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Research Article

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Immunomodulatory Potentials of Ethanolic Leaf Extract of *Phyllantus amarus* in Wistar Rats

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Abstract The immunomodulatory effect of the ethanolic leaf extract of *Phyllantus amarus* on wistar rats was investigated. Twenty eight (28) male animals were used for the study. The animals were divided into four (4) groups of seven (7) animals each. Group 1 was the normal control and received distilled water, animals in group 2 were given low dose (7.1 mg/kgBW) of the extract, group 3 were given medial dose (14.1mg/kgBW) and group 4 were given high dose (28.3 mg/kgBW) of the extract. The treatment was administered twice daily for twenty eight (28) days after which the animals were sacrificed and blood samples used for analysis where obtained via cardiac puncture. The phytochemical screening of the plant was carried out and results shows that the plant contains flavonoids, saponins, alkaloids, terpenes, phenols, steroids, glycosides and tannin. Results reveal that the group treated with low (102.41±7.41), medial (101.84±11.72) and high dose (100.94±30.47) recorded an elevated WBC value when compared with the control (83.20±11.30) at $p<0.05$ significant level. Similar trend was observed for lymphocytes. The extract recorded no effect on monocytes and eosinophils counts. More so, the plants extract lowers the CD4, T-cells and B-cells count at $p<0.05$ significant level compared with the control, suggesting that the extracts have anti-microbial properties to lower infections and hence the decreased values observed. Owing to this phytochemical constituents, it also suggests that the extract have the ability to suppress inflammatory diseases and auto-immune diseases occasioned by CD_4. Conclusively, ethanolic *Phyllantus amarus* leaf extract is a good immunomodulatory agent at recommended dose range of 7.1 to 28.3mg/kg.

Keywords Phyllantus amarus, immunomodulatory-agent, medicinal potentials

Introduction

Mechanistic studies and the identification of bioactive compounds in medicinal plants could lead to the discoveries of new biological active compounds. Biomedical sciences and modern translational research on herbal medicines beyond basic science and clinical perspectives could contribute immensely to the development of new therapeutics. Polysaccharides, one of main classes of bioactive substances from fungi, algae, and higher plants, have been demonstrated to exhibit a wide range of pharmacological activities, including broad immunomodulatory and antitumor effects [1-2] while mushroom and plant polysaccharides identified through complementary and alternative medicine are undergoing scientific analysis and development to treat cancer [3-6].

Many herbal plant preparations are prescribed to strengthen host resistance and many useful plants fall under this category [7] which exhibit immunomodulatory activities. One of such plant *Phyllanthus amarus*, commonly called 'Guduchi' (means to rejuvenate dead cells). It is widely used in veterinary folk medicine and has also been claimed

to be beneficial according to 'Ayurveda' for the cure of jaundice, skin diseases, diabetes, anemia, emaciations and various infections for its anti-spasmodic, anti- inflammatory, anti-arthritic and anti-allergic properties [8]. Kolte *et al.,* [9] studied the effect of feeding *Phyllanthus amarus* in broiler birds which were immunosuppressed with cyclophosphamide. They had found a significant rise in antibody titer against Newcastle disease (N.D.) virus with augmentation of inflammatory reaction to skin contact sensitivity test. Rege *et al.,* [10] and Bishavi *et al.,* [11] have also proved the hepato-protective potentials of *Phyllanthus amarus*. Manjrekar *et al.,* [12] also found that aqueous extract of *Phyllanthus amarus* is capable of increasing leukocyte count in mice.

The beneficial effects of *Phyllanthus amarus* could therefore be due to its direct or indirect effect on the immune system. The immune system protects the body against invading pathogens by generating several cells and molecules which eliminate the invaders [13]. Immunomodulators are being used as supportive adjunct to specific antibiotic therapy in immunodeficient patients and studies have also shown that some herbs are capable of stimulating the immune system; especially the innate immunity and this could be beneficial in immunotherapy and also serve as alternative to conventional chemotherapy [13-15].

However, with respect to *Phyllanthus amarus* very little work has been done to determine its effect on the immune system especially with regard to its specific mode of action and the pharmacological mechanism of *Phyllanthus amarus* and the signaling pathways involved in the immunomodulation remain unclear. In view of the above, this study was designed to investigate the immunostimulatory effects of ethanolic leaf extract of *Phyllanthus amarus* on Wistar rats.

Materials and Methods

Collection, Preparation and Extraction of Plant Material

Phyllanthus amarus plants were collected from the premises of Otuoke community secondary school Otuoke, Bayelsa state, Nigeria. The leaves were removed and dried in the shade for two weeks to dry before being pulverized into fine powder using blender and kept in an air-tight container; this was as described by Odey *et al.,* [16]. One hundred and fifty gram (150g) of the pulverized leaves was weighed and soaked in 450ml of 80% ethanol for twenty four (24) hours at 45^{o}C.

The preparation was later sieved with clean cheese cloth and double filtered with whatman No. 1 filter paper number 1 to obtain a filtrate which was concentrated using rotary evaporator and then water bath to evaporate it to dryness.

Experimental Animals

Twenty-five mice (males) weighing between 25-30g and twenty-eight albino rats weighing 120-180g were used for this study. The animals were obtained from the multidisciplinary Animal House of the college of medical sciences, University of Calabar, Cross River State, Nigeria. The mice were used for the determination of Medial Lethal Dose (LD_{50}), while the rats were used for the immunomodulatory property of the plant extract. The animals were given one week acclimatization period, there after the animals were reweighed and housed in a wire-mesh cages under monitored environmental conditions of temperature (28±2 ^{o}C), relative humidity (50±5%) and a 12 hour light/dark cycle. The animal facility was properly ventilated and the animals where place on the commercial rat pellet and water provided *ad libitum* during the experimental period. Ethical approval was obtained from Federal University Otuoke research ethical and quality control committee. Administration of the extract was done twice daily for a period of twenty eight days via orogastric intubation. Group A (Normal Control) received distilled water, group B was the low dose group receiving 7.1mg/kg body weight of the extract, group C was the medium group receiving 14.1mg/kg body weight of the extract while group D (High Dose) receiving28.3mg/kg body weight of the extract as determined by LD_{50} using Lorke method of 1983 in line with previous researches using *Phylantus amarus.*

Collection of Blood Samples for Analysis

The animals were sacrificed twelve hours after the last treatment, whole blood was collected from the heart via cardiac puncture using sterile syringe and needle. The blood sample was divided into two fractions: One fraction was put into plain sample tubes while whole blood samples were put in Ethylene di-amine tetra acetate (EDTA) treated sample tubes. The serum was collected from the clotted sample in the sample container by letting it stand for

2 hours at room temperature to clot prior to centrifugation at 3000 rpm for 20 minutes using MSE England bench top centrifuge.

Sera obtained from each sample were gently separated using Pasteur pipettes and dispense into respective dry specimen bottles that were labelled accordingly. These were kept frozen in a refrigerator until when needed for various biochemical assays.

The blood samples collected into the EDTA bottles were corked immediately, shaken gently to allow the blood to mix with the anticoagulant and prevent clotting and cell haemolysis. The haematological analyses were carried out as soon as the blood sample was collected.

Estimation of total white blood cell, B-cell and CD4 count

The total white blood cell, B-cell and CD4 count where estimated based the method of Tietz, 1994 using different approach in each case.

Statistical Analysis

The data obtained were analyzed using one-way analysis variance (ANOVA), statistical package for social sciences (SPSS) and presented as mean ± standard error of mean (SEM). The level of significance was considered at $p < 0.05$ in comparison with control.

Results

The results of the immunomodulatory property of the ethanolic leaves extract of *Phyllanthus amarus* investigated is presented in table 1.

From table 1, the values obtained show that the CD4, T-cell (total T-cells) and B-cell count ofgroup 4 (high dose) were significantly ($p<0.05$) lower when compared with the control (group 1). However, the changes compared with other treated groups though not significant at $p< 0.05$. This implies that at increased concentrations of the extract, decreases the values of the CD4, T-cells and B-cells.

Data evaluated for WBC, neutrophils, and lymphocytes count in table 2 reveals a significant ($p<0.05$) increased for animals in group 2 (low dose). The lymphocytes estimation recorded an elevated significant ($p<0.05$) values in all treated groups compared with control. However, the extract recorded no effect on eosinophils and monocytes.

Table 1: The effect of the ethanolic leaf extract on CD4, T-cells and B-cells count of wistar rats.

Group	CD4 X 10^9/L	T-Cell X 10^9/L	B-Cell X 10^9/L
Control (A)	50.32±1.76	2.83±1.87	1.98±2.05
Low Dose (B)	47.25±6.5	2.18±3.09	1.61±3.35
Medial Dose(C)	42.82±2.23	1.82±0.55	1.05±3.15
High Dose (D)	38.71±2.47*	0.96±0.13*	0.74±1.07*

Values are expressed as mean ± SEM, n = 7

* = Significant at P<0.05 compared to group 1(control)

a= Significant at P<0.05 compared to group 2(low dose)

Table 2: The effect of the ethanolic leaf extract on the components of the immune system of wistar rats

Group	White blood cell X 10^9/L	Neutrophils X 10^9/L	Lymphocytes X 10^9/L	Monocytes X 10^9/L	Eosinophils X 10^9/L
Normal Control	83.20±11.30	48.80±2.28	34.60±2.70	0.00±0.00	0.00±0.00
Low Dose	102.41±7.41*	31.23±16.74*	69.64±15.45*	0.00±0.00	0.00±0.00
Medium Dose	101.84±11.72	25.64±7.82*	75.22±13.48*	0.00±0.00	0.00±0.00
High Dose	100.94±30.47	38.86±11.94	62.19±20.62*	0.00±0.00	0.00±0.00

Values are expressed as mean ± SEM, n = 7

* = Significant at P<0.05 compared to group 1

Discussion

The immune system is the vital defense mechanism against tumor, cancer growth, and infectious diseases. A strong immune system comprises elements that are in balance with one another; if the balance is distorted, the immune system becomes incapable of protecting the body against harmful substances and invasive pathogens. The side effects linked with allopathic drugs along with their high cost have imposed the need for search of immunomodulatory agents that exert less adverse effects and can be administered for a longer duration to attain a continuous immune activation for the prevention of various diseases at little or no cost. Immunomodulation using medicinal plants can provide a substitute to conventional chemotherapy for a range of disease conditions, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is needed in situations such as autoimmune disorders. There are several diseases where immunostimulating drugs are needed to overcome the immunosuppression induced by drugs or environmental factors, and immunosuppressants are required where there is undesired immunopotentiation. There is a strong necessity for the drugs that can enhance the immune system to combat the immunosuppressive consequences caused by stress, chronic diseases, and conditions of impaired immune responsiveness.

Though medicinal plants have been investigated for diverse pharmacologic activities, the immunomodulatory potential oncytotoxic T-cells (TC) still remains unknown. This investigation deals with the TC that was used for its immunomodulatory activity and in this study, the ethanolic leave extract of *Phyllanthus amarus* at the indicated therapeutic doses determined via LD_{50} study was conducted, and from the results no cytotoxic effect was observed suggesting that this extract is safe for human use. However more toxicity tests is needed to assure its safety

To elucidate the mechanism of *Phyllanthus amarus* extract as an immunomodulatory agent, the effects of *Phyllanthus amarus* extract on CD4+ spleen T-lymphocyte populations in the animals were analyzed by flow cytometric assay. T-lymphocytes, the vital part of the defensive immune system, are differentiated into two different subsets according to their specific membrane molecule, which are CD4+ and CD8+ T-lymphocytes. Since T-cells play a key role in regulating the immune responses by being responsible for cell-mediated immunity, in a stable immune system, fast T-cell proliferation following antigen stimulus is governed by their differentiation into effector cells. Being main protectors of the host against diseases, lymphocyte activation and proliferation are vital during onslaught of infection or other pathological conditions. The agents responsible for the proliferation of the CD4+ and CD8+ cells have significant therapeutic potential for patients who are suffering from CD4+ lymphopenia caused by numerous infectious diseases, drugs, or cancer.

The proliferating T-helper cells (Th cells) are capable of developing into effector T-cells, which additionally differentiate into two main functionally distinctive subtypes of cells called Th1 and Th2 cells. It is well known that T-cells and cytotoxic T lymphocyte responses are linked with the enhancement of CD4+ and CD8+ T-lymphocytes, respectively.

The medicinal properties of the plant could be attributed to the presence of various bioactive compounds in ethanol extracts under study. The extracts have shown the presence of cardiac glycosides, flavonoids, steroids, terpenes, alkaloids, tannins and saponin. This is in accordance with the work of Imaran *et al*. [17] who reported the results of phytochemical analysis of *Phyllanthus amarus* leaves by using different solvent such as Petroleum ethar, chloroform, methanol and ethanol shows the presence of triterpenes, glycosides and fatty acids. Saponins have the property of precipitating and coagulating red blood cells, it is used to stop bleeding and in treating wounds [18]. Tannins have been found in the extracts of *Phyllanthus amarus* and it accounts for the astringent properties of the plant in wound healing and suppressing inflamed mucous membrane [19]. Tannins are also reported to be a potential metal ion chelator, protein precipitating agents and biological antioxidant while Ellagitannins have free radical scavenging activity [20]. Phytochemicals act as antimicrobial compounds and have made great contribution for quick and effective management of diseases both of microbial and non-microbial origin. The findings in this work conforms to the report of [21] in which similar constituents was found to exhibits antiprotozoal and antibacterial activities. Flavonoid has also been reported to have greater potential benefit to human Health [22]. It displays protective properties including anti-inflammatory, anti-oxidant, antiviral, and anti-carcinogenic properties.

Phenols**:** is a crystalline aromatic organic compound. Dilute solutions of phenol are useful antiseptics, but strong solutions of phenols are caustic and scarring to tissues. Phenols are widely used in the manufacture of resins, plastics, insecticides, explosives, dyes and detergents and as raw materials for the productions of medicinal drugs such as aspirin.

Phyto compounds such as saponins, steroids, flavonoids and alkaloids have been shown to have immunostimulatory effects. The presence of alkaloids, flavonoids and saponins in *Phyllanthus amarus* therefore supports the use of *Phyllanthus amarus* as immunostimulants as earlier reported by Manjrekar *et al* [12].

The plant extract lowers the CD4, T-cells and B-cells count at $P<0.05$ significant level compared with the control. Owing to this phytochemical constituents, it also suggests that the extract have the ability to suppress inflammatory and auto-immune diseases such as asthma and rheumatoid arthritis.

Conclusion

Phyllanthus amarus contains many secondary metabolites such as Flavonoid, Alkaloids, Tannin, Phenol, Glycosides, Saponin, and steroids. These phytochemicals points at its medicinal potentials as immuno-inflammatory suppressant as well as immunomodulatory agent.

The results demonstrate that the extract of *Phyllanthus amarus*may have been able to activate the cell-mediated immunity by enhanced chemotactic and phagocytic proliferation activity of lymphocytes along with enhanced expression of proinflammatory integrin. The present findings suggest that the ethanolic extract of *Phyllanthus amarus* possesses potent immunostimulatory properties. As the study demonstrates that the ethanol extract of *Phyllanthus amarus* could improve immune function,and can could serve as potential immune-therapeutic agent possibly beneficial in enhancing the immune response in immune-compromised diseases. However, the specific pharmacologically active ingredients in *Phyllanthus amarus* and the signaling pathways involved in the immunostimulation also remain to be further elucidated.

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