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Original Research Article

Evaluation of the Immunomodulatory Activity of *Gnetum africanum* (Gnatacae) Leaf Extracts

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ABSTRACT

Gnetum africanum (Gnataceae) is used by different communities to treat various ailments including modulation of the immune system without any scientific proof. The immunomodulatory activity of ethanol leaf extracts of *G. africanum* was evaluated in Wistar rats and mice. Fourteen experimental groups of five rats each were used. The immune systems of animals in Groups II to XIV were suppressed with 70 mg/kg Cyclophosphamide at the beginning of the study while that of the animals in group I was not suppressed. Group II received 2.5 mg/kg of levamisole. Group II1 received 10 mL /kg distilled water orally. Groups IV to XIV received 100 and 200 mg/kg of the extracts daily. Delayed-type hypersensitivity reaction (DTH), hemagglutination antibody titer (HT), Immunoasssay for IL-2, IL-4, and IFN-γ and percentage leukopoiesis were determined using standard methods and procedures. Treatment with *G. africanum* extracts produced a significant ($p<0.05$) dose- dependent inhibition of the DTH response. The mean HT to sheep red blood cell showed a dose-dependent increase ($p<0.05$) with 200mg/kg ethyl-acetate fraction having the highest percentage boost of 70%. The extracts and levamisole showed a dose-dependent significant increase in interleukin-2($p<0.05$) but not in interleukin-4 and interferon-γ. Cyclophosphamide administration caused 80% reduction in leucopoiesis, but this was restored to100% by the extracts and levamisole. The extracts also showed significant ($P<0.05$) increase in the WBC count when compared to the negative control animals. The extracts of *G. africanum* caused significant immunostimulatory effects on both cell-mediated and humoral immune systems of Wistar rats and mice.

***Keywords*:** Immune system, Immunomodulation, *Gnetum africanum*, cytokines, Levamisole, Cyclophosphamide.

Introduction

The immune system is at the epicentre known to be involved in the aetiology as well as the pathophysiologic mechanism of many diseases.[1] The frequency of life-threatening infections has increased dramatically among cancer patients, transplant recipients, AIDS patients, and those receiving broad-spectrum antibiotics, corticosteroids, and cytotoxic drugs.[2] Immune function disorder is responsible for these and other diseases. Agents that alter the immune system either by stimulating or suppressing it are of great significance in managing immunological disorders and are known as immunomodulators. Plant materials have been a major source of natural therapeutic remedies and are used to treat various infectious diseases in many developing countries.[3]

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According to the World Health Organization, 80% of the world population uses medicinal plants in the treatment of diseases and in African countries, this figure is much higher.[4] Equally, medicinal plants are rich sources of substances that are claimed to induce para-immunity[5] and relieve oxidative stress.[6] Immunomodulators of plant origin are preferred because they are mostly presumed to be less toxic and therefore safer.[7] Phytochemical constituents such as terpenoids, steroids, proteins, tannins, alkaloids and flavonoids are considered responsible for immunomodulatory properties exhibited by plants, which remain alternative sources for the development of new drugs.[8,9] *G.africanum* popularly known as 'afang'(Efik), 'okazi'(Igbo) is one of the most popular leafy vegetables in Nigeria and other African countries such as Cameroon, Gabon, Congo, and Angola.[10]*G.africanum*, a lone genus belonging to the family Gnataceae is a dioecious wild understory liana that grows on trees in the humid forest of Africa.[10] Its leaves are eaten as a vegetable, either raw or finely chopped and cooked. They are also widely used as an ingredient in soups and stews and are much in demand for their nutritional and therapeutic properties.[11] The leaves of *G.africanum* are traditionally used in the treatment of enlarged spleen, sore throat, and as cathartic in Nigeria.[12] Pharmacological studies on the plant have shown that it has anti-inflammatory[13], antimicrobial and antifungal,[14,15,16] antioxidant,[17,18] haematological[19] and anti-sickling properties.[20] Many therapeutic effects of plant extracts have been suggested to be due to their wide array of immunomodulatory effects

and influence on the immune system of the human body.[21] This study therefore aimed at evaluating the immunomodulatory effect of the ethanol leaf extracts,n-hexane fraction, aqueous fraction, ethylacetate fractions and butanol fractionsof *G.africanum* in rats.

Materials and Methods

Plant material

The leaves of *G.africanum*were obtained from a local market in AforNnobi, Idemili South Local Government Area, Anambra State, Nigeria in March 2020. They were identified by Mrs Onwunyili Amaka of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University, Agulu Campus and a voucher specimen PCG/474/A/066 was deposited in Pharmacognosy herbarium.

Solvents and reagents

Drugs used were levamisole (Medrel Pharm India), Cyclophosphamide (Zuviuslifesciences Pvt Ltd, India). Other chemicals include ethanol (80%) (Guangdong Sci.-tech Co.Ltd, Shantou China),ethyl acetate(Guangdong Sci.-tech Co.Ltd, Shantou China), n- hexane (Guangdong Sci.-tech Co.Ltd, Shantou China), butanol(Guangdong Sci.-tech Co.Ltd, Shantou China).

Experimental animals

Equal number of male and female adult Wistar rats and Swiss albino miceof both sexes weighing between (180-200 g) and (20-30g) were procured from the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were nulliparous, and male animals were separated from female animals in their various cages to avoid matting and pregnancy.The animals were fed with normal feeds (Guinea feed Nigeria Ltd) and had unrestricted access to clean drinking water. A healthy Male Sheep (for Sheep Red blood cells) was taken from the herd in the Experimental animal facility of the Faculty of Veterinary Medicine, University of Nigeria Nsukka.The sheep had a normal pasture in the Faculty of Pharmaceutical Sciences animal house environment atatNnamdiAzikiwe university, Agulu campus.Ethical approval wasobtained from the ethical committee of Chukwuemeka University Teaching Hospital, Awka with approval no, COOUTH/CMAC/ETH.C/VOL.1/FN:04/0064 before commencement of the study. The guide for the care and use of laboratory animal procedures was followed in the study.[22]

Preparation and extraction of plant materials

Fresh leaves of *G. africanum*were air -dried under shade for five (5) days. The dried leaves were pulverized into a fine powder using a grinding machine. Then 1 kg of the powdered leaves of *G. africanum* was extracted using cold maceration in 5 L of ethanol for 72 h with intermittent shaking. The supernatant was decanted after 24 h, and fresh ethanol was used to make up the original mark and was left for another 48 h after which the mixture was sieved with a muslin cloth. It was further filtered three times with No1 Whatman filter paper. The filtrate was concentrated using a rotary evaporator at40^{o}Cas well as water bath at 45^{o}C. The weight of the concentrated extract was obtainedusing weighing balanceand it was stored in a closed container for further use.

Solvent fractionation of extract of G.africanum

The ethanol extract of *G. africanum* was fractionated into n-hexane, ethyl acetate, n- butanol and aqueous fractions by liquid-liquid fractionation method with the aid of a separating funnel[23]. The concentrated extract was reconstituted with 300 mL of distilled water and poured into the separating funnel. Then 500 mL of n-hexane was added and shaken vigorously releasing the pressure at intervals. The mixture was then allowed to stand for one hour for proper separation was done using a separating funnel knob, and care was taken to ensure that the n-hexane fraction did mix with other fraction. The n-hexane fraction was collected into a clean beaker. Another 500 mL of n-hexane was added to the extract in the funnel and the fraction was collected again. The above procedure was repeated two more times. Then 500 mL of ethylacetate was added to the residue in the separating funnel and shaken vigorously and then allowed to stand for one hour. The ethylacetate fraction was then collected into a clean beaker. Another 500 mL of ethylacetate was added, and the fraction was collected, and this was done for one more time. The above procedure was repeated using butanol (500 mL three times), and thereafter, the aqueous layer was collected. The various fractions were further concentrated for 3 daysusing a rotary evaporator and their weights were obtained.

Acute toxicity (LD_{50}) test

The acute toxicity of the ethanol extract was done using the method of Lorke.[24] A total of 12 mice were used. The test involved two phases. In phase 1, 9 mice were divided into groups 1, 2, and 3 with each groups receiving 10, 100 and 1000 mg/kg respectively. Animals were observed behavioral changes and mortality for the first 4 h and thereafter at the end of 24 h. Absence of death in phase 1 led to the selection of 1600, 2900 and 5000 mg/kg in phase 2 for three animals of 1 mouse per group. They were also observed for 4 and 24 h behavioural changes and mortality. At the end of phase 2, LD_{50} was calculated using the geoemetric mean of the minimal lethal dose and the maximum non-lethal dose.

Qualitative and quantitative phytochemical analysis

The phytochemical analyses of the leaves of *G. africanum*were carried out using a standard method[25] to identify their active constituents. All reagents for the phytochemical tests were freshly prepared.

Determination of antioxidant activity using DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging assay

Antioxidant activity of the leaf extract of *G.africanum* was measured as its ability to scavenge stable DPPH radicals according to the procedure described by previous workers.[26]

1 mL of 0.3 mM of freshly prepared DPPH solution in methanol was added to 2.5 mL solution of the extract solutions and allowed to react in the dark at room temperature for 30 min. Absorbance of the resulting solution was measured at 518 nm. 1 mL of methanol was added to 2.5 mL of each extract concentration and was used as blank, while 1 mL of 0.3 mM DPPH solution was added to 2.5 mL of methanol, and it served as a negative control. Ascorbic acid was prepared in the same concentrations as the test extracts and was used as reference standards (positive controls) for comparison.

Percentage DPPH scavenging activities of the extracts and reference standards was determined using the formula:

$$\% \text{ Scavenging activity} = 100 - \left[\left(\frac{As - Ab}{Ac}\ x\ 100\right)\right]$$

Where A_s = Absorbance of sample (extract or reference standard), A_b = Absorbance of blank and A_c = Absorbance of negative control.

Immunomodulatory studies on extract and solvent fraction

This test was carried out using methods previously employed by other researcehers.[27,28] A total of 70 adult rats weighing (180-200g) were used. They were separated into 14 groups of five rats each. The immune system of all the animals in groups 2 to 14 was suppressed by a single intraperitoneal administration of 70 mg/kg of cyclophosphamideon day one. Group one animals at each stage was left unsuppressed and were termed normal control. The animals were left for 72 h for proper immune suppression. The experiment was carried out in two stages and the animals received treatment as follows:

Stage I: The ethanolic extract was used.

Group I: received distilled water, served as normal control
Group 11: received 50 mg/kg crude extract orally
Group 111: received 100 mg/kg crude extract orally
Group 1V: received 200 mg/kg crude extract orally
Group V: 2.5 mg/kg levamisole orally
Group V1: 10 mL /kg distilled water orally

Stage II: The different fractions of the extract were used

Group VII received 100 mg/kg n- hexane fraction per oral
Group VIII received 200 mg/kg n-hexane fraction p.o

Group IX received 100 mg/kg of butanol fraction p.o
Group X received 200 mg/kg of butanol fraction p.o
Group XI received 100 mg/kg of aqueous fraction p.o
Group XII received 200 mg/kg of aqueous fraction p.o
Group XIII received 100 mg/kg of ethyl acetate fraction p.o
Group XIV received 200 mg/kg of ethyl acetate fraction p.o

Antigen Preparation
Fresh sheep blood (10 mL) was aseptically taken from the jugular vein of a healthy male sheep and transferred to heparinized tube. The blood samples were washed thrice in 5-10mL pyrogen-free sterile normal saline by centrifugation at 3000rpm for 10min on each occasion. The washed SRBCs was adjusted to a concentration of $1x10^9$cells/mL with sterile normal saline and used as antigen for immunization and challenge.[28]

Delayed- type hypersensitivity response
The method described in a previous study was used.[27] A total of 70 adult rats were randomly distributed into fourteen groups of five rats each. On day 3 of post treatment with the extracts and standard drug the paw volume of the animals were measured and termed basal, one hour after extracts administration the rats were sensitized by subplantar injection of 0.02mL of 1 $x10^9$cells/mL of sheep red blood cells (SRBCs) subcutaneously into the sub-plantar of right hind foot paw. The animals were challenged on day 7 post treatment by injecting same amount of antigen SRBCs subcutaneously into the subplantar of left hind foot paw. The oedema produced by antigenic challenge in the left hind paw was measured as the difference in the paw thickness before and 24 hours after the challenge. Increase in paw volume was calculated as an index of cell- mediated immunity (delayed type hypersensitivity reaction).

Haemagglutination antibody titre
Animals were pretreated for 3 days with extract and Levamisol. At the end of 3 days (day 0), 1 mL of the 10^9 cell/mL SRBCs was administeredby intra-peritoneal injection to all groups for immunization. As treatment continued from day 0 to day 5, animals were challenged again on day 5 after determination of antibody titre by similar intra-peritoneal injection of same amount of 10^9 cell/mL SRBCs. Treatment continued from day 6 to day 10, when the second antibody titrewas determined. Blood sample were obtained by retro-orbital puncture into test tubes and allowed to clot. The clotted blood was centrifuged and 100µL of serum was obtained from it and was heat-inactivated. Then, 100 µL of serum was heated/inactivated at 56^{o}C in water bath for 30 min. Thereafter, 50µL of PBS was added to all the wells of microtiter plate row. First well was served as control and no serum was added, but only phosphate buffer saline (PBS). The next well received 50 µL of heat inactivated serum. From the same well, 50 µL of the mixture was taken, and was completely mixed and then serially diluted by 2 folds in the subsequent wells. Finally, 50 µL of SRBC with a cell density of $0.5x10^9$/mL was added to all the wells. The plate was gently tapped to mix the cells and was incubated at 37^{o}C for 2 h. The value of antibody titer was assigned to the highest serum dilution showing at least 50% of visible hemagglutination.[28]

Immunoassay for IL-2, IL-4, and IFN-γ
Immunoassaysfor IL-2, IL-4, and IFN-γ were carried out under standard laboratory practice using Enzyme- Linked ImmunoSorbent Assay (ELISA).

Effect of ethyl acetate fractionon Leukopoiesis in Immunocompromised mice
This assay was done using Cyclophosphamide-induced myelosuppression mice model as described by previous study.[29] The study involved five groups of 5 mice each. Group 1 served as the immunocompetent vehicle control group that was not administered cyclophosphamide but received 10 mL/kg of 5% Tween 80orally while group 2 was the immunosuppressed vehicle control group that received 10 mL/kg of 5% Tween 80 P.O. Group 3 received reference immunostimulatory drug levamisole at 2.5 mg/kg p.o. Groups 4 and 5 received 100 and 200 mg/kg of the most active fraction (ethyl acetate fraction) orally. The baseline total leukocyte counts of the mice were determined using improved Neubauer counting chamber on the first day of the experiment. Thereafter, the immune system of the animals was compromised by a single bolus intraperitoneal injection of cyclophosphamide (70 mg/kg) to each of the animals except animals in group 1. Daily oral administration of treatments was done for 30 days. Toelucidate the kinetics of the response following extract administration, the total leukocyte counts was determined every 3 days in the course of the treatment and percentage leucopoiesis calculated using the formula:

$$\text{Leukopoiesis (\%)} = (100 - [\frac{N.WBC - T.WBC}{N.WBC} \; x \; 100])$$

Statistical analysis
This was determined using one-way analysis of variance (ANOVA;Post hoc- Dunnett comparisons) in a computer-aided software-GraphPad Prism, version 3.0. The results were expressed as mean ± standard error of the mean. The values of the treated groupswere compared with those of the controlsand $p<0.05$ was considered significantly different.

Results and Discussion

The study explored the immunomodulatory activity of the ethanol leaf extract and different fractions of *G. africanum* by evaluating their effects on DTH reactions, hemagglutination antibody titres, and on the complete blood count.

Phytochemical analysis
The resultsof phytochemical analysis of the ethanol extract of *G.africanum* showed the presence of cardiac glycosides, flavonoids, terpenoids, alkaloids, tannins, and saponins (Table 1). This is similar to the findings of previous works.[14,19] Flavonoids and tannins are phenolic compounds, and they act as primary antioxidants or free radical scavengers. Although no constituents could be linked to the immunomodulatory properties of the plant at this stage, studies have shown that different types of flavonoids augment the humoral response by stimulating both the macrophage and B-lymphocytes.[30] Flavonoids have also been found to cause increase in the helper T cells, interleukin 2 (IL-2), interferon and macrophages; hence their use in the treatment of diseases of immune disorders.[31] The terpenoids help to regulate metabolism and also have antioxidant properties.[32] Carbohydrate and reducing sugar were also found to be present. Carbohydrate supplementation has been found to play an important role in immune system by reducing post-exercise stress hormone levels, inflammation, fatty acid mobilization and oxidation.[33]

Acute toxicity
The acute toxicity study did not indicate signs of toxicity such as sedation, pains, salivation, diarrhea, pilo-erection, convulsion or mortality up to a dose of 5000 mg/kg body weight. This is in line with previous study done on the plant which also recorded no toxicity at a dose of 5000 mg/kg body weight.[34] This may indicate that it is safe for consumption.

DPPH scavenging activity
The immune system of humans is intricately interwoven with oxidative processes in the body. High oxidative stress usually impairs some function of the immune system, precipitates free radicals, and leads to several diseases. Antioxidants and cytokines play an important role in regulating the microenvironment in which they function. The DPPH assay measures the electron-donating ability of the compounds in a mixture and thus provides an estimate of the antioxidant activity due to free radical scavenging. The result of antioxidant effect of *G. africanum* using DPPH showed a dose-dependent increase in DPPH scavenging activity of various fractions of the extracts compared to ascorbic acid in this study.

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Table 1: Phytochemical constituents of the ethanol extract of *G. africanum*

Parameters	Results
Alkaloids	+
Saponins	+
Tannins	–
Flavonoids	+
Steroids	–
Terpenoids	+
Cardiac glycosides	+
Proteins	–
Carbohydrates	+
Reducing sugar	+

+ = Present, - = Absent.

The extracts and ascorbic acid showed concentration-dependent increase in percentage scavenging activity. The highest scavenging activity of 96.18% was shown by the ascorbic acid at the concentration of 100 ug/mL, 92.22% at the concentration of 50 ug/mL, 59.63% at the concentration of 12.5 ug/mL and 16.17% at the concentration of 6.25 ug/mL. This was followed by ethylacetate fraction which showed 76.22, 57.66, 46.11, 32.46, 23.60% at concentrations of 500, 250, 125, 62.5 and 31.25 ug/mL, respectively. Butanol fraction showed 49.17, 37.57, 31.0, 25.83, 24.8% at the concentration of 500, 250, 125, 62.5 and 31.25 µg/mL, respectively. The -hexane fraction showed 38.2, 28.83, 20.97, 11.16 and 27.11% at the concentration of 500, 250, 125, 62.5 and 31.25 µg/mL respectively. The ethanol extract showed 24.05, 25.26, 21.75, 17.54, and 16.59% at the concentration of 500, 250, 125, 62.5 and 31.25 µg/mL, respectively (Figure 1).

Previous studies done by various authors using DPPH assay had shown that *G. africanum* has high radical scavenging activites.[35,36] The high scavenging activity of *G.africanum*on 2,2-diphenyl-1-picrylhydrazyl radical could be attributed to its content of flavonoids and other phenolic compounds. These compounds have varying levels of antioxidant activity, as a result of the hydrogen donating ability of their OH-groups. This might justify the traditional usage of *G. africanum*in the management of diseases that have a free radical origin.

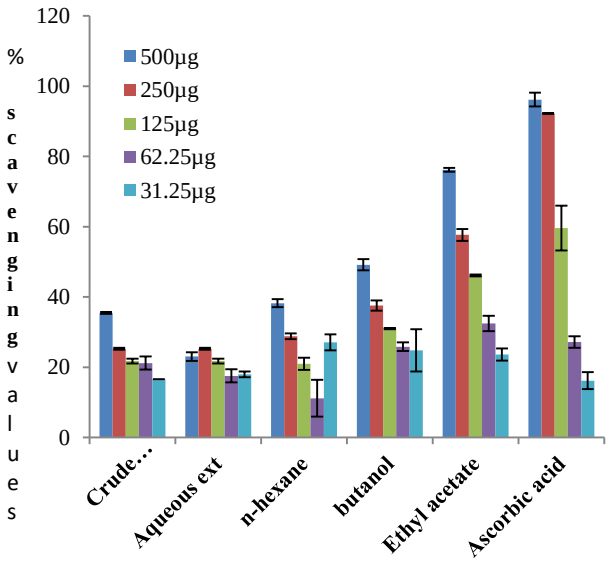

Figure 1: DPPH scavenging activity of the extracts**.**

The highest percentage of scavenging activity of 96.18% was shown by ascorbic acid at the concentration of 100 µg/mL. This was followed by the ethylacetate fraction with the percentage scavenging activity of 76.22% at the concentration of 500 µg/mL.

Effect of extracts of G. africanum on both total and differential white blood cell counts

Single dose injection of cyclophosphamide suppressed the immune system of the animals as shown ($p<0.05$) reduction in the WBC count compared to the pre-induction values.

The results of the mean effect of extracts of *G. africanum* on total white blood cell count and differentials showed a significant increase ($p<0.05$) in total white blood count of different groups of the animals treated with100 and 200 mg/kg of ethanol extract, 2.5 mg/kg levamisole, 100 and 200 mg/kg aqueous fractions, 100 and 200 mg/kg ethylacetate fractions while different doses of n-hexane and butanol fractions showed no significant differences ($p>0.05$) in total white blood cell count. No significant increase ($p>0.05$) in neutrophils were recorded in the groups treated with different doses of n-hexane, butanol and 100 mg/kg aqueous fractions of the extracts while significant increases ($p<0.05$) were recorded in the groups treated with 100 and 200 mg/kg ethanol extracts, 200 mg/kg aqueous fraction, 100 and 200 mg/kg of ethylacetate fractions. There was also significant statistical increase ($p<0.05$) in lymphocytes in different groups treated with 100 and 200 mg/kg of butanol, aqueous, ethylacetate fractions of the extracts, 200 mg/kg ethanol extract as well as 2.5 mg/kg levamisole while no significant increase was recorded in the groups treated with different doses of n- hexane. Different groups treated with both ethanol extracts and different fractions of *G. africanum* showed no statistical difference ($p>0.05$) in the values of monocytes and basophiles (Table 2).

Administration of cyclophosphamide caused about 80% reduction in leucopoiesis across groups. Levamisole and the ethyl acetate fraction at 200 mg/kg were able to restore 50% and 100% leucopoiesis on the 15th and 30th day of treatment. The above results demonstrated that both the ethanol extract and fractions from *G. africanum* leaf contains compounds that can stimulate the production of antibodies in an immunocompromised animal.

The current reduction of the total WBCs was consistent with previous study[29]. This reduction might be as a result of impaired erythropoietin production, resulting in the decreased stimulation of bone marrow erythropoiesis.

The immunostimulatory effect might be attributed to the presence of phytochemicals in the leaf of *G. africanum*. As earlier stated, flavonoids, terpenoids, carbohydrates and reducing sugar have varying effects on the immune systems. This may also justify the common usage of the herb as an immune stimulant.

Effect of extracts of G. africanum on cytokines

Cytokines play a key role in haemopoiesis, immunity, infectious disease, tumorigenesis, homeostasis, and tissue repair. They also provide a link between organ-systems, providing molecular signals for maintaining physiological stability.[37] Because of their major role in nearly all pathophysiologic processes and their therapeutic potential, this work investigated the immunomodulatory effects of solvent fractions of *G. africanum* by assessing the expressions of interleukin-2, interleukin-4, and interferon-γ in the test animals. The different fractions of the extracts studied showed a significant increase ($p<0.01$) in the value of IL-2 when compared to the negative control. Group treated with levamisole also showed a significant increase ($P<0.05$) in the value of IL-2 (Figure 2)**.** Interleukin -2 is known to greatly enhance the proliferation and function of T-lymphocytes and natural killer cells and promotes the production and release of other cytokines. There was no significant increase ($p>0.05$) in of IL-4 and interferon -γ in the groups treated with different fractions of extracts and the group treated with levamisole, when compared to the negative control. Significant increase in the value of IL-2 showed by the extract when compared to the negative control suggests that the ethanol leaf extracts of *Gnetum africanum* could improve lymphocyte proliferation and immune function.

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Effect of the ethanol leaf extracts of G. africanumon Delayed Type Hypersensitivity Response (DTHR)

The DTH reaction is a type IV cell-mediated immune response, and it provides a functional *in vivo* assessment of the cell-mediated immunity.[38] It is usually used to assess the skin response following intradermal inoculation of the antigen which is dependent on antigen - specific memory T-cells. Activation of the T cells leads to the release of lymphokines which causes the activation and accumulation of macrophages, increases vascular permeability, vasodilatation, inflammation.[39] It also produces a boost in phagocytic activity and increases the concentration of lytic enzymes for the more effective killing of microorganisms[37]. The mean Delayed -Type Hypersensitivity (DTH) response was measured by an increase in the rat paw volume (oedema) and was found to be relatively high. Treatment with ethanol leaf extracts of *G. africanum* produced a significant ($p<0.05$) dose- dependent suppression of the DTH response with ethylacetate fraction at 100 and 200 mg/kg showing the highest inhibition of 87.5% and 95.8% respectively while the standard immunostimulant drug levamisole produced 75% inhibition. The least percentage inhibition was recorded by 100 mg/kg and 200 mg/kg fractions of n-hexane (0%and 4.6%) respectively (Figure 3). The inhibition of delayed type hypersensitivity response by *G.africanum* extracts in this study could be attributed to the ability of the extract to activate lymphocytes and their accessory cell types leading to enhancement in the production of antibodies in the immunosuppressed rats thereby increasing cell-mediated immunity. The dose-dependent increment in paw size in response to antigen suggests that the extracts of *G. africanum* can be used to boost the immune system.

Table 2: Effect of extracts of *G. africanum* on total white blood cell and differential counts

	mg/kg	Total WBC	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Basophils (%)
Extract	100	2.93 ± 0.29*	38.67 ± 2.03*	64.67 ± 2.01	1.00 ± 0.00	1.33 ± 0.33
	200	3.30 ± 0.10**	39.33 ± 1.86*	73.00 ± 1.73**	1.67 ± 0.33	1.67 ± 0.33
NHF	100	2.03 ± 0.09	32.00 ± 1.16	44.33 ± 1.20	0.00 ± 0.00	0.00 ± 0.00
	200	2.23 ± 0.01	34.00 ± 1.16	46.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
BF	100	2.07 ± 0.13	35.33 ± 1.20	49.33 ± 0.88*	0.00 ± 0.00	0.00 ± 0.00
	200	2.30 ± 0.10	40.67 ± 0.89	50.00 ± 0.56**	0.00 ± 0.00	0.00 ± 0.00
AQF	100	2.97 ± 0.09**	41.67 ± 0.89	49.67 ± 0.33*	0.00 ± 0.00	0.00 ± 0.00
	200	3.63 ± 0.09**	44.67 ± 0.89**	54.67 ± 0.33**	0.33 ± 0.33	0.67 ± 0.33
EAF	100	3.30 ± 0.26**	42.33 ± 2.01*	51.00 ± 0.58**	0.00 ± 0.00	0.00 ± 0.00
	200	4.23 ± 0.26**	47.00 ± 1.53**	54.33 ± 2.85**	0.00 ± 0.00	0.33 ± 0.33
Lev.	2.5	2.93 ± 0.67*	41.00 ± 2.52	65.67 ± 0.88**	1.33 ± 0.00	1.67 ± 0.67
Norm cont.		2.40 ± 0.10	52.00 ± 1.73**	50.00 ± 1.16**	0.33 ± 0.33	0.33 ± 0.33
Neg. cont.		1.83 ± 0.09	34.00 ± 3.06	41.67 ± 0.88	0.00 ± 0.00	0.00 ± 0.00

Results are presented as the mean ± standard error of mean, n = 5. *P<0.05; statistically significantly different from the control group. **P<0.01; highly statistically significantly different from the control. **NHF:** N-hexane fraction, **BF:** Butanol fraction, **AQF:** Aqueous fraction, **EAF:** Ethyl acetate fraction **Lev:** Levamisol, **Norm. cont:** Normal control. **Neg. cont:** Negative control.

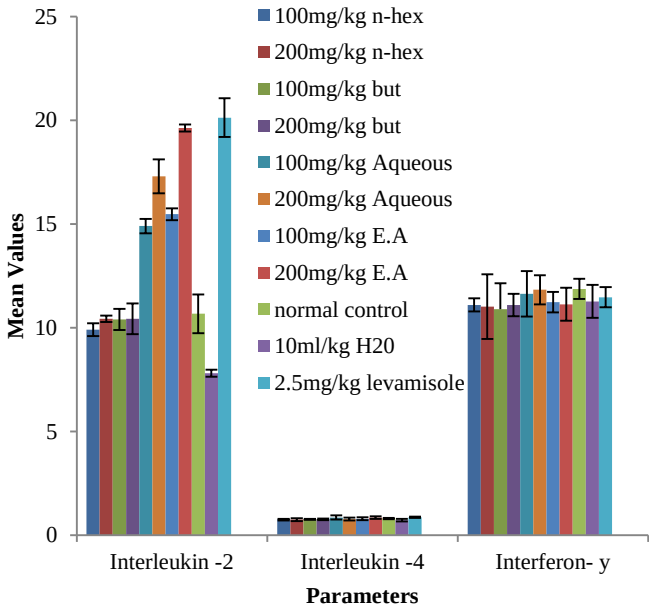

Figure 2: Mean cytokine values for different doses of the fractions in cyclophosphamide immuno-suppressed Wistar rats.

The different solvent fractions and levamisole showed a dose-dependent significant increase in interleukin-2 but not in interleukin-4

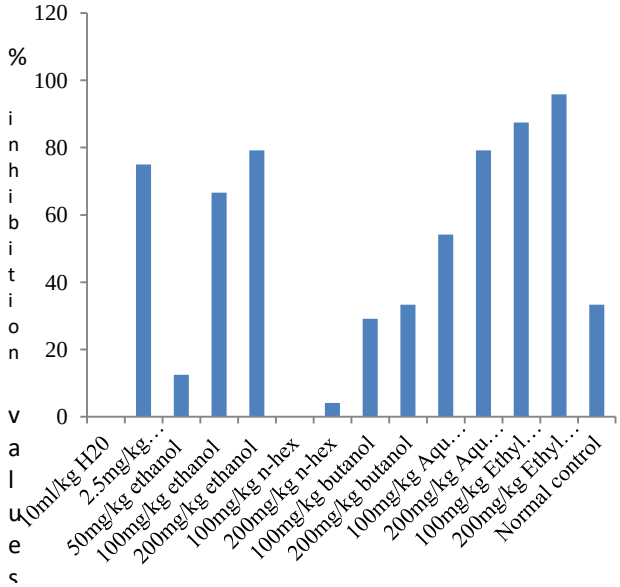

Figure 3: A Histogram showing Percentage Inhibition of the Paw Volume in different groups treated with different doses of the extracts and levamisole.

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Effect of the ethanol leaf extracts of G. africanum on haemoagglutination antibody titre

Haemagglutination antibody (HA) titre was determined to establish a humoral response against SRBC. A hemagglutination test was performed to determine the effect of both the ethanol extract and fractions of *G. africanum*on the humoral immune response. Humoral immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into plasma cells that secrete antibodies. Antibodies thus function as the effectors of the humoral response by binding to the antigens and neutralizing them or facilitating their elimination by cross- linking to form clusters that are ingested by phagocytic cells.[40] The study results demonstrated that both the ethanol extracts and fractions of *G. africanum* had stimulatory effects on the humoral immune response. There was a significant increase ($p<0.05$) in the haemoagglutination antibody titre value in the group treated with 200mg/kg of ethyl acetate fraction with percentage haemoagglutination antibody titre boost of 70% and the group treated with levamisole, which showed the highest percentage haemoagglutination antibody titre boost of 75%. There was also a significant increase in HA value ($p<0.05$) in the groups treated with 200 mg/kg ethanol extract,100 mg/kg ethylacetate extract and 200 mg/kg of the aqueous extracts with percentage boosts of 64.98, 60 and 60% respectively (Figure 4). The increase in antibody titresevoked by *G. africanum* clearly indicates stimulation of the immunity. By stimulating antibody production, *G. africanum* may augment the body's immunity against infectious diseases.

Effects of the most active fraction on WBC count and rate of leucopoiesis recovery

Multiple comparisons between groups showed significant ($P<0.05$) reduction in WBC count of the immunosuppressed animals compared to theimmunocompetent control group. Treatment with the ethyl acetate fraction at 200 mg/kg was able to offset this difference from the 27th day of treatment ($P <0.05$) just like the reference drug (levamisole). This same effect was achieved at the lower dose of the ethyl acetate fraction on day 30. Treatment of the immunosuppressed animals with the ethyl acetate fraction at 100 and 200 mg/kg showed significant ($P<0.05$) increase in WBC count from the 9th day when compared to the immunosuppressed vehicle treated control animals.

The extract at 200 mg/kg also showed similar activity as 2.5 mg/kg levamisole with significant ($P<0.05$) difference recorded only from day 9 – 15.

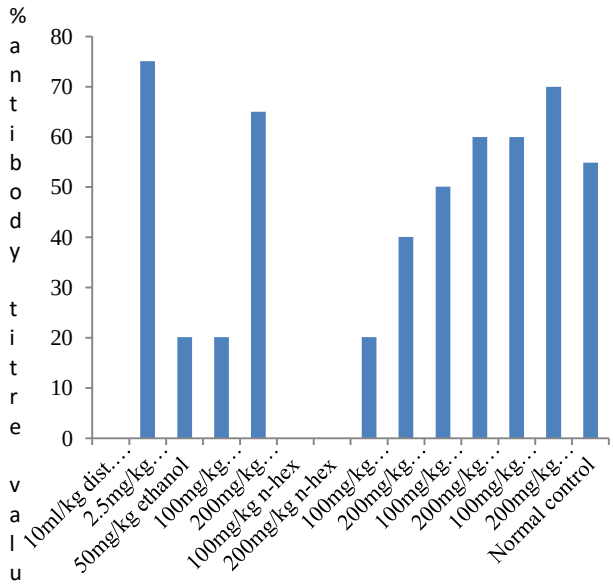

Figure 4: Percentage antibody titre boost with treatments.

Cyclophosphamide administration caused about 80% reduction in leucopoiesis across groups. levamisole and the ethyl acetate fraction at 200 mg/kg were able to restore 50% leucopoiesis on the 15th day of treatment (Figure 5).

Further treatment with these agents were able to restore normal leucopoiesis just like in the immunocompetent control group at day 30. The immunosuppressed vehicle control group was unable to achieve 50% recovery at the 24th day post-induction. However, at day 30, 63% recovery was recorded for this control group against full recovery shown by the ethyl acetate fraction at 200 mg/kg (Figure 6). From the study, outstanding activity produced by ethyl acetate fraction could be attributed to its ability to extract polyphenol and other semi-polar compounds such as flavonoids, which have been reported in previous studies to produce antioxidant, anti-inflammatory and immunomodulatory activities.[41,42]

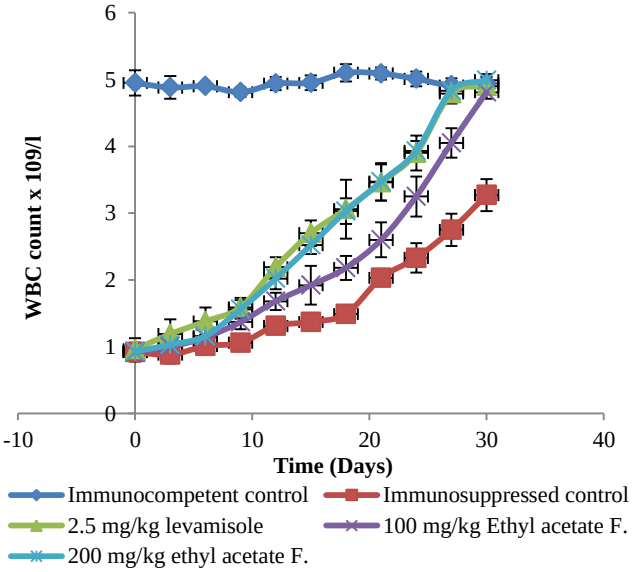

Figure 5: Time-response curves of various treatments on total WBC count

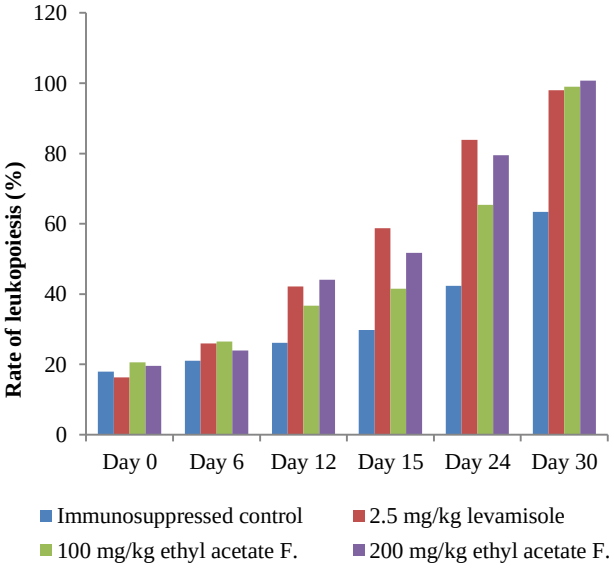

Figure 6: Effects of the most active fractions on rate of leucopoiesis recovery

Conclusion

Based on the findings from the study, the extracts of *G. africanum* may increase cell-mediated and humoral immune responses in rats. This may be attributed to the different nutrients and phytochemicals present in the plant. The different leaf extracts of *G. africanum*, therefore have a potential therapeutic values in several immunostimulation and hence the reason for the use of *G. africanum* in local communities to treat various disease conditions. Research on structural elucidation of the active ingredients necessary for its action is ongoing.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

We hereby affirm that the work presented in this article is original and that we are responsible for any liability for claims relating to the content of this article.

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