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Immunomodulatory Effects of *Phyllanthus muellerianus*: A Mechanistic Approach

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Abstract

Background: *Phyllanthus muellerianus* is used in folkloric medicines to treat infectious diseases (both bacterial and viral), inflammatory disorders and skin diseases. This study evaluated the modulatory effects of methanol leaf extract and fractions of *Phyllanthus muellerianus* on some specific and non-specific immune responses.

Methods: The powdered dried leaves were extracted with analytical grade of methanol using a soxhlet apparatus and the extract concentrated in vacuo to obtain the methanol extract (ME). The ME was fractionated using the following solvent; n-hexane, ethylacetate and methanol in the order of increasing polarity. The specific immunomodulatory potentials were investigated using delayed-type hypersensitivity reaction (DTHR) for cellular responses and antibody synthesis for humoral immune responses. The effects of the ME and ethylacetate fraction (EF) which was the most effective fraction, on leucopoiesis in cyclophosphamide-induced immunosuppressed rats were further investigated.

Results: The ME (50 mg/kg, 100 mg/kg and 200 mg/kg produced non-dose dependent inhibition of foot-pad swelling in mice with 50 mg/kg causing the highest inhibition in delayed-type hypersensitivity reaction (DTHR) tests. The highest stimulation and inhibition in DTHR occurred with EF at the lowest and highest dose respectively. Similarly, there was a non-dose dependent increase in antibody titre with 50 mg/kg of ME eliciting a significant increase ($p<0.05$). The EF caused 100% increase in antibody titre while the other fractions (hexane (HF) and methanol (MF) fractions) elicited different degrees of inhibition. In cyclophosphamide-induced myelosuppression, the ME and EF evoked greater reduction in total leucocyte count (TLC) than cyclophosphamide. The ME also caused greater reduction in lymphocyte proliferation and significant ($p<0.05$) increase in neutrophil proliferation.

Conclusion: The results suggest that the extracts of *Phyllanthus muellerianus* exhibit both immune-boosting and immunosuppressing actions at different doses and can be employed in both immunodeficiency and over reactive immune conditions at appropriate doses.

Keywords: Antibody titre; Delayed-type hypersensitivity reaction; Cyclophosphamide; leucopoiesis; myelosuppression; Immune-boosting; Immunosuppressing

Introduction

The immune system is the body's natural guard against foreign invaders. It is composed of a system of biological structures and processes which helps to protect against a wide variety of pathogens [1]. An efficient immune system must be able to detect and attack a wide variety of noxious agents including viruses, bacteria, fungi, parasitic worms etc. The immune system employs both antigen-specific (adaptive) and non-antigen-specific (innate) systems to eliminate these threats [1]. A healthy and efficient immune system is needed to fight deadly diseases such as AIDS and Ebola virus that weaken the immune responses. Drugs like cytotoxic agents and antibiotics also weaken the immune system and as a result, there is a growing interest in the search for natural agents that can modulate the immune system, when desired.

Immunomodulators are substances capable of interacting with the body to stimulate or suppress specific aspect of the host immune response [2,3]. Immunostimulants are needed when the host defense mechanisms need to be strengthened as in conditions of impaired immune responses such as immunodeficiency diseases like HIV and for generalized immunosuppression following infection or drug treatment [4,5]. Immunosuppressants are important when the host defense responses need to be minimized in situations like autoimmune disorders as occur in rheumatoid arthritis, allergic reactions, inflammation, organ transplant rejection etc. [6]. Immunomodulators of plant origin are preferred because they are mostly less toxic and therefore safer [7]. They are also affordable and useful in cases where microbial resistance and emergence of more virulent diseases whose cures are yet to be found are present [8]. Many immunomodulatory agents have been reported in plants such as *Randia dumetorum* [9]; *Rubia cordifolia* [10]; *Morus alba* [11]; *Cassia auriculata* [12]; *Garcina kola* [13]; *Tinospora cordifolia* [14]; *Tinospora crispa* [15]; *Xanthium strumarium* [16]; *Myrsine seguinii* [17]; *Spinacia oleracia* [18]; *Stevia rebaudiana* [19] and *Calotropis procera* [20].

Phyllanthus muellerianus is being screened for its effects on the immune responses because of its wide application in folklore. In West Africa, various parts of the plant are traditionally used to treat intestinal troubles such as dysentery, constipation and diarrhea [21]. Its

anti-malaria, antidiabetic, antigonorrhoea, antiulcer and anti-jaundice activities have been reported [22]. Its antimicrobial, antiviral, antifungal and anti-inflammatory activities have also been reported [22-24]. The therapeutic potentials of this plant are enormous, and this sparked our interest to investigate its effects on the immune system using experimental animals.

Methods

Chemicals and drugs

Methanol (Sigma Chemical Co, St Louis USA), Levamisole (Retrax®, 40 mg) and cyclophosphamide (Cycloxan®, 500 mg) were used for the study, and are of analytical grade.

Animals

Ten weeks old male Sprague Dawley mice (20 g-25 g) and male albino rats (Wistar strain) (150 g-200 g) obtained from the animal House of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used. The animals were allowed free access to water and fed with Guinea Feed pellets. They were maintained at a temperature of 25 ± 2°C and 12 h light/dark cycle.

Antigen

Fresh sheep red blood cells (SRBCs, 0.20 mL) were obtained from healthy sheep reared in the Animal Farm of the Department of Veterinary Medicine, University of Nigeria, Nsukka. The cells were washed four times by centrifugation at 3000 rpm for 10 min using pyrogen-free normal saline (0.9%, w/v). The concentration of the washed SRBCs was adjusted to 1.0×10^9 cells per ml and used for immunization and challenge of the animals.

Plant material

The leaves of *Phyllanthus muellerianus* were collected from Nsukka in the month of June 2014, identified and authenticated by Mr. Alfred Ozioko, of the International Center for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Nigeria with voucher specimen number InterCEDD062014.

Extraction of plant material

The leaves were air-dried for 4 days and pulverized using electronic mill and the powdered leaves (2.2 kg) extracted in methanol (5 L) with a Soxhlet apparatus. The extract (ME) was concentrated at reduced temperature and pressure to a semi-solid paste and thereafter dried to obtain 250 g (11.30% w/w) of the extract. The ME (100 g) was subjected to solvent-guided fractionation in a silica gel (70 nm-230 nm) mesh, column (60 cm length × 7.5 cm diameter) successively eluted with n-hexane, Ethyl acetate and methanol in order of increasing polarity. The solvent fractions obtained were concentrated under reduced pressure in a rotary evaporator to obtain the hexane fraction (HF; 2.52 g; 2.52% w/w), Ethyl Acetate Fraction (EF; 32.15 g; 32.15% w/w) and Methanol Fraction (MF; 57 g; 57.00% w/w).

Phytochemical screening and acute toxicity testing

The ME and fractions were screened for the presence of secondary metabolites using standard methods [25], while the acute toxicity test (LD_{50}) was done on ME as discussed by Lorke [26].

Pharmacological evaluation

In each of the following experiments, animals were randomized into five groups *viz:* groups I and II received only the vehicle (20% Tween 80) and levamisole (2.5 mg/kg) or cyclophosphamide (30 mg/kg) as negative and positive control respectively while groups III, IV and V received 50 mg/kg, 100 mg/kg, 200 mg/kg or 400 mg/kg doses of the extract. The animal experiments were conducted in line with the National Institute of Health Guide for care and use of laboratory animals (Pub No.85-23, revised 1985) and in accordance with the University of Nigeria Ethics Committee on the use of laboratory animals, registered by the National Health Research Ethics Committee (NHREC) of Nigeria, with the number; NHREC/05/01/2008B. The study protocols were approved by our institution's Ethics Committee.

Delayed type hypersensitivity (DTH) response

On day 0 of the study, all the animals in groups (I-V) were immunized with SRBCs (0.2 ml of 10^9 cells/ml i.p) and challenged on day 14 with the same concentration of SRBCs in sub plantar region of right hind paw. Pedal edema/swell was used to detect cellular immune response. Foot pad reaction was assessed after 24 h in terms of increase in the thickness of footpad due hypersensitivity reaction to the antigenic challenge. The footpad reaction was expressed as the difference in the mean thickness (mm) of paw-size before the challenge and after the challenge. The thickness of the footpad was measured using a micrometer screw gauge.

Humoral antibody (HA) synthesis

Mice were immunized by intraperitoneal (i.p) injection of 0.2 ml of 10^9 cells/ml SRBC on day 0 and challenged by injecting the same concentration (i.p) on day 14. The extract was administered orally for three days prior to immunization and continued once daily for 7 days post-challenge. Primary and secondary antibody titers were determined on day 14 and day 21 respectively by the hemagglutination technique [27]. Blood samples were obtained from the retro-orbital plexus and allowed to clot, the serum collected and antibody levels were determined [28]. Briefly, two-fold serial dilution of 25 µl of collected serum was done in 96-U well micro titer plates using pyrogen-free sterile normal saline. The diluted sera were challenged with 25 µl of 1% (v/v) SRBC and then incubated at 37°C for 1 h. The highest dilution showing visible hemagglutination was taken as antibody titer. Antibody titers were expressed in graded manner, the minimum dilution (1/2) being ranked as 1.

Leucopoiesis in cyclophosphamide- induced myelosuppression

The baseline hematological parameters of the animals were determined after collecting blood by retro-orbital puncture. Afterwards, a single bolus injection of cyclophosphamide (30 mg/kg, i.p) was administered to all the animals. The extract was administered for 14 days. Blood samples were withdrawn on day 7 and day 14 and analyzed for total and differential leucocyte counts.

Statistical analysis

This was carried out using one-way analysis of variance (ANOVA; Post hoc- Dunnett comparisons) in a computer–aided software-Graph Pad Prism, version 5.0. The results are expressed as mean ± standard error of the mean. The values of the treated group were compared with those of the control and $p<0.05$ is considered significantly different.

Results

Acute toxicity and lethality test

The extract exhibited oral LD_{50} greater than 5000 mg/kg since no death or any sign of acute intoxication in any of the groups was apparent after 48 h observation.

Phytochemical analysis

The results of phytochemical analysis indicate the presence of Alkaloids, Flavonoids, Glycosides, and Tannins (Table 1)

	ME	HF	EF	MF
Alkaloids	+++	-	-	+++
Flavonoids	+	-	+++	+
Glycosides	+++	-	+++	+++
Saponins	+	-	+	+
Steroids	+	+	++	+
Tannins	+++	-	+++	+++
Terpenoids	+	+	-	-

Table 1: Phytochemical constituents of the extract and fractions.

Effect of the extract on SRBC-induced delayed-type hypersensitivity reaction

The extract caused non-dose related inhibition of foot-pad swelling (pedal edema) in mice. The inhibition was however, non-significant ($p>0.05$) (Figure 1). The EF evoked both significant ($P<0.001$) elevation and significant ($P<0.05$) inhibition in foot-pad swelling (pedal edema) at low and high doses respectively (Figure 2). These values were higher when compared to the stimulation and inhibition caused by other fractions (HF and MF), hence the remaining studies were carried out only on the ME and EF.

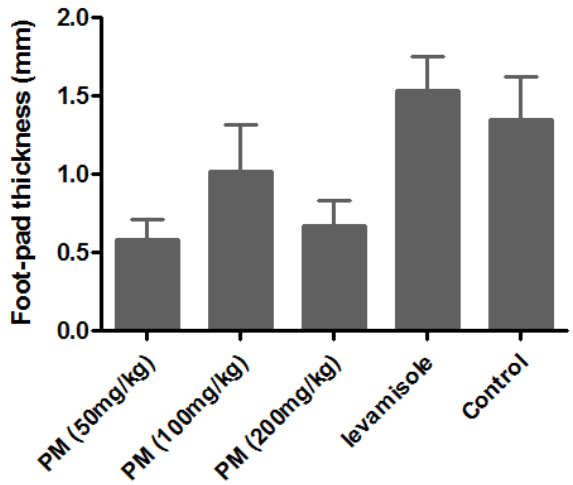

Figure 1: Effect of ME on DTH response in mice, n=6.

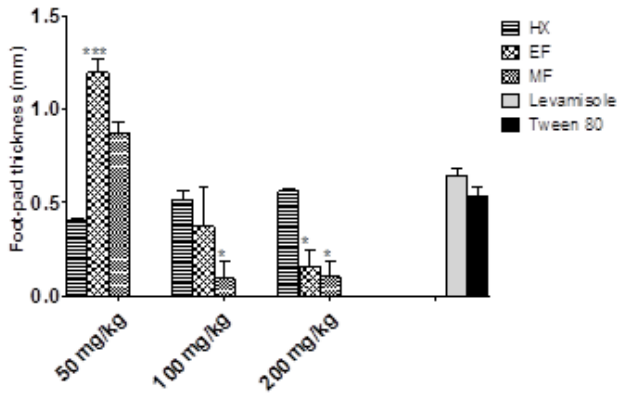

Figure 2: Effect of the fractions on delayed-type hypersensitivity reaction. $^{*}p<0.05$; $^{***}p<0.01$;

Effect of the extract on SRBC- induced antibody synthesis

The ME caused non-dose dependent elevation of secondary anti-SRBCs-specific titer with 50 mg/kg eliciting a significant ($p<0.05$) effect compared to the control. However, there was a progressive decline in the titer as the dose was increased with 200 mg/kg eliciting the least elevation (Figure 3). The EF (100 mg/kg) elicited significant increase ($p<0.05$) in both primary (100%) and secondary (42%) antibodies production (Table 2). The MF and HF, evoked different degrees of inhibition in both primary and secondary antibodies production with MF causing 100% inhibition at 50 mg/kg and 100 mg/kg.

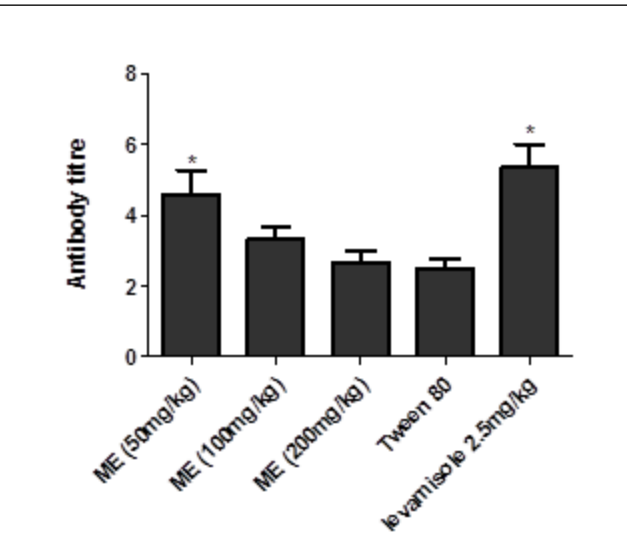

Figure 3: Effect of ME on secondary antibody synthesis in mice, * $P<0.05$, n = 6

Treatment	Dose (mg/kg)	Humoral antibody titre (mean ± SEM)	
		Primary	Secondary
HF	50	1.7 ± 0.23 (11.8)	2.4 ± 0.27* (-37.5)
	100	1.2 ± 0.20 (-25)	1.9 ± 0.3 (-73.7)
	200	1.1 ± 0.24 (-36.4)	1.6 ± 0.2* (-106.3)
EF	50	1.5 ± 0.29 (0.0)	2.8 ± 0.48 (-17.9)
	100	3.0 ± 0.6* (100)	5.7 ± 0.33* (42.1)
	200	1.7 ± 0.33 (11.8)	4.0 ± 0.58 (17.5)
MF	50	0.0 ± 0.0* (-100)	2.0 ± 1.00 (-65)
	100	0.0 ± 0.0* (-100)	2.5 ± 0.2 (-32)
	200	0.5 ± 0.5 (-66.67)	3.3 ± 0.25 (0.0)
Levamisole	2.5	1.5 ± 0.28 (0.0)	3.5 ± 0.87 (5.7)
Negative Control	0.3 ml Vehicle	1.5 ± 0.28	3.3 ± 0.48

Table 2: Effect of the fractions on SRBC-induced humoral antibody synthesis in rats *P<0.05; n=5, HF: Hexane Fraction; EF: Ethyl Acetate Fraction; MF: Methanol Fraction. Percentage humoral stimulation is shown parenthesis, negative sign indicates inhibition of antibody synthesis, and vehicle is 20% tween 80 in water.

Effect of ME and EF on leucopoiesis in cyclophosphamide-induced myelosuppression

There was a significant reduction (P<0.05) in TLC in all the treatment groups on day 7, Levamisole and cyclophosphamide only treated group overcame the reductive effect on day 14 (Table 2 and Figures 4a and 4b). The ME (100 mg/kg and 200 mg/kg) elicited a significant (P<0.05) increase (29.50% and 27.97% respectively) in Neutrophils on day 7 post cyclophosphamide and the increase continued on day 14 (43.51% and 30.15%) respectively. Cyclophosphamide and levamisole on the other hand elicited non-significant increase on day 7 (11.54% and 22.22%) respectively. Which decreased appreciably on day 14 (-0.74% and 5.97%) (Figure 5a). On Lymphocyte population, ME (100 mg/kg and 200 mg/kg), levamisole and cyclophosphamide elicited reduction on day 7 but on day 14, ME still caused reduction while levamisole and cyclophosphamide overcame the reductive effect and evoked increases (Figure 5b).

	% Neutrophil (% increase)			% lymphocytes (% reduction)			TLC 10^3/µL (% reduction)		
	days			days			days		
Treatment	0	7	14	0	7	14	0	7	14
A	27.2 ± 1.9	30.8 ± 2.2 (11.5%)	27.0 ± 1.3 (-0.74%)	69.2 ± 2.4	66.0 ± 2.1 (4.6%)	69.0 ± 1.8 (0.3%)	12.85 ± 1.0	9.28 ± 1.2 (25.8%)*	15.4 ± 0.7 (-19.8%)
B	25.2 ± 2.1	32.4 ± 1.4 (22.2%)	26.8 ± 1.9 (5.9%)	71.0 ± 2.8	65.2 ± 1.6 (8.2%)	68.4 ± 1.6 (3.7%)	13.8 ± 13	11.6 ± 0.8 (15.8%)	14.6 ± 0.5 (-5.95%)
C	27.0 ± 3.2	38.3 ± 5.7* (29.5%)	47.8 ± 5.3* (43.5%)	70.4 ± 3.4	59.3 ± 5.4 (15.8%)	5.8 ± 5.5 (27.8%)	17.9 ± 0.9	11.2 ± 0.7 (37.4%)*	16.7 ± 2.5 (6.8%)*
D	27.8 ± 2.2	38.6 ± 3.8* (28%)	39.8 ± 3.3* (30.1%)	69.2 ± 2.5	56.0 ± 3.2 (19.1%)	57.0 ± 3.7 (25%)	16.7 ± 2.5	0.7 ± 1.3 (35.8%)*	12.5 ± 1.0 (25.0%)*
E	27.4 ± 2.5	31.8 ± 1.3 (16.1%)	26.2 ± 1.5 (-4.4%)	69.8 ± 2.1	64.4 ± 1.3 (7.7)	70.4 ± 1.5 (0.9%)	18.5 ± 0.5	12.0 ± 1.0 (35.1%)*	15.5 ± 1.4 (16.2%)*
F	28.0 ± 1.6	27.0 ± 1.3 (-3.6%)	28.0 ± 1.0 (0.0)	69.2 ± 1.9	69.8 ± 1.3 (-0.87)	68.4 ± 1.4 (1.2%)	17.9 ± 1.4	9.6 ± 1.1 (46.4%)*	15.1 ± 1.3 (15.6%)*

Table 3: The Effect of ME and EF on leucopoiesis in immunocompromised rats; A=Cyclophosphasmide alone, B=Cyclophosphasmide +Levamisole, C=Cyclophosphasmide+ME (100 mg/kg), D=Cyclophosphasmide+ME (200 mg/kg), E=cyclophosphamide+EF (100 mg/kg), F=Cyclophosphamide+EF (200 mg/kg). Values are expressed as mean ± SEM, n=5, *p<0.05, values in parenthesis show percent increase or reduction in neutrophil and TLC/lymphocyte respectively.

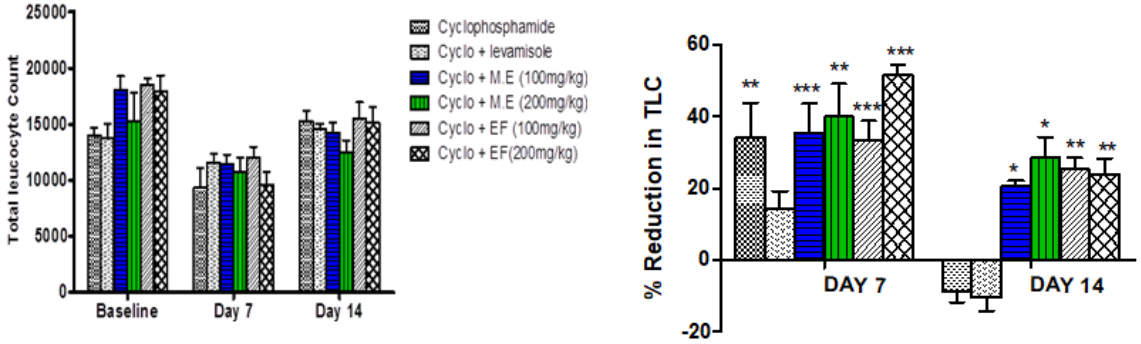

Figure 4: Effect of ME and EF on TLC in immunocompromised rats. *p<0.05, **p<0.01, ***p<0.001, n=5.

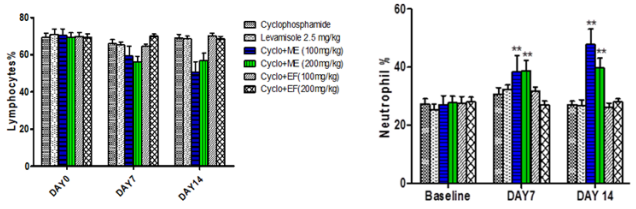

Figure 5: Effect of ME and EF on the proliferation of lymphocytes and neutrophils in immunocompromised rats. **p<0.01, ***p<0.001

Discussion

In this study, ME and fractions modulated the manifestation of delayed-type hypersensitivity reaction (DTHR) induced by SRBC. The EF evoked the highest stimulation at the lowest dose and inhibition at higher doses. Inhibition is evident by its ability to inhibit pedal edema. This result is in agreement with the report of Boakye [24] who reported the anti-inflammatory property of this plant extract. DTHR is a type IV hypersensitivity reaction that involves cell-mediated inflammatory reactions. The response is initiated by the migration of T helper 1 (TH1) cells to the site of antigen injection to react with the antigen. This interaction activates TH1 cells to secrete inflammatory cytokines such as IFN- γ and TNF- α [29]. These cytokines stimulate the expression of adhesion molecules on the endothelium and increase local blood vessel permeability, allowing plasma and other non-specific inflammatory cells to enter the site thus causing visible swelling [29, 30]. These cytokines mobilize macrophages to the site of antigen invasion and activate them, thus enhancing phagocytic activity with increased concentration of lytic enzymes for more effective killing [31]. The reaction of TH1 cells with the antigen (SRBC), manifests as swelling of the footpad as was evident in the study. The inhibition and stimulation of footpad swelling by different doses of the extract and fractions indicate that they were able to influence one or more of the steps involved in cell-mediated immune responses. They might cause inhibition by immune deviation which entails steering the T cells to produce TH2 cells instead of TH1 cells during differentiation [32]. The cytokines- transformation growth factor (TGF- β) and interleukin (IL-10) produced by TH2 cells prevent macrophage activation [33]. In addition, they do not stimulate the expression of adhesion molecules caused by IFN- γ and TNF- α secreted by TH1 cells that signal other inflammatory cells to migrate to the infection site to cause swelling. DTHR is the major mechanism of host defense against intracellular parasites and bacteria. It also effects transplant rejection and tumor immunity. Since the EF was able to inhibit and also stimulate DTHR at different doses, it could be considered as a lead material in developing agents that can prevent inflammation and organ transplant rejection and agents that can confer tumor immunity.

Humoral immune responses to infection may involve the production of antibodies by plasma cells derived from activated B lymphocytes, the binding of the antibodies to pathogen and the elimination of the bound pathogen by phagocytic cells and molecules of humoral immune system [29, 34]. In the study, the ME and EF, evoked a stimulatory response on the humoral immunity manifested as increase in anti-SRBC titer. The effect could be due to the up regulation of TH2 cells since they are the cells that cause activation of B cells before their proliferation and differentiation into antibody secreting cells. TH2 cells through the secretion of IL-5 and IL-6 cause the later stage activation of B cells and thus secondary antibody synthesis [29]. The EF causing both inhibition of DTHR and stimulation of antibody synthesis at 100 mg/kg, lends credence to the postulation that the inhibition of DTHR noted above might be because of steering of T cells to produce TH2 cells instead of TH1 cells. TH1 cells activate macrophages to cause cell-mediated immune response (DTHR) while TH2 cells inhibit macrophages activation to inhibit cell-mediated immunity but on the other hand activate B cells to enhance humoral immunity (antibody synthesis) [29]. Stimulation of antibody production by ME and EF suggests that it can assist the body's immune response and enhance its ability to suppress various infections of bacteria, viruses, parasitic worms, protozoa etc. It may also be a good adjuvant in vaccine development.

Passenger leucocytes (the leucocytes carried along with the transplanted tissue) are important in graft rejection. Removal of passenger leucocytes by treatment of the tissue prior to transplantation could reduce the immunogenicity of a tissue graft tremendously; it is therefore better, to attempt to alter tissue immunogenicity by treating the tissue donor rather than the recipient [35]. Cyclophosphamide is an immunosuppressant and acts by cross-linking DNA to prevent cell

replication and thus its function [36]. It affects particularly the proliferating lymphocytes. Cyclophosphamide pretreatment has been reported to sharply decreased the activity of all lymphoid cells, especially the $CD4^+$ T lymphocyte [37]. It was reported also that both destruction of donor antigen stimulated T cells in the periphery and elimination of donor reactive clones of T cells in the thymus were essential mechanisms of cyclophosphamide-induced tolerance [38, 39]. In line with previous reports [40,41]; cyclophosphamide group in this study exhibited a significant ($p<0.05$) reduction in TLC on day 7 when compared to its baseline value, however, this effect was overcome as the TLC level returned and increased a little above the pretreatment value on day 14. Similar trend occurred with levamisole group. However, in ME and EF treated groups, the reduction was higher on day 7 and persisted on day 14. This indicates that the extracts, especially the EF is a very potent lymphocyte suppressant. It is likely that the population of the lymphocytes suppressed is the TH1 cells. This is because from the DTHR and antibody synthesis studies, EF at the same 100 and 200 mg/kg inhibited foot-pad swelling which is a TH1 cell-mediated immune response; meaning that it down-regulated the TH1 cells while stimulating humoral antibody synthesis which is a B- cell mediated response with the assistance of TH2 cells. These findings suggest that the extract at these doses is a TH1-cell suppressant. The data from TLC and lymphocyte reduction assay show that the extract evoked more T cell suppressant activity than cyclophosphamide suggesting that it could be a useful tool in removing passenger leucocytes from a tissue graft prior to transplantation to afford reduction in its immunogenicity to the host, thereby preventing organ transplant rejection. By increasing neutrophil population, the extract may enhance non-specific immune responses like phagocytosis and intracellular killing of microbes. In some kind of cancer (lymphocytic leukemia), there is usually marked elevation in TLC with significant increase in neutrophil (neutropenia), thus the extract eliciting the opposite effects may be a useful lead in developing anti-leukemia agent.

The ME and EF exhibited biphasic effects with low doses having opposing effects with higher doses. This is in line with the previous reports that both exogenous and endogenous compounds can have opposing, dose-dependent biological effects [42]. Dhuley reported that 100 mg/kg of *Withania somnifera* caused immunostimulation by increasing TNF expression [43] while Davis et al. reported that at 20 mg/kg, *Withania somnifera* caused immunosuppression by lowering TNF expression [44]. Paradoxical response to *Tylophora asthmatica*, a herb used traditionally to treat asthma, allergies and autoimmune disorders have also been reported [45,46] where lower doses increased IL-2 levels while higher doses caused reduction. There is need therefore for a dose-response assessment in the use of these extracts so that the dose that will give the desired activity is chosen.

Conclusion

These results have established the modulatory effects of methanol leaf extract and fractions of *Phyllanthus muellerianus* on the immune responses in animal models. Our results indicated that PM has immunosuppressive effect but it is evident that it was a selective suppression of TH1-induced cellular immunity and a shift towards TH2-mediated humoral immunity rather than generalized immunosuppression. The extract as seen in appropriate (high) doses can be useful as immunosuppressive agent for unwanted/exaggerated immune responses. It can also be useful at lower doses in boosting the immune responses in cases of infectious diseases and conditions of impaired immunity as indicated by the increase in humoral antibody synthesis.

The ability of the extracts to elicit significant reduction in TLC and significant increase in neutrophil population presents it as a useful candidate for the development of agents for lymphocytic leukemia. The process of isolating the active constituents responsible for these activities has reached advance stage.

Competing Interest

There is no competing interest in the study.

Authors' Contribution

MNO designed the study and performed of the experiments, TCA performed some of the experiments, CSN performed some aspect of the experiment and analyzed the data, MOA extracted the plant material and prepared the manuscript for publication, PAA supervised the work and prepared the manuscript for publication.

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