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Original Research Article

Immunomodulatory potential and toxicological assessment of methanol extract and fractions of *Cryptolepis sanguinolenta* root

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Abstract

Purpose: *To evaluate the paramunity induction potentials of crude methanol extract (CME) and methanol fractions (MF) of Cryptolepis sanguinolenta (CS) root (Fam. Periplocaceae).*
Method: *A total of forty (40) albino rats were divided into two sets of four groups, with five animals per group. Groups 1 – 3 of both animal sets were orally administered 200, 300 and 500 mg/kg bw of CME and MF, respectively once daily for 14 days. Furthermore, Group 4 of both sets served as negative control. The effect of extracts on early-type hypersensitivity (ETH), delayed-type hypersensitivity (DTH), leukocyte count and haemagglutinin titer using sheep red blood cell (SRBC) as antigen were evaluated.*
Results: *Significant ($p < 0.05$) body weight gains were recorded in the animal groups treated with the ME while no changes were observed in the relative organ weight of all the animals in all the groups. Acute toxicity studies yielded no adverse effect. The CME and MF elicited significant ($p < 0.05$) increases and decreases in ETH and DTH, respectively, at all doses. They showed a significant ($p < 0.05$) dose-dependent, stimulatory effect on primary and secondary antibody titer across the treated groups. Packed cell volume and red blood cell count increased significantly ($p < 0.05$), while a dose-dependent increase occurred in neutrophil counts in rats receiving CME, but the MF-treated rats had significantly decreased ($p < 0.05$) neutrophil counts at 300 mg/kg compared to control. Both CME and MF did not affect lymphocyte counts.*
Conclusion: *Cryptolepis sanguinolenta extract and MF produce significant stimulatory effects on humoral immunity (antibody) with non-significant impact on cellular and innate immunity. Future studies will require isolation and characterization of the bioactive compounds in the plant extracts.*

Keywords: *Cryptolepis sanguinolenta, Hypersensitivity, Haemagglutinin titer, Immune response, Toxicity*

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INTRODUCTION

The immune system comprises special system made up of cells, including special proteins, tissues and organs that contribute to protection against diseases. Medicinal plants harbour bioactive substances with the ability to stimulate granulocytes, macrophages and natural killer cells, and complement functions to effect

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immune protection [1]. Owing to the numerous side effects of conventional drugs, the use of natural products as alternatives has been on the increase in the last few decades. Medicinal plants and their byproducts often serve as remedial alternatives and in some cases, the only effective treatment option [2]. Several recent studies have shown that many plants and their isolated constituents possess bioactive properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial and immunomodulatory properties [3-8].

The *Cryptolepis sanguinolenta* (Lindl.) Schlt plant, belonging to the family *Periplocaceae* and locally known by the Igbo people of Nigeria as "Akpa-oku", is made up of a thin twining stem and scrambling shrub that grows in the wild but can also be intently grown. Aboriginal to Africa, it is also found as a climbing liana in Central, East and West Africa. The plants' bitter roots are widely employed for different purposes in traditional medicine. The raw root is often chewed, while the extract is locally administered in therapies against fever, hepatitis, malaria, hypertension, urinary and upper respiratory tract infections, colic, amoebic dysentery and diarrhoea, wound, measles, hernia, snakebite, rheumatism, insomnia, as well as a tonic [9].

In Burkina Faso, the plant leaves are used as vegetables [10]. Some antimalarial medicines have been developed from this plant such as Phytolaria in Ghana and Malarial in Mali. The plant raw powder and the freeze-dried aqueous extract have also been formulated into tablets and suppositories [11]. The *C. sanguinolenta* roots and leaves are rich in cryptolepine, a bioactive alkaloid possessing anti-plasmodial, anti-cancer, anti-fungal, anti-bacterial, anti-pyretic, anti-inflammatory and anti-hyperglycemic activities [12,13]. It has also been recommended for effective treatment of *Candida albicans*, *Escherichia coli, Klebsiella pneumonia*, and *Bacillus subtilis*-implicated infections [14]. This study is aimed at investigating the immunomodulatory potentials of the methanol root extract and fraction of *Cryptolepis sanguinolenta*.

EXPERIMENTAL

Sample collection and processing

Root samples of *C. sanguinolenta* were collected from Orba in Udenu Local Government Area of Enugu State during the rainy season (June – July) of 2022. Identification and authentication of the plant sample (Voucher no: InterCEED 042) was ascertained by Mr Alfred Ozioko, a certified Taxonomist with the International Centre for Ethnomedicine and Drug Development (InterCEED), Nsukka, Enugu State, Nigeria. The harvested root samples were air-dried for one month, after which they were milled, powdered, transferred into clean containers and subjected to a cold maceration extraction process. Briefly, methanol (500 mL) was added into the container containing the 300 g of the powdered plant sample with continuous stirring and left for 24 h after which it was filtered with gauze and a funnel into a vessel. The filtrate was transferred into an evaporating dish and left to dry at 35 ºC, and the dried extract collected into a clean container, which was placed in the refrigerator (4 ºC). Thereafter, fractionation was done using a column containing silica gel. The tip of the column was first plugged with a small layer of cotton wool and silica gel was packed in the column using a funnel. Methanol was introduced into the column containing methanol extract (CME) and silica gel until the eluting methanol fraction (MF) became clear.

Animals grouping and dosing

Healthy albino rats (138 - 218 g) of both sexes were obtained from the Department of Veterinary Medicine, University of Nigeria, Nsukka. They were acclimatized at the Department's Animal House and fed with vital feed growers with free access to water. Two (2) sets of four (4) animal groups (Group 1 – 4) were set up for the experiment. One set (1 – 4) served CME, while the other served MF. Each animal group had five (5) animals. For CMF, the first Group 1 were orally administered 200 mg/kg of CME once daily, while Groups 2 and 3 received 300 and 500 mg/kg of CME, respectively for a total period of two weeks. Group 4 served as negative control and received normal saline (2 mL). For MF, Group 1 were orally administered 200 mg/kg of MF once daily, while Groups 2 and 3 received 300 and 500 mg/kg of MF, respectively, for a total period of two weeks. In addition, Group 4 served as negative control and received normal saline (2 mL).

The animals were adequately handled according to international guidelines for animal studies, and received ethical clearance (approval no. FRSRE/UNN/22/0022) from the Faculty Research Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. After 14 days of daily extract and fraction administration, blood samples were collected for analysis and the animals were sacrificed by cervical dislocation after chloroform anesthesia.

Antigen

The sheep red blood cells (SRBCs) were collected from an abattoir at an urban market (Ogige Market, Nsukka) in Enugu State, Nigeria. The cells were washed three times in a large volume of phosphate buffer saline (PBS) and subsequently used for immunization and challenge.

Acute toxicity (LD_{50}) test on extract

The acute toxicity profiles of methanol extract (CME) and methanol fraction (MF) were estimated in mice using the (LD_{50}). Healthy albino mice (18 - 25 g) of both sexes were also obtained from the Department of Veterinary Medicine, University of Nigeria, and acclimatized as previously described. Two sets of the procedure were carried out using the oral and intraperitoneal routes. Briefly, the test involved two phases. The first phase was the determination of the toxic range. The mice were placed in three groups of 3 mice each and were given 10, 100 and 1000 mg/kg of the extract and fraction solubilized in 25 % (v/v) propylene glycol in water, respectively. The treated mice were observed for 24 h for the number of deaths. The mortality patterns in the first phase determined the doses used for the second phase. Since no death was recorded in the first phase, four mice received 1000, 1600, 2900 and 5000 mg/kg of the extract and fraction at one dose per mouse for each route of administration. The animals were observed for 24 h for lethality or signs of acute intoxication. The LD_{50} was the geometric mean of the highest non-lethal and least toxic dose [15].

Preliminary screening of methanol extract and fractions of *C. sanguinolenta* for immunomodulatory activity

The CME and MF were screened for immunomodulatory activities in rats using different models and involving the effects on some specific and non-specific immune response mechanisms, hypersensitivity reaction, delayed type of hypersensitivity reaction, primary and secondary antibody synthesis, and total leukocytes and differentials as described below.

Early and delayed-type hypersensitivity

Hypersensitivity reaction to SRBC was induced in rats as follows: The CME and MF of *C. sanguinolenta* (in doses of 200, 300, and 500 mg/kg body weight) were orally administered to the respective groups of each plant material daily for three days, respectively, while the vehicle (normal saline) was administered to the negative control. On the third day, 0.2 mL of SRBC was injected subcutaneously into the right hind paws of all the rats. They were challenged on the seventh day by injecting the same amount of SRBC in the left hind paw. The edema produced by the antigenic challenge was calculated as the difference in paw thickness 4 and 20 h after the challenge [16]. The paw thickness was determined with a micrometer screw gauge.

Humoral antibody response

The CME and MF of *C. sanguinolenta* (in doses of 200, 300, and 500 mg/kg of body weight) were administered orally to the respective groups of animals for five days. On the 5^{th} day, the rats were immunized by injecting 0.2 mL of SRBC intraperitoneally. The animals were again fed for seven days and blood samples were collected through the media cantus of the eye to determine the primary titer. Thereafter, 0.2 mL of SRBC was injected intraperitoneally into the animals again and they were fed for another seven days. Finally, blood samples were collected to determine secondary titer. A haemagglutinin titer assay was performed using a microtiter plate. Blood was collected from the media cantus of the animals for serum preparation. The serum was diluted in 50 µL PBS (pH 7.2) with two-fold serial dilutions in 96 well microtiter plates and mixed with 50 µL of SRBC. The plates were kept at room temperature for 2 h. The value of antibody titer was the highest serum dilution that resulted in visible hemagglutination [17].

Body weight and relative organ weight determination

The body weight before and after extract administration were recorded and the relative organ (lungs, liver, kidney, heart and spleen) weight (W) for each animal was calculated using the equation (1) below:

$$W = (A/Bw)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where A: organ weight; Bw: Body weight of rat on sacrifice day

Blood parameters

Blood samples were collected into plain tubes without anticoagulants. The samples were centrifuged for 10 min and the supernatant (serum) was pipetted into Eppendorf tubes. Serum was used to determine the blood parameters including packed cell volume (PCV), red blood cell (RBC), white blood cell (WBC),

and differential leukocyte counts (DLC) using standard protocols.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was carried out using the T-test, one-way variance analysis (ANOVA) and Dunnett's multiple comparison test. Values of *p* less than 0.05 were considered statistically significant.

RESULTS

Effects of CME and MF of *C. sanguinolenta* on early and delayed-type hypersensitivity response

Figure 1 and Figure 2 show the effect of the CME and MF of *C. sanguinolenta* roots on the hypersensitivity response in rats. The results showed that at the beginning of the experiment, non-significant ($p > 0.05$) variations were observed in the hypersensitivity values of rats in all the test groups administered varying doses of the extract and fraction compared to that of the control group. But at 4 h, a significantly ($p < 0.05$) higher rate of hypersensitivity (ETH) was observed in all the animals in the test group compared to the negative control. Delayed-type hypersensitivity response, however, differed with the rats in all the treatment groups producing a significant ($p < 0.05$) inhibition of DTH compared to the negative control. The effect of both the crude extract and fraction on hypersensitivity response was, however, the same.

Effect of methanol extract and the fraction of *Cryptolepis sanguinolenta* roots on primary and secondary titers

The results of the effects of CME and MF on primary and secondary titer are represented in Figure 3 and Figure 4, respectively. Animals in Group 1 produced similar results for both CME and MF, however, there was a significantly ($p < 0.05$) higher dose-dependent elevation of the primary antibody titer by the CME at 300 and 500 mg/kg bw compared to the MF. The effect of *Cryptolepis sanguinolenta* in all the tested groups was found to be significantly ($p < 0.05$) more than the negative control. Similarly, an elevated secondary titer was produced by both CME and MF, especially at 300 and 500 mg/kg doses.

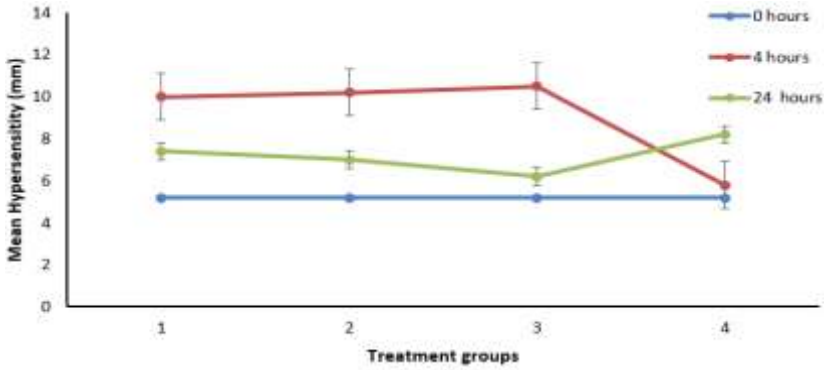


Figure 1: Effect of methanol crude extract of *Cryptolepis sanguinolenta* roots on hypersensitivity response in rats. Values are mean ± SD

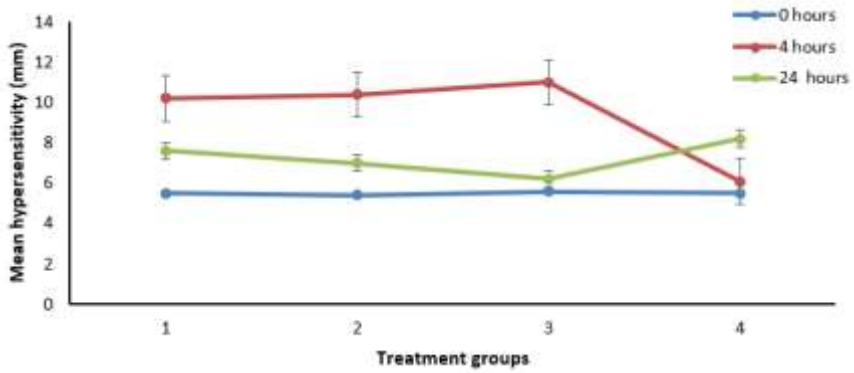


Figure 2: Effect of methanol fraction of *Cryptolepis sanguinolenta* roots on the hypersensitivity of rats. Values are mean ± SD

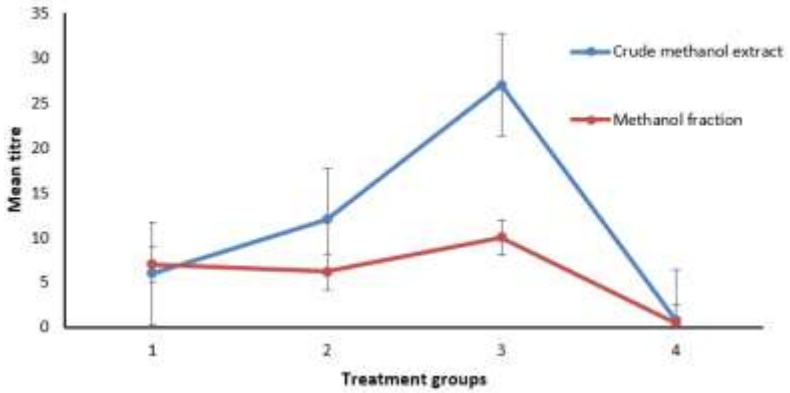


Figure 3: Effect of crude methanol extract and methanol fraction of *Cryptolepis sanguinolenta* roots on primary antibody titers in rats. Values are mean ± SD

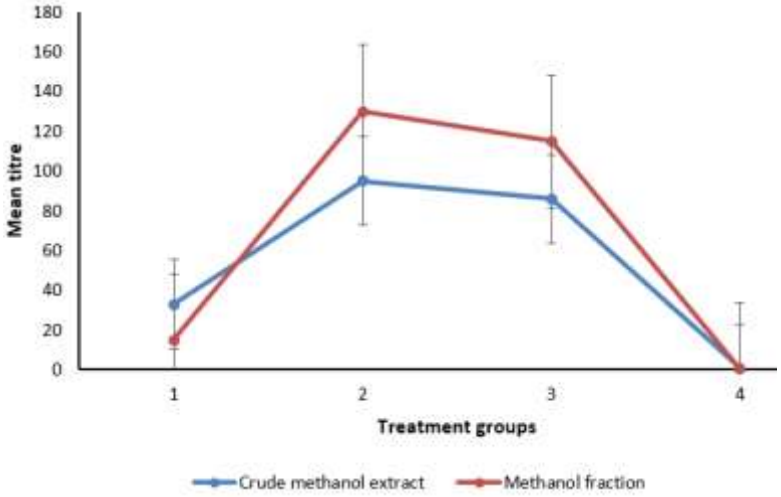


Figure 4: Effect of crude methanol extract and methanol fraction of *Cryptolepis sanguinolenta* roots on secondary antibody titers in Rats. Values are mean ± SD

Effect of crude methanol extract and fraction of *Cryptolepis sanguinolenta* roots on blood parameters

The CME and MF produced effect on the PCV, RBC and Hb count as the negative control (Figures 5 A – C). This was in contrast to the effect on the WBC count, as, there was a significant ($p < 0.05$) decrease in the WBC counts at doses of 200 and 300 mg/kg for the CME and MF, respectively (Figure 5 D).

Also, the effects of the CME and MF of the plant on differential leucocyte counts showed that at 200 mg/kg (CME and MF), 300 mg/kg (CME) and 500 mg/kg (MF), no significant difference ($p > 0.05$) in the neutrophil count was produced compared to the negative control. However, at 300 and 500 mg/kg doses of the fraction and extract, a significant ($p < 0.05$) decrease and increase in the neutrophil count, respectively, was observed, compared to the negative control. The extract and the fraction did not affect the lymphocyte counts (Figures 5 E and F).

Effect of methanol extract and fraction of *Cryptolepis sanguinolenta* roots on body weight and internal organs of rats

Figure 6 A - H show that both the CME and MF did not produce any significant differences in the sign of toxicity as shown by the body weight and weights of the internal organs measured. There were no differences in the weights of the organs pre- and post-administration of plant extract and fraction.

DISCUSSION

Though *C. sanguinolenta* is primarily described as an antimalarial herb in traditional medicine and implicated in the treatment of hypertension, here, it was shown to possess stimulatory effects on humoral immunity with an inhibitory effect on cellular immunity. The inhibition of delayed-type sensitivity response could be very beneficial in organ transplants as most transplants usually go with immune suppressants such as corticosteroids. T cells and monocytes/

macrophages are implicated in delayed-type hypersensitivity reactions. The body employs this mechanism during encounter with intracellular pathogens, fungi and certain parasites and also occurs in transplant rejection and tumour immunity [18]. The ability of the study plant samples to inhibit delayed-type hypersensitivity (DTH) shows that the plant extract and fraction can effectively be utilized during organ transplantation to reduce rejection.

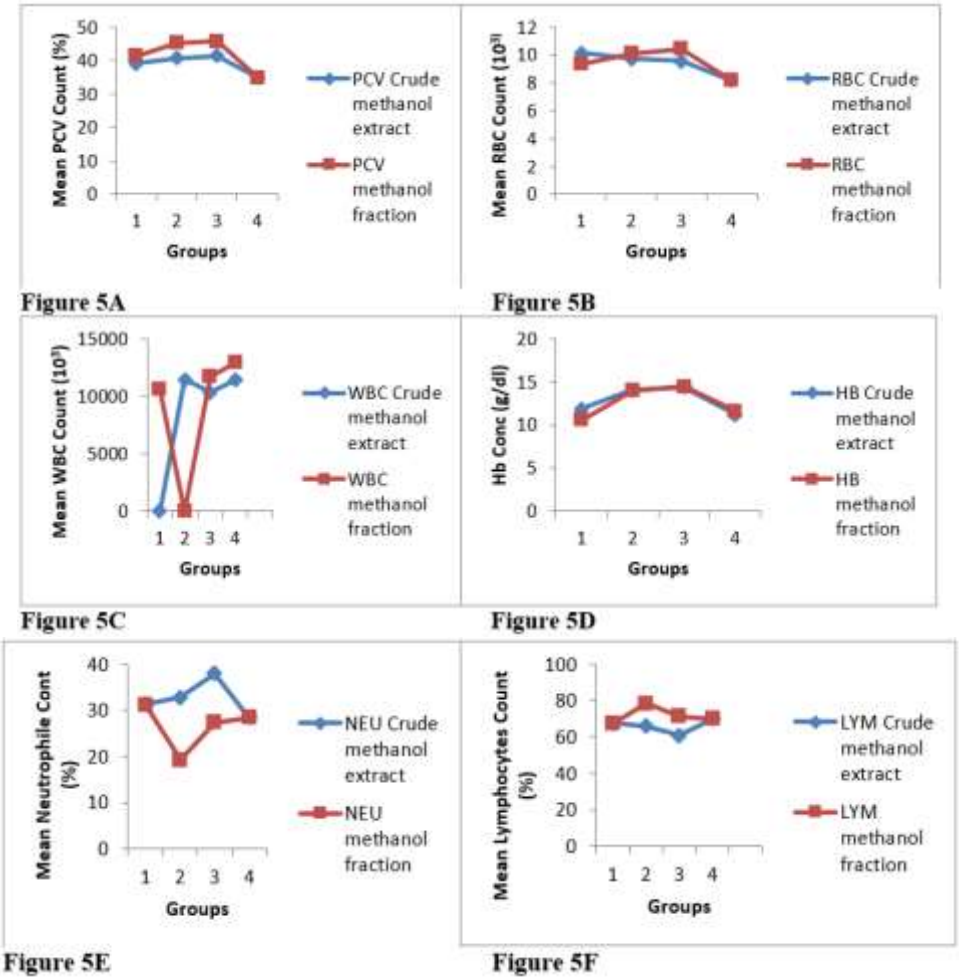


Figure 5: Effect of Crude Methanol Extract and Fraction of *Cryptolepis sanguinolenta* roots on blood parameters in rats. The PCV count (A), RBC count (B), WBC count (C) Hb count (D), Neutrophil (E) and Lymphocyte count (F) are represented above

Antibody molecules secreted by plasma cells mediate humoral immune response. The antibodies exert their immune response through neutralization, opsonization, and stimulation of complement proteins. The plant extract showed an increase in hemagglutination titer at doses 300 and 500 mg/kg bw of both crude and methanol fractions in animal studies. This augmentation of humoral response to SRBC indicated an enhanced responsiveness of the macrophage and T and B-lymphocyte subsets involved in antibody synthesis [19]. The extract and fraction's ability to effectively stimulate antibody production shows that they could be effective agents in antibody-mediated immunity.

Cryptolepis sanguinolenta extract and fraction produced no effects on the packed cell volume (PCV), red blood cell (RBC), hemoglobin (HB), neutrophil and differential leukocyte count (DLC). This shows that the plant does not interfere with the animal's blood parameters, an attestation of its safety. There was also no effect on body weight as observed and the extract did not alter the relative weight of all the organs tested, thus, showing that the extract has no negative effects on the vital organs tested. The plant extract contained polyuronides such as tannins that possess antioxidant properties and consequently protect the renal tissues [20]. The beneficial effects of tannins against nephrotoxicity are also well-known [21]. The result of the study showed that *C. sanguinolenta* CME and MF possess immunomodulatory potential and a high safety index.

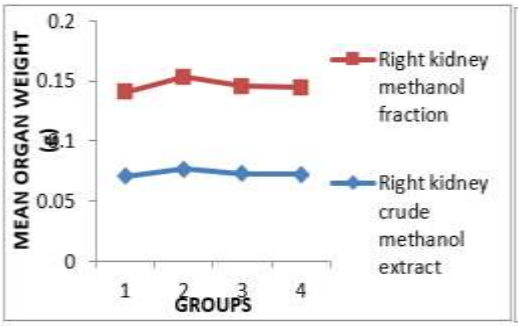
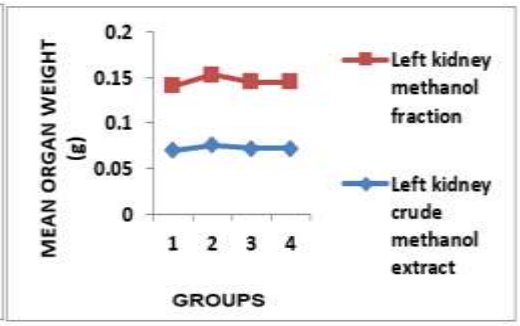


Figure 6E



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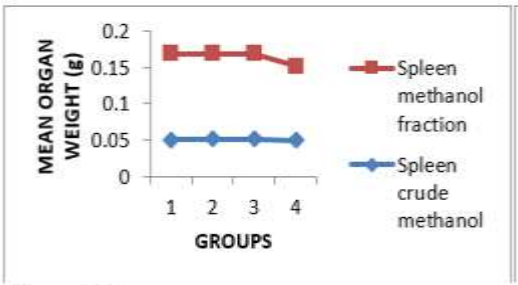

Figure 6G

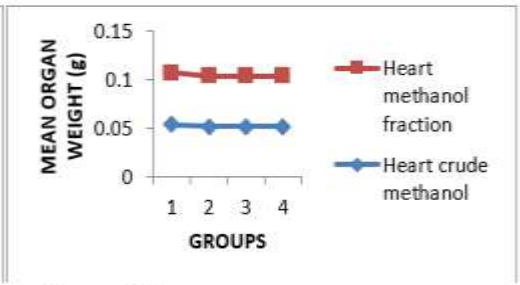

Figure 6H

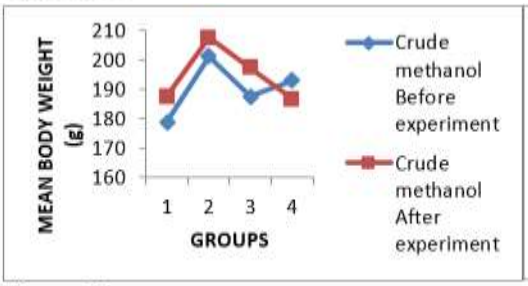

Figure 6A

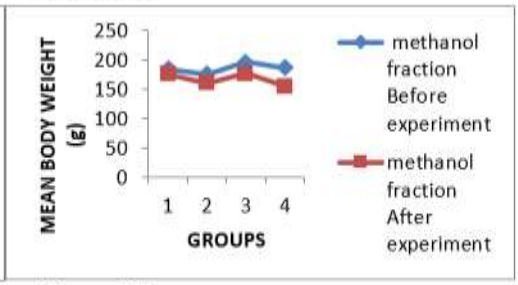

Figure 6B

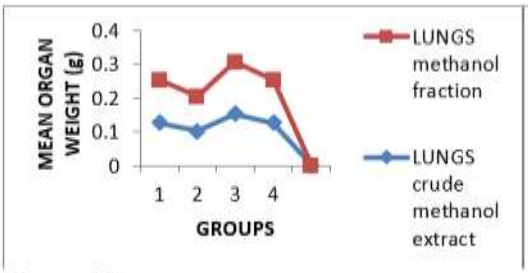

Figure 6C

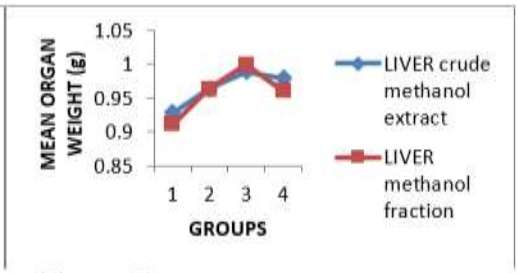

Figure 6D

Figure 6A – H: Effect of crude methanol extract and the fraction of *Cryptolepis sanguinolenta* roots on body weight and internal organs of rats. Organ shown are Values are mean ± SD

CONCLUSION

Cryptolepis sanguinolenta root methanol extract and fractions possess stimulatory effect on humoral immunity/antibody production. The study gives an insight into the immunomodulatory potentials of the methanol extract and fraction of *C. sanguinolenta*. However, this leaves room for further studies including the isolation and characterization of the bioactive compounds of the plant.

DECLARATIONS

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Ethical approval

The animals were adequately handled according to international guidelines for animal studies and received ethical clearance (approval no. FRSRE/UNN/22/0022) from the Faculty Research Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

All claims related to the material in this paper will be borne by the authors, and we certify that this work in question was performed by the author(s) of this article. Conceptualization: Chinelo C Eze, Martina C Agbo; Data curation: Chinelo C Eze, Martina C Agbo, Stephen C Emencheta; Formal analysis: Chinelo C Eze, Martina C Agbo, Stephen C Emencheta; Funding: Chinelo C Eze, Martina C Agbo, Stephen C Emencheta, Osita C Eze, Somtochukwu A Evurani; Investigation: Chinelo C Eze, Martina C Agbo, Stephen C Emencheta, Osita C Eze, Somtochukwu A Evurani; Methodology: Chinelo C Eze; Project administration: Chinelo C Eze; Validation: Chinelo C Eze; Writing – original draft: Chinelo C Eze, Stephen C Emencheta; Writing – review & editing: Chinelo C Eze, Martina C Agbo, Stephen C Emencheta, Osita C Eze, Somtochukwu A Evurani.

Use of Artificial Intelligence

Not applicable.

Use of research reporting tool

Not applicable.

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