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INVESTIGATING THE ACTIVITY OF ETHANOL EXTRACT OF *GONGRONEMA LATIFOLIUM* LEAF AS IMMUNOMODULATOR IN WISTAR ALBINO RATS

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ABSTRACT

Recently, immunomodulation by way of stimulation is an approach employed globally to surmount the problems posed by immunosuppressive diseases such as malnutrition, HIV/AIDS, cancer and infections caused by pathogenic microorganisms. A large proportion of the world population particularly in the Sub-Saharan Africa uses medicinal plants with immunostimulatory property to boost immunity in preference to conventional chemotherapeutic agents that are associated with high cost, adverse effects and risk of infection on prolonged use. *Gongronema latifolium* leaf is used in traditional medicine as an immunomodulator in South Eastern Nigeria. The aim of this study was to investigate the activity of ethanol extract of *Gongronema latifolium* leaf as an immunomodulator in Wistar albino rats. This was done by determining the total and differential blood cells count, neutrophil adhesion to nylon fibres, hemagglutination antibody titre and delayed-type hypersensitivity response. From the results, the ethanol extract of the plant in dose-dependent manner, significantly ($p<0.05$) increased the total blood cells count, neutrophil adhesion to nylon fibres, hemagglutiation antibody titre and foot pad thickness in delayed-type hypersensitivity response. These findings therefore validate the tradomedicinal claim on *Gongronema latifolium* as an immunomodulator.

KEYWORDS: Immunomodulator; Infection; Extract; *Gongronema latifolium*; Tradomedicine.

INTRODUCTION

The importance of immune system to humans is to prevent or protect the body against infections by pathogenic microorganisms which are ubiquitous in our environment. Globally, particularly in the developing countries, there is increase in the incidence of infectious diseases that tremendously compromise immune system response in the affected individuals.[1] Control of these infectious diseases requires efficient and effective body defense mechanism that can be achieved through immunomodulation. The term immunomodulation is generally used to describe pharmacological manipulation of immune system.[2] Immunomodulators either increase or decrease the magnitude of immune responses, and increase in immune responses is referred to as immunostimulation/immunopotentiation while decrease in immune responses is referred to as immunosuppression.[3] From therapeutic point of view, immunomodulation refers to the process and/or technique by which an immune response is altered to a desired level.[4] In the past years, it had been of medical interest to modulate immune system response to alleviate diseases.[5,6,7] Recently, there is stimulated interest in search of agent that can effectively modulate the immune system, especially in immunocompromised conditions such as HIV/AIDS, tuberculosis, stress and malnutrition.[6] because the conventional immunomodulators are associated with some constraints such as unavailability, high cost, adverse effects, resistance by microorganisms and risk of infection on prolonged usage. Consequently, attention of people particularly in the developing countries has shifted and they have resorted to the use of herbal plant as an alternative. Medicinal plants have been used for a long time before now as immunomodulators in management of various diseases.[4,8,9] A number of plants documented for their immunomodulatory activity include *Withania somnifera*,[10] *Morus alba*,[11] *Sophora subprosrate*,[12]

Acacia catechu,[13] *Jathropha curcas*.[14] and *Allium sativu*.[15]

Gongronema latifolium of family, Asclepiadaceae, is a flora of Tropical Africa[16] claimed by tradomedicine in Eastern Nigeria as a remedy for wide range of ailments including contagious infections and immune disorders. *G. latifolium* is locally known in Nigeria as "utazi" among the Igbos, "utasi" among the Efiks/Ibibios,and "arokeke" among the Yorubas.[17] *G. latifolium* is used as vegetable in soup flavoring.[18] Ethnomedicinally, *G. latifolium* is used as an antitussive,[19] antidiabetic[20,21] and antimalarial.[22] Some documented scientific reports on the activities of *G. latifolium* include anticancer,[23,24] antioxidant[25] and antimicrobial.[26] Various studies have reported the immunomodulatory activity of *G. latifolium*, but the studies were done in different geographical regions which affect the nutritional, phytochemical composition and consequently, the effectiveness of the plant.[27] This study therefore, investigated the immunomodulatory activity of ethanol extract of *G. latifolium* leaves collected from South Eastern Nigeria.

MATERIALS AND METHODS

Harvesting and Authentication of Plant material

From its natural habitat in a farm land in Okigwe, South Eastern Nigeria, matured fresh leaves of the plant were collected, identified and authenticated as *Gongronema latifolium* Benth in the herbarium of Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria, were voucher specimen was deposited under the identification number UPH/P/1471.

Processing and Extraction of Plant Material

About three kilograms (3.0 kg) of the harvested leaves of *G. latifolium* were thoroughly washed with clean tap water and air-dried under shade to a constant weight. Extraction procedure reported in our earlier study.[28] was adopted, where about 500g of the pulverized plant material was macerated in two litres of 80% ethanol for 72 hours, with occasional agitation every six hours. Using Whatmann number 1 filter paper, the resulting mixture was filtered, and the filtrate was poured into previously weighed clean empty beaker. The beaker and its content were kept in hot–air oven at 40^{o}C until ethanol was completely evaporated and a dried residue obtained. The weight of the extracted residue was determined as final weight of beaker and residue minus the initial eight of empty beaker.[28]

Animal ethics permit

Ethics permit (MAU/SREC/A/22) to this study was granted by Senate Research and Ethics Committee of Madonna University, Nigeria. Care and handling of animals were followed in accordance with international guidelines for animal studies.[29]

Experimental animals

Healthy adult Wistar albino rats (14 – 16 weeks old; 210-230g) and healthy adult Swiss albino mice (12 -14 weeks old; 22 - 24g) were obtained from Animal Facility Centre of Madonna University, Nigeria. The animals were housed in laboratory cages, properly floored with dry wood shavings. The animals were allowed to acclimatize for two weeks under adequate ventilation with free access to food and clean drinking tap water.

Drugs/reagents and source

The following drugs and reagents were employed in the study: Levamisole (GlaxoSmithKline, India), 96% Ethanol (Gungsdong Guandgua Chemical Factory China), Ferric Chloride (Super Tek Chemical, Germany), Sodium tetraoxocarbonate IV (Sigma Aldrich Chemie, Germany), Tetraoxosulphate VI acid (Hi Media Laboratories Pvt Ltd, India) Ethylacetate (Rankem, Mumbai, India), Glacial acetic acid (Sigma Aldrich Chemie, Germany), Hydrochloric acid (Nice Laboratories Reagent, Kevala, India), Sodium Hydroxide (Rankem Mumbai, India), Chloroform (Super Tek Chemicals, India)

Phytochemical Screening

The procedure as described by[30,31] was employed in this study to qualitatively analyze for the presence or absence of phytochemical constituents in the plant

Acute Oral Toxicity Study

Acute oral toxicity study was carried out for crude extract of *G. latifolium* using the procedure as described by,[32] using Swiss albino mice.

Preparation of Sheep Red Blood Cells (SRBCs) as antigen

From a healthy sheep, fresh blood was collected and mixed with Alsever's solution (1:1 ratio) in a sterile container. The mixture was centrifuged at 3000 rpm for 5 minutes to settle the red blood cells at bottom of the tube, and then the supernatant was discarded. By centrifugation, the sheep red blood cells in the tube were washed three times with sterile phosphate buffered saline of pH 7.2. Using Neubauer counting chamber, the total SRBCs was enumerated, and then stored in the refrigerator until when needed for immunization and challenge study in rats

Evaluation of Immunomodulatory Activity

Immunomodulatory activity of the plant was investigated by determining complete and differential blood count, neutrophil adhesion to nylon fibre, hemagglutination antibody titre and delayed-type hypersensitivity response in healthy adult Wistar albino rats.

Determination of complete blood count

This study was conducted using thirty healthy adult Wistar albino rats (age: 14-16 weeks old; weight: 210 230g) randomized into six groups (A to F) of five rats per group. Rats in each group were treated as follows:
Group A received 100mg/kg Extract daily x 14 days p.o
Group B received 200mg/kg Extract daily x 14 days p.o.
Group C received 400mg/kg Extract daily x 14 days p.o.

Group D (positive control) received 50mg/kg Levamisole daily x 14 days p.o.
Group E (disease control) received 15mg/kg Prednisolone daily x 14 days p.o
Group F (negative control) received 5ml/kg 3% v/v Tween 80 daily x 14 days p.o.

On the 15th day of the study, 2ml of whole blood was drawn from each rat in groups A to F by intraventricular puncture into clean EDTA-containing vacutainers. The blood was analyzed using automated hematology analyzer (Dynamind DH36) for red blood cell (RBC) count, platelet count, white blood cell (WBC) count and differential WBC count

Determination of neutrophil adhesion
This study used thirty healthy adult Wistar albino rats (age: 14-16 weeks old; weight: 210-230g) randomized as per treatment groups above. The method employed in this study was as described by[33] where at the end of 14 days treatment (i.e. on the 15th day) 2ml blood sample was collected from each animal by intraventricular puncture and analyzed for total and differential WBC counts using an automated hematology analyzer (Dynamind DH36). After the initial count, blood samples were incubated with 80mg/kg nylon fibre for 15 minutes at 37°C. At the end of incubation period, the nylon fibre was removed and blood samples analyzed again for total and differential WBC counts. Neutrophill index (NI) and percent neutrophil adhesion were calculated as reported by.[34]

NI= Total WBC count x percent neutrophil

$$\% \text{ Neutrophil adhesion} = \frac{NIu - NIt}{NIu} \times \frac{100}{1}$$

Where NIu = Neutrophil index of untreated blood sample
NIt = Neutrophil index of treated blood sample.

Determination of humoral antibody response to sheep red blood cells (Hemagglutination antibody titer)
This study used thirty healthy adult Wistar albino rats (age: 14-16 weeks old; weight: 210-230g) randomized as per treatment groups above. The procedure used for this study was as described by[35,36] where on the 7th day of treatment, each rat was immunized by injecting intraperitoneally 0.2ml of $5x10^9$ SRBCs/ml and the treatment was continued for another 7 days (i.e. until day 14). On the 15th day, blood samples were drawn from the rats by intraventricular puncture, centrifuged at 1500 rpm for 10 minutes to obtain serum. Normal saline was used to make serial two-fold dilutions (0.025ml) of the serum in microtitre plates of 96-well capacity. SRBCs (0.025ml of 1% v/v SRBC prepared in normal saline) were added to each of the dilutions and then hemagglutination plates were incubated for 1 hour at 37°C. After incubation, the plates were visually examined for hemagglutination.

The reciprocal of the highest dilution of the serum showing agglutination was recorded as hemagglutination antibody titre.[37] Graded manner was used to express the antibody titre as HA unit/uL, minimum dilution (1/2) ranked as 2

Determination of of delayed-type hypersensitivity (DTH) response
Thirty healthy adult Wistar albino rats (age: 14-16 weeks old; weight: 210-230g) employed in this study were randomized and treated in groups as explained above. The procedure employed in this study was as described by[38] in which rats were sensitized on the 7th day of drug treatment by subcutaneous injection of 20 microlitres (0.2ml) suspension containing $1x10^9$ SRBCs in the right hind foot pad. The drug and extract treatment was continued to day 14 when the rats were challenged by subcutaneous injection of 20 microlitres of $1x10^9$ SRBCs into the left hind foot pad. Vernier caliper was used to measure the thickness of the foot pad before and after 12, 24 and 48 hours of antigenic challenge to ascertain the degree of delayed-type hypersensitivity reaction. The difference in thickness (mm) of the left and right hind pads was used as a measure DTH response.

Statistical Analysis
The values are presented in tables as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used in the assessment statistical significance followed Duncan's (Post-Hoc) multiple comparison. P value less than 0.05 is considered significant.

RESULTS

Yield
When compared to the amount (500g) of plant material soaked for extraction, quantitative yield of ethanol-extracted residue from *Gongronema latifolium* leaves was low (29.63g; 5.93%).

Phytochemical analysis
From the result of phytochemical analysis in table 1, carbohydrates, proteins, alkaloids, flavonoids and tannins are present in high amounts while anthraquinones was found to be absent.

Oral acute toxicity test
From the result of oral acute toxicity test, the ethanol-extracted residue from *Gongronema latifolium* leaves at limit dose of 4000mg/kg, did not produced signs of toxicity nor mortality within 24 hours and 14 days observation.

Effect of extracts and standard drugs on total and differential blood count
When compared to the negative control, the groups that received levamisole and different doses of the extract showed significant ($p<0.05$) increase in total blood cell count while the group that received prednisolone showed

decreased total cell count (table 2). The converse is observed in the case of differential count for lymphocytes where the proportion decreases with increasing dose of the extract.

Effect of extract and standard drugs on neutrophil adhesion

As shown in table 3, decreased neurtophil count was observed in the nylon fibre-treated blood due to adhesion of neutrophils to the nylon fibre. Percent neutrophil adhesion increases significantly ($p<0.05$) with increasing dose of the extract.

Effect of extract and standard drugs on hemagglutination antibody (HA) titre

Relative to negative control, the groups that received prednisolone (immune suppressant) produced decreased HA titre while the group that received levamisole (immune stimulant) and plant extract produced increased HA titre as shown in table 4

Effect of extract and standard drugs on delayed-type hypersensitivity (DTH) reaction

Significant ($p<0.05$) increase in the thickness of the foot pad was observed in the group that received levamisole and the extract (table 4).

Table 1: Phytochemistry of the ethanol extract.

Test	EE(crude)
Carbohydrates	+++
Proteins	+++
Resins	+
Alkaloids	+++
Anthraquinones	_
Glycosides	++
Flavonoids	+++
Steroids	+
Saponins	++
Phenols	+
Tannins	+++
Terpenoids	+

EE = ethanol extract
+ = Present in low amount
++ = Present in moderate amount
+++ = Present in high amount
– = Absent

Table 2: Effect of different doses of extract and standard drugs on total RBC count, platelet count, WBC count and differential count.

Group	Treatment	Total blood count			Differential blood count (%)				
		RBC ($x10^6$/uL)	Platelet ($x10^3$/uL)	WBC (10^3/uL)	Neut	Lymp	Mon	Eos	Bas
A	100mg/kg Extract	5.18±1.18	611±67.08	6.64± 0.62	18.84±1.51	77.64±2.26	1.42±1.14	0.36± 0.35	0.68±0.38
B	200mg/kg Extract	5.72±0.82	634±61.71	6.94±1.02	19.48±1.14	76.54±1.60	1.36±0.62	0.40±0.40	0.64±0.28
C	400mg/kg Extract	7.46±0.87	647±57.24	7.84±1.09	21.76±1.29	75.42±1.06	1.54±028	0.38±0.38	0.80±0.34
D	50mg/kg Levamisole	7.68±0.79	675±67.53	8.46±1.11	22.26±1.02	74.32±1.07	1.20±0.56	0.40±0.27	0.52±0.43
E	15mg/kg Prednisolone	5.02±0.64	424±42.04	4.38±0.83	12.86±1.13	71.64±1.84	1.46±0.42	0.88±0.47	1.24±0.89
F	5ml/kg 3% Tween 80	5.14±0.95	662±108.2	6.26±1.24	18.70±0.98	75.52±1.10	1.44±0.39	0.76±0.71	1.50±0.63

RBC = red blood cell; WBC = white blood cell; Neut = neutrophil; Lymp = Lpmphocytes; Mon = monocytes; Eos = osinophil; Bas = Basophil.

Values represent mean ± SEM of five rats per group

* Significant relative to negative control at $p<0.05$.

Table 3: Effect of different doses of extract and standard drugs on neutrophil adhesion to nylon fibre.

Group	Treatment	Total WBC ($x10^3$/uL)A		Neutrophil % B		Neutrophil index (A x B)		
		Untreated blood	Nylon fibre treated blood	Untreated Blood	Nylon fibre treated blood	Untreated blood	Nylon fibre blood	% neutrophil
A	100mg/kg Extract	6.64±0.62	6.22±0.74	18.84±1.51	17.14±1.89	124.84±2.41	106.44±1.13	14.76±1.78*
B	200mg/kg Extract	6.94±1.02	6.34±0.81	19.48±1.14	17.92±2.30	135.12±1.45	113.44±1.51	16.06±1.66*
C	400mg/kg Extract	7.84±1.09	6.90±0.67	21.76±1.29	19.08±1.15	170.16±1.56	131.66±1.31	22.64±0.68*
D	50mg/kg Levamisole	8.46±1.11	6.26±0.59	22.26±1.02	20.12±1.94	188.24±1.69	125.96±1.80	33.10±1.48*
E	15mg/kg Prednisolone	4.38±0.83	4.18±0.93	12.86±1.13	12.36±1.56	56.20±0.94	51.66±1.29	8.08±1.27
F	5ml/kg 3% Tween 80	6.26±1.24	5.72±1.27	18.70±0.98	18.28±1.01	116.82±1.64	104.56±2.02	10.50±1.61

Values represent mean ± SEM of five rats per group

* Significant relative to negative control at $p<0.05$

Table 4: Effect of different doses of extract and standard drugs on hemaggluination titre and on foot pad thickness.

Group	Treatment	HA titre (unit/uL)	Foot pad thickness (mm) at different time interval		
			12hrs	24hrs	48hrs
A	100mg/kg Extract	20.8±10.02*	0.28±0.21*	0.30±0.36*	0.34±0.23*
B	200mg/kg Extract	51.2±40.07*	0.30±0.13*	0.34±0.16*	0.36±0.32*
C	400mg/kg Extract	70.4±32.91*	0.34±0.19*	0.36±0.17*	0.38±0.25*
D	50mg/kg Levamisole	89.6±34.35*	0.36±0.14*	0.38±0.16*	0.38±0.15*
E	15mg/kg Prednisolone	12.0±5.37	0.06±0.08	0.10±0.13	0.18±0.11
F	5ml/kg 3% Tween 80	17.6±6.44	0.04±0.05	0.10±0.07	0.10±0.28

Values represent mean ± SEM of five rats per group

* Significant relative to negative control at $p<0.05$

DISCUSSION

A disease-free state in humans can be achieved by immunomodulation via stimulation or suppression of immune responses. Agents that can activate the immune mechanisms can provide supportive therapy to conventional chemotherapy.[39] Research interest is increasing in the identification of herbal plants that possess immunomodulatory activity.[40] This work therefore collected fresh matured leaves of *Gongronema latifolium* from natural habitat, extracted and examined the phytochemical constituents of the plant, conducted oral acute toxicity test in mice and immunobioassays which included total blood cells count, neutrophil adhesion, hemagglutination antibody titre and delayed-type hypersensitivity response in Wistar albino rats.

Extraction of plant material

Ethanol was employed as extracting solvent not only on the account of its availability but because higher proportions of plants constituents are soluble in ethanol, hence, most of the traditional herbal preparations are done with ethanol (kai-kai, a locally distilled gin in South Eastern Nigeria).

Yield

Judging from the amount (500g) of soaked powdered leaves *Gongronema latifolium*, the quantitative yield of ethanol extract were low. As reported by[41], bioactive compounds are usually present in plants in low amounts.

Phytochemical analysis

Plants have been reported as rich source of potent phytochemicals that produce numerous biological activities in human and animals.[42] Phytochemical analysis of ethanol extract of *Gongronema latifolium* leaves reveals the presence of various phytoconstituents in small, moderate and high amounts. Similar findings were reported on the same plant by[43] Although this study did not pin-point the exact constituent(s) responsible for immunostimulatory activity, study by[44] on other immunostimulatory plants reported the presence of similar phytoconstituents, particularly alkaloids, flavonoids, steroids, phenols and tannins, which are also found in ethanol extract of *Gongronema latifolium* leaf.

Total blood cells count

The result of this study in table 2 shows that the extract of the plant increased blood cells count. The same observation was reported on the same plant by.[43] This observed increment may be attributed to the presence of various nutritional and phytochemical components present in the plant that can stimulate various hematopoietic systems with consequent proliferation of various blood cells in animal group treated with the extract. Similar assertion was made on other plants by some studies.[7,9,36,45-47]

Neutrophil adhesion

Neutrophils are immune cells that contribute to elimination of foreign particles, such as pathogenic organisms, from host's body by recognizing and migrating towards the foreign pathogens, then phagocytose and eventually kill the pathogens.[48-50] One of the earliest response to immunological injury is the cell adherence ability of neutrophils[33] where cytokines are secreted by activated T-helper cells and macrophages for margination and exravasation of phagocytes, mainly polymorphonuclear neutrophils.[51] Adhesion of significant proportion of neutrophils to nylon fibres which correlates to margination process may be due to up regulation of $beta_2$ integrin found on the surface of neutrophils through which they firmly adhere to nylon fibre.[51,52] The result of this study in table 3 shows a dose-dependent increase in neutrophil adhesion to nylon fibres in the animal groups treated with plant extract when compared to the negative control group. The significant increase in neutrophil adhesion can be interpreted as a boost in immunity caused by nutrients and phytochemicals present in the plant.

Hemagglutination antibody titre

B-cells play a central role in the mediation of humoral immunity by secreting antibodies. B-cells, on interaction with antigens results to their differentiation and proliferation into memory cells and antibody-secreting plasma cells. The secreted antibodies which function as

effector of humoral immune response, bind, neutralize and/or facilitate the antigen elimination by phagocytosis.[53-56] Production of antibody to thymus-dependent antigens such as SRBCs requires the participation of B- and T-lymphocytes and macrophages. From the result of this study shown in table 4, the mean hemagglutination (HA) titre of the groups administered with the extract significantly ($p<0.05$) increased with increasing doses (100, 200, 400mg/kg), hence, suggesting that *Gongronema latifolium* leaf possesses stimulatory activity on humoral response to SRBCs probably by acting as antigen that stimulates B-lymphocyte sub type to produce antibody in wistar albino rats. This finding correlates with the report by[57] on *Achillea wilhelmasi*.

Delayed-type hypersensitivity response

Delayed-type hypersensitivity (DTH) response is a protective cell-mediated immunity directed towards numerous intracellular infectious microorganisms, particularly those that cause chronic diseases like tuberculosis and leprosy. DTH reaction is characterized by large influxes nonspecific inflammatory cells including macrophages, memory T-cell, and $CD8^+$ T-cells, with macrophages constituting the majority.[58,59] DTH reaction is initiated by recognition and activation of T-lymphocytes by exposure to a specific antigen such as SRBC used in this study, giving rise to sensitized T-cells. Activation of T-cells by antigens presented through appropriate antigen-presenting cells (APCs) results in secretion of various cytokines including interleukin-2, gamma interferon, macrophage migration inhibition factor and alpha tumor necrosis factor.[60,61] These in turn increase vascular permeability, vasodilation and attract scavenger phagocytic cells to the site of reaction, hence, leading to localized inflammation (i.e. edema of the foot pad) that characterizes delayed-type hypersensitivity observed in this study. The result of this study in table 4 shows a dose-dependent increase in foot pad thickness in the group administered with the extract as compared to the negative control group. The observed increase in foot pad thickness in previously immunized Wistar albino rats may be pointing to the ability of ethanol extract of *Gongronema latifolium* leaf to activate the adaptive immune cells including lymphocytes, monocytes and/or macrophages that participate in cell-mediated immunity. Similar finding was reported on the same plant by.[43]

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