Immunomodulatory Activity on Specific Immune Function in Albino Mice Exposed to Extract and Fractions of *Zapoteca portoricensis* (Jacq) HM. Hernández Roots

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TCA and BCO conceived and designed the experiments. Authors ODW and UCA performed the experiments. Authors BCO, ODW and TCA analyzed the data. Authors BCO and UCA prepared the manuscript draft. Author TCA made manuscript revisions. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Zapoteca portoricensis (Jacq) HM. Hernández popularly called "Elugelu" belongs to the Fabaceae family. It is reportedly used in traditional medicine as anti-diarrhoea, anticonvulsant, antispasmodic and in management of autoimmune disorders. The immunomodulatory activity of methanol root extract and fractions of *Zapoteca portoricensis* was evaluated using three experimental models: neutrophil adhesion, haemagglutination antibody (HA) titre and delayed hypersensitivity (DTH) of an immune response in mice. Five (5) groups of mice (n=5) were used; group I was the normal control; group II was the standard control (levamisole, 2.5 mg/kg) while groups III[A–C], IV[A–C] and V[A–C] received graded doses (25, 50, 100 mg/kg) of methanol extract (ME), ethyl acetate fraction (EF) and methanol fraction (MF), respectively. Sheep red blood cells (SRBCs; 0.1 ml) were injected

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subcutaneously to sensitize the animals. The study results showed that the methanol fraction (MF) exhibited the highest percentage (46.12%) in neutrophil adhesion followed by the ethyl acetate fraction (EF) (37.06%) at 100 mg/kg dose, respectively, compared with the normal control. The highest percentage increase in both primary and secondary antibody titre was found to be ME (85.19%, 43.20%), EF (91.53%, 102.67%) and MF (128.31%, 111.89%) at 50 mg/kg dose, respectively compared to the normal control. The EF at the doses of 25 and 50 mg/kg produced the highest percentage inhibition (56.57%, 58.33%) in DTH response, respectively, followed by MF (42.46%) at a dose of 25 mg/kg. Oral administration of *Z. portoricensis* exhibited immunomodulatory effects on specific components of the immune system in mice.

1. INTRODUCTION

Immunomodulation is a process used to describe any change in the immune response that can involve enhancement or suppression of any part or phase of an immune response [1]. Protection of the host against pathogenic microbial agents is one of the vital roles of the body immune system. The two major subsystems of the immune system: nonspecific immune response (also known as innate or natural immunity) and specific immune response (also known as acquired or adaptive immunity) work together to eliminate pathogenic microorganisms and other foreign agents. The cells responsible for both nonspecific and specific immunity are the white blood cells (leukocytes). The presence of foreign antigens produces immediate response by the activation of immune component cells and the production of cytokines, and a variety of inflammatory proteins that function to protect the host [2]. Chemical substances that can influence a specific immune function or modifies one or more components of the immunoregulatory network are clinically used as immunomodulatory agents. Generally, immunomodulators act by achieving immunostimulation (as in the treatment of AIDS) or immunosuppression (e.g., the treatment of autoimmune disease) [3,4].

Medicinal plants, since times immemorial, are known to have therapeutic effects and have been used in the treatment of human and animal diseases. In recent times, much success has been attained in the screening of different medicinal plants for possible immunomodulatory properties. A few of such plants include *Phyllanthus emblica*, *Momordica charantia*, *Buchholzia coriacea*, *Moringa oleifera*, *Dendropanax morbifera*, *Bougainvillea xbuttiana*, *Mahonia aquifolium*, *Terminalia catappa*. With regards to new and reemerging infectious diseases (Ebola, SARS, MERS, Dengue, Zika, tuberculosis, HIV/AIDS), and with the limitations of synthetic drugs, agents from natural sources that can activate host defence mechanisms can provide supportive therapy to conventional treatments. *Zapoteca portoricensis* (Jacq) HM. Hernández (commonly known as white stickpea) is a perennial glabrous shrubby plant belonging to the Fabaceae family [5,6,7]. The immunomodulatory activity of extracts of various plants belonging to the Fabaceae family is well documented. Extracts of plants contain secondary metabolites which could act on the immune system by either suppressing or stimulating innate or adaptive immune cells/molecules [8]. *Z. portoricensis* is widely distributed in Southeast Asia, West Indies, Atlantic Coast of America and West Tropical Africa. It has slender unarmed branches and long cream coloured flowers, in axils of small and oval green leaves. The flowers are perfect, pentamerous and radially symmetrical with flat fruits. It is popularly called "Elugelu" in eastern Nigeria and its leaves and roots have a long history of medicinal uses that are still employed today. It is reportedly used in traditional medicine for wound healing, tonsillitis, gastrointestinal disorders, and as an anticonvulsant and antispasmodic [9]. Additionally, the plant has been exploited extensively in terms of its pharmacological effects and the results indicate potent antifungal and antibacterial activity [10], antiulcer activity [11] anti-inflammatory activity [12], antimicrobial activity [13], antimalarial activity [14], anti-BPH activity [15], among other ailments *in vitro* and/or in animal models. Nwodo et al. [16] isolated two dipeptides, saropeptate (aurantiamide acetate) and anabellamide from the methanol extract of *Z. portoricensis* root which possessed antitrypanosomal activity. Notwithstanding, its long term folkloric use for therapeutic purposes, no information is available on its effect on immune response. The present study, therefore, investigated the immunomodulatory activity of *Z. portoricensis* using animal models.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction Procedure

Fresh roots of *Z. portoricensis* were collected from Orba, Nsukka Local Government Area, Nsukka, Nigeria. The plant was identified and authenticated by Mr A. O. Ozioko, Botanist, International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka (reference specimen no: InterCEDD/16043). The roots were air-dried and pulverized using electric grinder machine. About 200 g of the powdered material was subjected to extraction using 10 L of methanol by cold maceration and filtered after 48 hours. The resulting filtrate was concentrated using a rotary vacuum evaporator (40 °C) under reduced pressure to obtain the methanol extract (ME; 160 g; 5.33% w/w). The methanol extract (ME, 150 g) was subjected to solvent-guided fractionation in a silica gel (70-230 nm mesh size) column (60cm length x 7.5cm diameter) using n-hexane, ethyl acetate and methanol as solvents. The fractions were concentrated to obtain the n-hexane fraction (HF; 0 g, 0% w/w), ethyl acetate fraction (EF; 3.34 g, 2.22% w/w) and methanol fraction (MF; 46.25 g, 30.80% w/w). The n-hexane fraction yield was very poor and insignificant for testing.

The yield (%) of the extraction and fractionation process was calculated as follows:

Yield of extract (%) =

$$\frac{\text{Weight of final extract(g)}}{\text{Weight of plant material macerated}} \times 100$$

Yield of fraction (%) =

$$\frac{\text{Weight of final fraction (g)}}{\text{Weight of extract fractionated (g)}} \times 100$$

2.2 Red Blood Cell Antigen

Fresh sheep red blood cells (SRBCs) were obtained from Veterinary Medicine Teaching Hospital, University of Nigeria, Nsukka. Before used, blood plasma cells were washed three times in a copious volume (15 ml) of pyrogen-free sterile normal saline by centrifugation at $3000 \times g$ for 10 minutes on each occasion. The settled SRBCs were then suspended in normal saline and concentration adjusted to 0.5×10^9 cells/ml [17].

2.3 Evaluation of Immunomodulatory Activity

2.3.1 Experimental animal design

For all the studies, twenty-five (25) adult albino mice (18-25 g) of either sex were used. The mice were purchased from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. They were housed under standard conditions of temperature (25°C), 12 h/12 h light/dark cycles and fed with standard laboratory pellets (Guinea Feeds Nigeria Limited) and tap water *ad libitum*. The animals were divided into five groups (n=5) as follows:

Group I: received vehicle (distilled water, 10 ml/kg) as normal control.
Group II: received levamisole (2.5 mg/kg) as a standard control.
Group III[A] – III[C]: received methanol extract (ME, 25, 50, 100 mg/kg).
Group IV[A] – IV[C]: received ethyl acetate fraction (EF, 25, 50, 100 mg/kg).
Group V[A] – V[C]: received methanol fraction (MF, 25, 50, 100 mg/kg).

All animal experiments were conducted according to the institutional principles on the use of laboratory animals and in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (Pub No. 85–23, revised 1985).

2.3.2 Neutrophil Adhesion test

Neutrophil adhesion test in mice was done using the modified method [18,19]. Mice were pre-treated with extract, fraction or standard orally for fourteen days. On day 14, blood samples were collected from the retro-orbital plexus in heparinized vials and analyzed for total leukocyte count (TLC). The differential leukocyte count (DLC) was performed by fixing the blood smear and stained with Leishman's and the neutrophils percentage in each sample were determined. After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 minutes at 37 °C. The incubated blood samples were re-analyzed for TLC and DLC, respectively. The product of TLC and per cent neutrophils gave the neutrophil index of the blood sample. The percentage neutrophil adhesion was calculated using the formula:

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

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

2.3.3 Haemagglutination antibody (HA) titre

Haemagglutination antibody titre test in mice was done using the method of Sharma [20], with slight modifications. Animals received graded doses of the extract and fractions of *Z. portoricensis* roots as described in the experimental protocol. One hour later, about 0.1 mL of suspension containing 40% of sheep red blood cell (SRBC) was injected subcutaneously into the subplantar region of the right hind paw to sensitize the animals. The day was recorded as day zero. The treatment was continued once daily for fourteen days. On day 7 of post-treatment, blood samples were collected from the retro-orbital plexus and serum separated to estimate primary hemagglutination antibody titre. Two-fold diluted serum in saline (25 µl) was challenged with 25 µl of 1% (v/v) SRBC in U-shaped microtitre plates and incubated at 37°C for 1 hour and then observed for hemagglutination. The value of the highest serum dilution that showed visible hemagglutination was taken as the primary antibody titre. The secondary antibody titre was determined on day 14 (7 days after challenge with 0.1 ml of 40% SRBC).

2.3.4 Delayed-type hypersensitivity (DTH) test

The method of Sharma [20] was adopted with slight modifications. On day 7, DTH was induced in mice using SRBCs as antigen after pre-treatment with the extract and fractions of *Z. portoricensis* roots. About 0.1 mL of suspension containing 40% of sheep red blood cell (SRBC) was injected subcutaneously into the subplantar region of the right hind paw to sensitize the animals. The day was recorded as day zero. The administration of the extract and fractions of *Z. portoricensis* roots was continued once daily until the 14th day. On day 14, the sizes of the left hind paw of the mice were measured using micrometre screw gauge and the animals challenged by subcutaneously injecting 0.1 ml of 40% SRBC into the left subplantar of the paw. The DTH was determined by measuring the volume of the paws of each mouse after 24 h of challenge using vernier callipers. The percentage inhibition was calculated using the formula:

$$\text{Percentage inhibition (\%)} = \frac{\text{PVu} - \text{PVt}}{\text{PVu}} \times 100$$

Where PVu = Paw volume of untreated group. PVt = Paw volume of treated group.

2.4 Statistical Analysis

The data obtained were analysed using One-Way Analysis of Variance (ANOVA) followed by Post Hoc Dunnett's test. Results were expressed as mean ± SD (n=5). Differences between mean values were considered significant at 5% level of significance (i.e. p<0.05).

3. RESULTS

The effect of *Z. portoricensis* on neutrophil adhesion is shown in Table 1. The neutrophil adhesion to nylon fibre significantly ($p < .05$) increased in the group that received ME (25, 50 and 100 mg/kg) compared to the control. At 50 mg/kg, a maximum percentage neutrophil adhesion (19.76%) was observed. Similarly, EF and MF significantly ($p < .05$) increased neutrophil adhesion to nylon fibre compared with the control, and maximum percentages of 37.06% and 46.12% observed at 100 mg/kg, respectively. The standard, levamisole (2.5 mg/kg) showed lower neutrophil adhesion (28.46%) than *Z. portoricensis* treatment.

The effect of *Z. portoricensis* on HA titre is shown in Table 2. The extract and fractions of *Z. portoricensis* showed significant ($p < .05$) stimulatory effect on humoral immune system at the different doses (25, 50, 100 mg/kg) administered. The highest percentage increase in HA titre was observed at 50 mg/kg dose for ME (85.19%, 43.20%), EF (91.53%, 102.67%), and MF (128.31%, 111.89%) whereas levamisole (2.5 mg/kg) showed the least percentage increase (15.87%, 12.86%), respectively, for both primary and secondary response.

The effect of *Z. portoricensis* on DTH antibody response is shown in Table 3. DTH response was non-significantly ($p > .05$) lower in the group that received varying doses of ME (25, 50, 100 mg/kg) compared with the control group. However, the DTH response of the group that received EF and MF was significantly ($p < .05$) lower compared with the control. The highest percentage inhibition in DTH response was observed as 58.53% and 42.46% for EF (50 mg/kg) and MF (25 mg/kg), respectively. Similarly, the DTH response for levamisole treated group was significantly ($p < .05$) lower with a percentage inhibition of 20.05%.

Table 1. Effect of methanol root extract and fractions of *Z. portoricensis* on neutrophil adhesion test

Treatment	Dose (mg/kg)	Neutrophil index (AxB)		Neutrophil adhesion (%)
		UB	FTB	
Control	-	172.62±.7.96	151.52±.5.16	[12.22]
Levamisole	2.5	305.53±24.71*	223.46±16.54*	[26.86]
ME	25	292.06±18.15*	247.06±11.75*	[15.40]
	50	312.00±20.53*	250.36±16.54*	[19.76]
	100	291.35±17.37*	247.40±15.69*	[15.08]
EF	25	391.87±23.44*	326.65±19.07*	[16.64]
	50	322.76±27.65*	212.29±23.48*	[34.23]
	100	340.30±23.36*	214.18±27.53*	[37.06]
MF	25	365.08±18.68*	303.83±6.08*	[16.78]
	50	384.46±29.77*	266.32±8.70*	[30.73]
	100	397.22±30.25*	214.02±9.29*	[46.12]

Values are expressed as mean ± SEM; n=5, *p < .05 when compared with the control (One way ANOVA; Dunnett's post hoc). UB - untreated blood; FTB -fiber treated blood; ME=methanol extract; EF= ethyl acetate fraction; MF= methanol fraction. Values in parenthesis represent neutrophil adhesion (%) relative to control

Table 2. Effect of methanol root extract and fractions of *Z. portoricensis* on haemagglutination antibody (HA) titre

Treatment	Dose (mg/kg)	Haemagglutination Antibody Response	
		Primary	Secondary
Control	-	3.78±0.33	4.12±0.39
Levamisole	2.5	4.38±0.64 [15.87]	4.65±0.64 [12.86]
ME	25	5.28±0.23 [39.68]*	4.22±0.38 [2.43]
	50	7.00±0.91 [85.19]*	5.90±0.48 [43.20]*
	100	5.46±0.87 [44.44]*	4.84±0.52 [17.48]
EF	25	5.62±0.34 [48.68]*	7.18±0.61 [73.79]*
	50	7.24±0.73 [91.53]*	8.35±0.97 [102.67]*
	100	5.16±0.51 [36.51]*	6.20±0.64 [50.49]*
MF	25	5.20±0.46 [37.57]*	5.88±0.45 [42.72]*
	50	8.63±0.31 [128.31]*	8.73±0.77 [111.89]*
	100	4.47±0.25 [18.25]	5.00±0.69 [21.36]

Values are expressed as mean ± SEM; n=5, *p < .05 when compared with the control (One way ANOVA; Dunnett's post hoc). ME=methanol extract; EF= ethyl acetate fraction; MF= methanol fraction. Values in parenthesis represent change (%) in hemagglutination antibody titre relative to control

4. DISCUSSION

Many natural products have been reported to modulate immune reaction either by stimulation or suppression. With regards to new and re-merging infectious diseases, agents from natural sources that can modulate the host defence mechanisms by suppression of hyperactive responses or enhancement of impaired immune responses may be beneficial in management of autoimmune disorders. The present study investigated the immunomodulatory effects of the extract and fractions of *Z. portoricensis* root using three experimental models (neutrophil adhesion, HA titer and DTH) of an immune response in mice. Immune responses were initiated by injecting sheep red blood cells (SRBCs) in mice, subcutaneously. SRBC, a non-replicating particulate antigen, induces a T-cell dependent immune response mediated by CD4 T cells [21].

Neutrophil, as the first defense line against invading pathogens, play an important role in both innate and adaptive immunities through different mechanisms. They are capable of a wide range of functions including chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing of a pathogen [22]. Neutrophils circulate in the vasculature in a

Table 3. Effect of the methanol root extract and fractions of *Z. portoricensis* on Delayed-Type Hypersensitivity (DTH) reaction

Treatment	Dose (mg/kg)	Paw volume (24 mm) (mg/kg)	Inhibition (%)
Control	-	6.24±0.04	-
Levamisole	2.5	4.96±0.18*	[20.05]
ME	25	5.56±0.29	[10.90]
	50	5.61±0.16	[10.10]
	100	5.92±0.11	[5.13]
EF	25	2.71±0.22*	[56.57]
	50	2.60±0.14*	[58.33]
	100	3.20±0.16*	[48.71]
MF	25	3.59±0.22*	[42.46]
	50	4.11±0.42*	[34.13]
	100	4.10±0.31*	[34.29]

*Values are expressed as mean ± SEM; n=5, *p < .05 when compared with the control (One way ANOVA; Dunnett's post hoc). ME=methanol extract; EF= ethyl acetate fraction; MF= methanol fraction. Values in parenthesis represent inhibition (%) in DTH response relative to control*

passive state and become more adhesive upon stimulation at sites of inflammation [23]. In this study, the stimulation and/or release of neutrophils in the SRBCs-sensitized animals was determined. An indication of inflammation is an increase in neutrophil adhesion to nylon fibre. Pre-treatment with *Z. portoricensis* extract and fractions increased significantly the neutrophil adhesion to nylon fibre when compared with the control group. The methanol fraction (MF) exhibited the highest percentage (46.12%) in neutrophil adhesion followed by the ethyl acetate fraction (EF) (37.06%) at 100 mg/kg dose, respectively, as compared with the standard, levamisole (26.86%). This confirms the stimulation of margination of polymorphonuclear cells in the blood vessels, thereby protecting the animals against SRBCs-induced lethal inflammation. This is consistent with previous reports that increased neutrophil adhesion may be attributed to the upregulation of β2 integrins through which neutrophils are capable of adhering firmly to nylon fibres [17,24,25].

The haemagglutination antibody (HA) titre is a more sensitive test used to determine the level of antibodies generated in response to antigenic SRBCs. Elevated HA titre indicates an augmentation in humoral immunity which is exclusively mediated through the various actions of synthesized immunoglobin (IgG and IgM) and involves opsonization, direct neutralization of antigen and activation of complement system which results to lysis and death of antigenic cells

[26,27]. *Z. portoricensis* extract and fractions showed a stimulatory effect on primary and

secondary antibody formation. The highest percentage increase in primary and secondary antibody titre was found to be ME (85.19%, 43.20%), EF (91.53%, 102.67%) and MF (128.31%, 111.89%) at 50 mg/kg dose, respectively, as compared to the standard control, levamisole with the least percentage (15.87%, 12.86%). The primary response consisted mainly of immunoglobin-M while the secondary response consisted mainly of immunoglobin-G (sometimes IgE and IgA) [28]. In SRBCs-sensitized animals, the secondary titre was expectedly higher than the primary titre due to subsequent antigenic stimulation of priory-sensitized animals resulting in higher antibody production [29]. Solanke and Jain [30] also evaluated the immunomodulatory activities of *Vigna mungo* L. extract and observed that on re-exposure to the antigen; a secondary response is elicited that is characterized by a rapid onset and highly amplified level of antibody production. This is in contrast to the report of Mubashir et al. [31] which showed a pronounced production of primary antibodies as compared to secondary antibodies in SRBCs-sensitized animals. However, interestingly, no particular pattern in the activation of antibodies was observed except in the consistent enhancement of antibody titres in the test groups as compared with the corresponding values from the control group. A similar result was observed in the standard pre-treated group, levamisole. Thus, the differences in HA titres for both primary and secondary response could be a consequence of differential physiology of the animals. The augmentation, therefore, of the humoral immune response validates the stimulation of lymphocyte

proliferation, which in turn may lead to the production of cytokines that subsequently activate T and B lymphocyte subsets involved in antibody synthesis [32].

Delayed hypersensitivity (DTH) is recognized as an essential parameter in determining a cell-mediated immune response. It is mediated by mononuclear leukocytes and plays a major role in defense against viral, bacterial, parasitic infections or malignant cells and any other perceived foreign body [33]. The formation of oedema after injection of SRBC's into the hind paw of animals indicates recruitment of cells to the inflammatory site. This is dependent on the release of vasoactive and chemotactic factors that increase regional blood flow and leukocytes migration from the intravascular space into the tissues [31]. *Z. portoricensis* extract and fractions were not able to enhance the DTH response, in terms of the size of paw volume, as compared with the control group. The EF at the dose of 25 and 50 mg/kg produced the highest percentage inhibition (56.57%, 58.33%) in DTH response, respectively, followed by MF (42.46%) at a dose of 25 mg/kg. This inhibition in DTH response which is comparable to that of the standard, levamisole suggests that *Z. portoricensis* possesses immunomodulatory activity. This may be attributed to a suppressing effect on lymphocytes and accessory cell types required for the expression of cell-mediated immunity. From previous studies, it is evident that plant extract exhibited significant immunomodulatory activity on specific components of the immune system in animals challenged with SRBCs [34,35], which corroborate with the result of this study. Although the exact mechanism for this activity was not elucidated, it is, however, likely to be associated with secondary metabolites that have been reported to confer plants with several therapeutic potentials [36]. Recently, we observed the presence of saponins, steroids, flavonoids, terpenoids, glycosides and alkaloids in *Z. portoricensis* roots similar with the reports of Ukwe et al. [11] and Agbo et al. [12]. The immunomodulating effects of saponins, flavonoids and alkaloids are prominent and well documented [37,38]. More so, the effect of terpenoids on the immune system was reported to be two-fold; first to enhance antibody production and second to suppress T-cell response [39]. Hence, it is suggested that the presence, absence or synergy which occur among these secondary metabolites may have resulted in the amplification or inhibition of the

participating cells to produce the required response.

5. CONCLUSION

The results of the present study indicate that *Z. portoricensis* possesses immunomodulatory effects on specific components of the immune system. This effect may be attributable to specific secondary metabolites that are important in regulating normal immunological functioning. Further studies will be conducted on identification of the exact bioactive constituent(s) responsible for the immunomodulatory effect and also establish the molecular mechanism of action.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All animal experiments were conducted according to the institutional principles on the use of laboratory animals and in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (Pub No. 85–23, revised 1985).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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