by the traditional healer under the supervision of the researchers for the purpose of this study. The fresh *Cnidioscolus aconitifolius* leaves (iyana ipaja-Chaya) were plucked on daily basis and confirmed at the Department of Agricultural Technology, The Oke-Ogun Polytechnic Saki-Nigeria. The leaves were washed, drained and squeezed for the raw liquid extract.

Administration of *Cnidioscolus aconitifolius* leaf (iyana ipaja-Chaya) Extract

Sixty milliliters (60ml) of the liquid was administered to each of the 23 anaemic patients and the 50 normal control volunteers 3 times on daily basis for 14 days when the PCV was found to have increased appreciably.

Methods

HIV-1 p24 Antigen ELISA using Zeptrometrix retrotek kit

Principle: Microwells are coated with a monoclonal antibody specific for the p24 gag gene product of HIV-1. Viral antigen in the specimen is specifically captured onto the immobilized antibody during specimen incubation. The captured antigen is then reacted with a hightitered human anti-HIV-1 antibody conjugated with biotin. Following a subsequent incubation with Streptavidin-Peroxidase, color develops as the bound enzyme reacts with the substrate. Resultant optical density is proportional to the amount of HIV-1 p24 antigen present in the specimen.

Anti HCV ELISA Assay

This was assayed using Anti-Hepatitis C Virus Core Antigen antibody (ab50288) Abcam kit. The hepatitis C virus (HCV) core protein represents the first 191 amino acids of the viral precursor polyprotein and is cotranslationally inserted into the membrane of the endoplasmic reticulum. Hepatitis C virus (HCV) core is a viral structural protein; it also participates in some cellular processes, including transcriptional regulation. However the mechanisms of core-mediated transcriptional regulation remain poorly understood. Hepatitis C virus (HCV) core protein is thought to contribute to HCV pathogenesis through its interaction

with various signal transduction pathways. In addition, HCV core antigen is a recently developed marker of hepatitis C infection. The HCV core protein has been previously shown to circulate in the bloodstream of HCV-infected patients and inhibit host immunity through an interaction with gC1qR. Hepatitis C Virus is a positive, single stranded RNA virus in the Flaviviridae family. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids. The polyprotein is processed by host cell and viral proteases into three major structural proteins and several non structural proteins necessary for viral replication. Hepatitis C virus (HCV) causes most cases of non-A, non-B hepatitis and results in most HCV infected people developing chronic infections, liver cirrhosis and hepatocellular carcinoma. T cell responses, including interferon-gamma production are severely suppressed in chronic HCV patients.

HBsAg ELISA Test

This was assayed using Diagnostic automation/ Cortez Diagnostics, INC kit by ELISA method

HBsAg ELISA Kit Background Information

Hepatitis B infection is spread through infected blood or body secretions of infected individuals. Since Hepatitis B virus (HBV) is known as one of the major causes of blood transmitted hepatitis infections, blood screening using the HBsAg ELISA test is one of the most effective ways of preventing the spread of HBV. When using this kit, it is important to classify hepatitis B infection through three phases of the infection - incubation, acute, and convalescent. This can be done by identifying serological markers through each phase. Knowing the serological markers of HBsAg is an excellent method for the diagnosis and treatment of infected individuals. Symptoms of HBV infection can range from mild to severe, including chronic liver disease (cirrhosis and carcinoma). HBV is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family. The outer envelope surface antigen of the Hepatitis B virus is HBsAg. It contains the determinant "a" and is identified in two subgroups (ay and ad). HBV has four HBsAg subtypes (adw, ady, ayw, and ayr) and has 10 major serotypes. HBsAg can be identified two to four weeks before the ALT levels are abnormal, and three to five weeks before symptoms appear. HBsAg elisa assay is one of the best methods available for either screening blood donors or in the clinical diagnosis of hepatitis B-infected individuals.

HBsAg ELISA Assay Kit Principle

The HBsAg ELISA Test kit employs an antibody sandwich ELISA technique where monoclonal antibodies unique to HBsAg, are pre-coated on polystyrene microwell strips. The serum or plasma sample is added together with a second antibody, the

HRP Conjugate, (horseradish peroxidase) and directed against a different epitope of HBsAg. Throughout the time of incubation, specific immunocomplex that may have formed (indicating presence of HBsAg) is captured on the solid phase. After washing, to eliminate serum proteins and unbound HRP-conjugate, chromogen solutions containing tetramethyl-benzidine (TMB) and urea peroxide are added to the wells. Next, the colorless chromogens are hydrolyzed by the bound HRP-conjugate to a blue-colored product while in the presence of the antibody-antigen-antibody (HRP) sandwich immunocomplex. Halting the reaction with sulfuric acid, the blue color then turns yellow. The color intensity can be gauged proportionally to the amount of antigen captured in the wells, and to the amount in the sample, respectively. The wells remain colorless if the HBsAg result is negative.

Identification of plasmodium spp using thick blood films.

This was carried out as described by Chessbrough [12]

Urine and stool microscopy for parasite

Urine and stool microscopy for parasites was carried out as described by Chessbrough [12]

Serum Glutamic Pyroovate Transaminase (SGPT/ALT) using Randox kit

Principle: SGPT/ALT catalyses the formation of L glutamate and pyruvate by transferring L alanine to X-oxaloglutamate. The formed oxaloglutamate reacts with 2,4 dichlorophenyl hydrazine to form pyruvate hydrazone which develop to a brownish colour in alkaline medium. The intensity of the colour formed is directly proportional to the enzyme activity.

METHOD OF STATISTICAL ANALYSIS

The results obtained were subjected to statistical analysis using SPSS 18.0 to determine mean, standard deviation, and student “t” test and probability value to determine level of significance at 0.05.

RESULTS

The result obtained showed a significantly higher plasma value of TNF-α and ALT in Anemic Patients After they were given Raw liquid Extract of *Cnidoscopus aconitifolius* (iyana ipaja) leaf (Supplement) compared with the result obtained in the Control (before extract supplementation) and also with the same anemic patients when they were not given *Cnidoscopus aconitifolius* (iyana ipaja) leaf (Supplement) with p<0.05 (Table 1, 2 figure 1).

There was also a significantly higher PCV in both the anemic patients and normal controls following the supplementation with raw liquid extract of *Cnidoscopus aconitifolius* (iyana ipaja) leaf than when they were not supplemented with the raw liquid extract with p<0.05 (Table 1, 2 figure 2)

There was no significant difference in the plasma value of IL-4, IL6 and AST in the anaemic patient and control subjects before and after supplementation with p>0.05

The result also showed no significance difference in the plasma ALT value in Patients Before Extract Supplement compared with control subjects before and after supplementation with *Cnidoscopus aconitifolius* (iyana ipaja) leaf extract and also in control either before or after supplementation with the extract compared with anemic patients supplemented with the extract. with p>0.05 (table 1 and 2 figure 1).

Table 1: Mean and Standard Deviation of Values Obtained in Control and Subject Groups

Parameter	Control Before Extract Supplement	Control after Extract Supplement	Anemic Patients Before Extract Supplement	Anemic Patients After Extract Supplement
TNF-α pg/mL	1.6±0.1	1.7±0.1	1.7±0.2	2.5±0.1
IL-4 pg/mL	2.0 ±0.1	2.0 ±0.2	2.1±0.1	1.7±0.3
IL-6 pg/mL	1.2±0.2	1.3±0.1	1.5±0.3	1.4±0.2
ALT (IU/L)	9±1.0	10±2.0	8.0±0.5	14.0±1.0
AST (IU/L)	8±2.0	9±1.5	8.0±2.0	13.0±2.5
PCV (%)	42±3.0	44±3.0	19±1.0	29±1.0
HIV-1 p24 Antigen ELISA	Negative	Negative	Negative	Negative
ELISA Anti HCV ELISA	Negative	Negative	Negative	Negative
HBsAg ELISA	Negative	Negative	Negative	Negative
Urine Microscopy	No Parasite seen	No Parasite seen	No Parasite seen	No Parasite seen
Stool Microscopy	No Parasite seen	No Parasite seen	No Parasite seen	No Parasite seen
Giemsa Thick blood film test	No Parasite seen	No Parasite seen	No Parasite seen	No Parasite seen

Table 2: Comparative analysis of Values Obtained in Control and Subject Groups

Parameters		Control Before Extract Supplement Vs Control after Extract Supplement	Control Before Extract Supplement Vs Aneamic Patients Before Extract Supplement	Control Before Extract Supplement Vs Aneamic Patients After Extract Supplement	Control after Extract Supplement Vs Aneamic Patients Before Extract Supplement	Control after Extract Supplement Vs Aneamic Patients After Extract Supplement	Aneamic Patients Before Extract Supplement Vs Aneamic Patients After Extract Supplement
TNF- α pg/mL	"t"	-0.71	-0.44	-3.36	0.00	-5.66	-3.58
	"p"	0.28	0.35	0.01*	0.50	0.02*	0.04*
IL-4 pg/mL	"t"	0.00	-0.70	0.95	-0.45	0.83	1.26
	"p"	0.50	0.28	0.22	0.35	0.26	0.17
IL-6 pg/mL	"t"	-0.2	-0.83	-0.71	-0.75	-0.6	0.28
	"p"	0.43	0.25	-3.53	0.26	-1.79	0.40
ALT (IU/L)	"t"	-0.44	0.89	0.04*	0.97	0.11	-5.37
	"p"	0.35	0.23	0.08	0.22	0.15	0.02*
AST (IU/L)	"t"	-0.4	0.00	-1.94	0.4	-1.8	-1.95
	"p"	0.36	0.5	0.10	0.36	0.11	0.10
PCV (%)	"t"	-0.47	7.27	4.11	7.91	4.74	-7.07
	"p"	0.34	0.009**	0.03*	0.008**	0.02*	0.01*

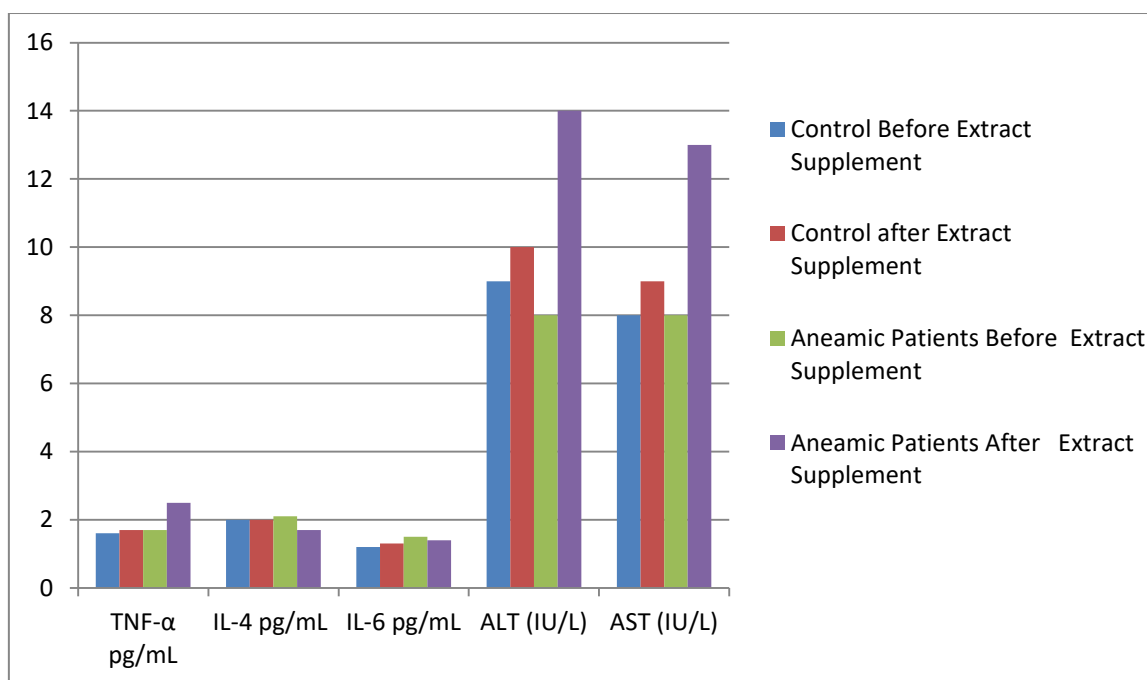


Fig-1: Descriptive Analysis of Plasma TNF- α , IL-4, IL-6, ALT, AST Obtained in Subjects and Control Groups

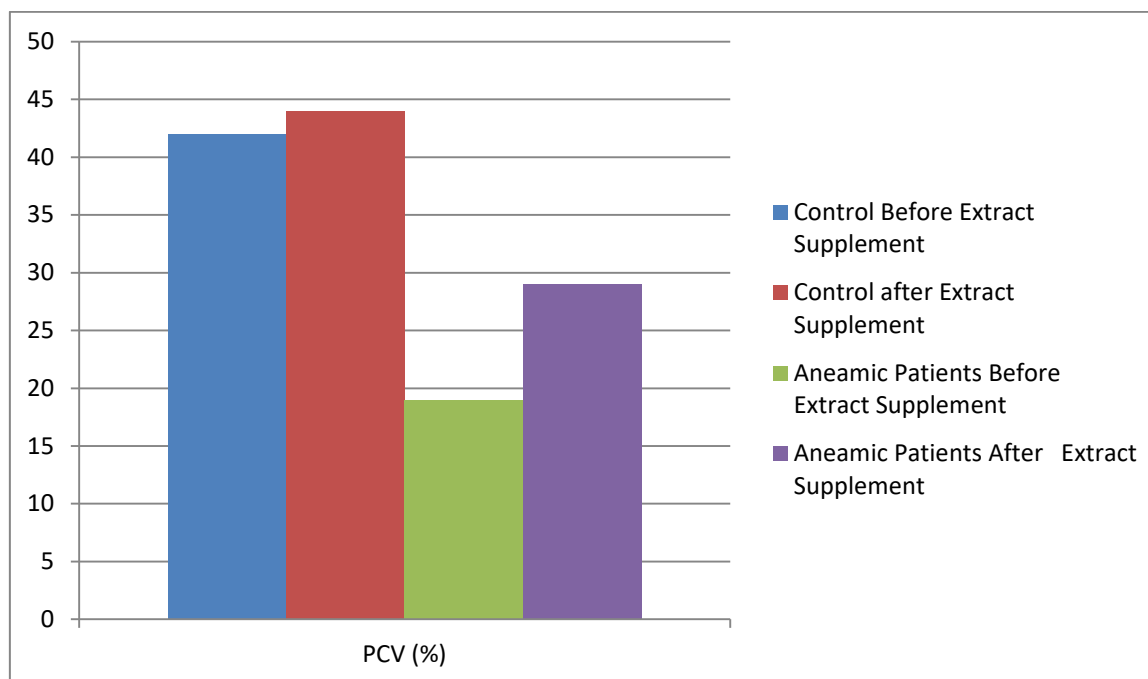


Fig-2: Descriptive Analysis of PCV Obtained in Subjects and Control Groups

DISCUSSION

This work has been used to determine pro and anti-inflammatory responses in the traditional application of raw liquid extract of *Cnidoscolus aconitifolius* leaf (iyana ipaja-chaya) in the treatment of anaemia.

The result obtained showed a significantly higher plasma value of TNF- α and ALT in Aneamic Patients After they were given Raw liquid Extract of *Cnidoscolus aconitifolius* (iyana ipaja/ chaya) leaf (Supplement) compared with the result obtained in the Control (before extract supplementation) and also with the same aneamic patients when they were not given *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf (Supplement). The significant increase in the plasma value of TNF- α and ALT in the anaemic patients as a result of the administration of Raw liquid Extract of *Cnidoscolus aconitifolius* (iyana ipaja) leaf could be attributed with inflammatory response which was manifested by plasma increase of the two biochemical parameters. Alanine aminotransferase (ALT) or serum glutamic pyruvic transaminase (SGPT) is found mainly in the liver, but also in smaller amounts in the kidneys camera.gif, heart, muscles, and pancreas. ALT catalyzes the transfer of an amino group from L-alanine to α -ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate. Increase in plasma level of ALT could indicate liver disease, especially cirrhosis and hepatitis- inflammation caused by alcohol, drugs, or viruses[6,7].

Increase in plasma ALT in this work could be as a result of inflammation due to the administration of

raw liquid extract of *Cnidoscolus aconitifolius* as indicated by a plasma increase in a proinflammatory cytokine - TNF- α . Pro inflammatory cytokines (inflammatory cytokines) are excreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote inflammation. protein [3,4,13-15]. Upon inflammation like hepatitis caused by chemical agents the local inflammatory cells which include neutrophil granulocytes and macrophages secrete cytokines into the bloodstream in most cases interleukins IL1, IL6 and IL8, and TNF α . The liver will in turn respond by producing a large number of acute-phase reactants such as increase in C-reactive protein [3,4,13-15].

Increase in plasma ALT and TNF- α . following the administration raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf (Supplement) may be due to the fact that raw *Cnidoscolus aconitifolius* leaves are toxic because they contain a glucoside capable of releasing toxic cyanide which is similar to cassava which could be inactivated by cooking [16]. This finding could be as a result of the extract toxicity causing inflammation of the liver as the organ of metabolism including detoxification.

There was also a significantly higher PCV in both the anemic patients and normal controls following the supplementation with raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja) leaf than when they were not supplemented with the raw liquid extract. This could be linked with the phytonutrient constituents of *Cnidoscolus aconitifolius* (iyana ipaja) leaf as it is a good source of protein, vitamins, calcium, and iron; and

is also a rich source of antioxidants [10,17]. Haematinic property of the leaf extract has also been reported by Azeez *et al.*, [9] and Onuoha *et al.*, [8] who reported increase in PCV and haemoglobin concentration following the administration of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf extract which is also consistent with the findings of this study.

CONCLUSION

This work revealed increase in Packed Cell Volume, plasma ALT and TNF- α . following the administration raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf which a possible indication of proinflammatory response and a potential natural supplement in the treatment of anaemia.

RECOMENDATION

Plasma cytokines and liver enzymes should be monitored in the administration of raw liquid extract of *Cnidoscolus aconitifolius* (iyana ipaja/chaya) leaf to serve as supplement in the treatment of anaemia.

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