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Immunomodulatory Activity of the Methanolic Extract of *Buchholzia coriacea* Seeds on *Trypanosoma brucei Brucei* Infected Mice

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Abstract: The current study was carried out to assess the immunomodulatory activity of methanolic extract of *Buchholzia coriacea* seeds on *Trypanosoma brucei brucei* infected mice comparing with Levamisole as drug reference. Delayed hypersensitivity reaction, humoral antibody response and *in vivo* leucocyte mobilization were assessed in three different experiments. A total of 75 mice at 25 mice per experiment were used for the study. The extract was able to inhibit delayed hypersensitivity reaction at 250 and 500mg/kg. However, the dose of 1000mg/kg significantly increased the paw size when compared with the control. The Levamisole group did not differ from the infected control. The extract caused elevation of secondary sheep RBCs specific antibody titre at all doses of the extract tested. The increased antibody titre was not dose dependent. The elevation was more pronounced at 500mg/kg than in 250 and 1000mg/kg. However, all the extract treated groups were significantly higher than the control but not with the Levamisole treated group. The effect of the extract on *in vivo* leucocytes mobilization led to increase in total leucocytes count. The dose of 1000mg/kg produced the highest leucocytes mobilization and was significantly higher than Levamisole and control groups. In differential leucocytes mobilization, all the doses tested improved the neutrophil and lymphocyte counts. However, the basophil, monocyte and eosinophil of the extract treated group showed decrease in number when compared with the control group. The results of the present study concluded that, *Buchholzia coriacea* seeds possess immunostimulatory activity on *Trypanosoma brucei brucei* infected mice.

Key words: *Buccholzia corieca* · Delayed Hypersensitivity Reaction · Antibody Response · Trypanosomosis

INTRODUCTION

The immune system has a fundamental role in protecting the body against the pathogenic microbial agents [1]. It is a part of body that detects the pathogen by using a specific receptor and produces immediate response by the activation of immune component cells, cytokines, chemokines and also release of inflammatory mediator [2]. The immune system can be manipulated by the use of immunomodulators in disease conditions by achieving immunostimulation (as in the treatment of AIDS) or immunosuppression (suppression of normal or excessive immune function (e.g. the treatment of graft rejection or autoimmune disease)) [3].

Animal trypanosomiasis constitutes a major threat to food security in several parts of sub-Saharan Africa including Nigeria [4, 5, 6]. FAO [7] reported that almost more than any other disease affecting both people and livestock, trypanosomiasis straddles the ground between human health, livestock health, agricultural production and rural development; consequently tackling trypanosomiasis has the potential to impact on all eight millennium development goals of the Organization which includes eradication of extreme poverty [8]. Direct losses due to trypanosomosis are estimated to amount to between US$ 1-1.2 billion each year whereas the indirect impact of AAT on agriculture in sub-Saharan Africa exceeds this amount [2, 9, 10].

Modulation of immune response to alleviate disease conditions has long since been of interest and increasingly recognized as a key component of effective disease control. Plant extracts have been widely investigated in this recent time in different parts of the world for their possible immunomodulatory properties [2, 11].

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Since most of the drugs currently available for treatment of African trypanosomosis are toxic, costly and no longer effective [12], attempts are being made in laboratories around the world to discover new, safe and cost effective molecules from medicinal plants with an ethnomedical history. The ethanol extract of *B. coriacea* seed was shown to have antitrypanosomal activity in mice experimentally infected with *Trypanosoma brucei* [13]. The plant has also been shown to possess antiplasmodial activity [14], antibacterial activity [15], larvicidal effect [16], antispasmodial and anti-diarrhoea properties [17], analgesic activity [18] and anthelminthic activity [16, 19]. The biological activities and mode of action of this plant extracts are poorly understood and may act directly or indirectly.

Phytochemical constituents of *Buchholzia coriacea* include; Alkaloids, Anthraquinones, Cardiac glucosides, Flavonoids, Saponins and Tanins [16, 20]. In this view, many plant extracts with immunomodulatory and antioxidant activities can be of great help in the control of trypanosomosis. Plants such as: *Caesalpinia bonducella, Rhododendron spiciferum, Curcuma longa linn, Azadiracta indica, Boerhaavia diffussa, Ocimum sanctum etc* are known to posses immunomodulatory activity [21]. Research has shown that some immunomodulators and antioxidants are known to be beneficial in control of trypanosome infection [1].

This work was therefore designed to study the immunomodulatory activity of ethanolic extract of *Buchholzia coriacea* seeds on *Trypanosoma b.brucei* infected mice with the aim of having a better understanding of the antitrypanosomal activity of the extract.

MATERIALS AND METHODS

Plant: *Buchholzia coriacea* is an evergreen under storey tree of the forest, 30 – 60ft high; Slash deep red, flowers cream, fruits yellowish when ripe, seeds blackish, spicy tasted. Common names - Wonderful cola, Musk tree and Elephant kola. It is found in Eastern and Western Nigeria, extending from the Ivory Coast to Gabon [22]. It was named after Buccholz who collected the plant in the Cameroons in the late 19th century.

Experimental Animals: A total of 75 adult male albino mice weighing (23-28g) were used for the study. The animals were housed in a fly proof laboratory animal house and given pelleted chick grower feed and water *ad libitum*. Animal studies were in compliance to the ethical procedure of the Animal Use and Care Committee, Faculty of Veterinary Medicine, University of Nigeria, Nsukka which corresponds with NIH guidelines [23].

Trypanosome Parasite: *Trypanosoma brucei brucei* was used for this study. It was isolated from a clinically sick dog. The trypanosome was maintained in the Department by serial passages in mice.

Preparation of the Plant Extract: Ground *Buchholzia coriacea* seed were extracted according to Alanis *et al* [24]. Seeds were defatted with hexane at room temperature by cold maceration with intermittent shaking in a shaker for 72hours. In the methanol extract, nitrogen gas was used to evaporate the solvent. To further ensure that all the water was removed, the extract was freeze dried (Edward's high vacuum crawley, England) the extract was stored at 4^0c until use.

Acute Toxicity Test: In the acute toxicity test, graded doses (250, 500, 1000, 2000mg/kg) of the extract was administered intra peritoneally to 4 groups of 6 mice each. They were monitored for acute toxicity signs like behavioral changes or death for a period of 24hours (Nweze *et al* 2009).

Technique for Estimating Trypanosomes: This technique was described by Herbert and Lumsden [25]. A drop of blood from the tail of a mouse was examined under x 40 magnification of a table microscope and the number of trypanosomes in each field was counted. Each counting per field was matched with log. figures obtained from the reference table [25]. The log figures were converted to anti-log and then subsequently converted to absolute numbers which reflected the number of trypanosomes per ml.

Red Blood Cell Antigen: Fresh sheep red blood cells (SRBCs) used as antigen were obtained from blood collected by venipuncture of a West African dwarf sheep. Three milliliters of sheep blood was collected. Before use, the red blood cells were washed three times with 15ml of phosphate buffered saline (PBS), PH 7.2, by centrifugation at 3000xg for 10 minutes on each occasion using desktop centrifuge. After the final wash, the SRBSCs were suspended in PBS as a 2% suspension (based on packed cell volume) for the serological tests and as a 10% suspension for immunization of the mice. A 1m1 amount of the 2% suspension contained approximately 10^a red blood cells [26].

Delayed Hypersensitivity Reaction: Delayed hypersensitivity reaction was induced in mice using SRBCs as antigen [26]. Twenty five adult male mice divided into five groups for 5 mice each were used for the study. The mice were infected with 1.00 x 10^6 *Trypanosoma b brucei* intraperitoneally. On day 7 post infection, groups A, B and C were given 250, 500 and 1000mg/kg of the extract respectively while group D received 7.5mg/kg body weight of Levamisole and group E was left untreated as control sheep RBCs (0.2ml of 109 cells ml) were administered (subcutaneous) on the planter region of right hind foot pad on day 0 to sensitize the animals. On day 5, subcutaneous injection of the same amount of antigen was administered in the left hind pad as a challenge. Edema was produced by antigenic challenge in the left foot pad and was measured as the difference in the paw thickness before and 24hours after the challenge. This was done with a vernier caliper [27]. *B. coriacea* (250, 500 and 1000mg/kg) was administered 3 days prior to sensitization and continued till challenge [27]. Also, the parasitaemia were estimated the day the administration of *B coriaca* started and again on the day the administration stopped 8 days later using Herbert and Lumsden [25] method.

Humoral Antibody Response: Twenty – five adult male mice divided into five groups of 5 mice each were used for the study. The mice were infected with 1.00 x 10^6 *Trypanosoma b. brucei* intraperitoneally. On day 7 post infection, groups A, B, C were given 250, 500 and 1000 mg/kg of the extract respectively while group D received 7.5 mg/kg body weight of Levamisole and group E was left as a control. Sheep RBCs Co.1m1^{-1} of 10^9 m1^1) were used to immunized by intra peritoneal injection on day 0 and challenged by similar IP injection of the same amount in day 5 post immunization (PI). Primary antibody titre on day 10 PI by the hemagglutination technique [28].

***In vivo* Leucocytes Mobilization:** Leucocytes mobilization described by the method of Ribeiro *et al.* [29] the effect of the *Buchholzia coriacea* extract on *in vivo* leucocytes migration induced by inflammatory stimulus was investigated. Twenty-five adult male mice divided into five groups of 5 each were used for the study. The mice were infected with 1.00 x 10^6 *Trypanosoma b. brucei* intraperitoneally. On day 7 post infection, groups A, B and C were given 250, 500 1000mg/kg weight of Levamisole, intraperitoneally and group E was left untreated and used as the control. Oral administration of the *B. coriacae* (250, 500, 1000 mg/kg) was given to the mice. One hour later, each mouse in group (A, B, C, D and E) received intraperitoneal injection of 0.5 ml of 3% agar suspension in normal saline. Four hours later, the mice were sacrificed under anesthesia and the peritoneum washed with 5ml of phosphate buffer saline containing 0.5ml of 10% EDTA. The peritoneal fluid was recovered and total leucocytes counts (TLC) determined with hemocytometer and the differential cell count was determined by microscopic counting of Giemsa stained perfusate smear on glass slide.

Statistical Analysis: Results were analysed using one way Analysis of Variance (ANOVA; Fischer LSD post hoc test) and expressed as mean±standard error of mean. Differences between means of treated and control groups were considered significant at $p<0.05$.

RESULTS

The result of the acute toxicity tests showed that the extract did not cause clinical signs or death within 24 hours post treatment at all the dose levels tested. The extract was able to inhibit delayed hypersensitivity reaction at 250 and 500mg/kg (Table 1). However, at 1000mg/kg, the paw size increased significantly when compared with the control. The Levamisole treated group showed no difference from the infected control. The extract was able to reduce the parasite level in the treated groups with the 500 and 1000 mg treated group being significantly ($p \leq 0.01$) lower than other groups (Table 2). The extract caused elevation of secondary sheep RBCs specific antibody titre (Table 3) at all doses of the extract tested. The increased antibody titre was not dose dependent. The elevation was more pronounced at 500mg/kg than in 250 and 1000mg/kg. However, all the extracts treated groups were significantly higher than the control but not with the Levamisole treated group. The effect of the extract on *in vivo* leucocytes mobilization led to increase in total leucocytes count. The 1000mg/kg produced the highest leucocytes mobilization and was significantly higher than Levamisole and control group. In differential leucocytes mobilization, all the tested doses improved the neutrophil and lymphocyte count. However, the basophil, monocyte and Eosinophil of the extract treated group showed decrease in number when compared with the control group (Table 4).

Table 1a: Effect Of Methanolic Extract Of Buchholzia Coriacea seeds On Delayed Hypersensitivity Reaction Mice Estimated As Paw Swelling (Mm)

Treatment	Dose(mg/kg)	Paw swelling (mm)	Inhibition (%)
Extract	250	0.84±0.22[a]	20.8
Extract	500	0.84±0.07[a]	20.8
Extract	1000	2.14±0.02[b]	-101.9
Levamisole	7.5	1.06±0.09[a]	0
Control	-	1.06±0.09[a]	-

Mean±SEM with different superscript letters are significantly different ($p<0.05$)

Table 2: Effect of Methanolic extract of *Buchholzia coriacea* seeds on the parasitaemia (10^6 parasites/ ml) before and after treatment.

Treatment	Dose(mg/kg)	Day 0 of the treatment Parasites (10^6)	Day 8 of the treatment Parasites (10^6)
Extract	250	22.38±42321.09	28.32±43425[a]
Extract	500	19.02±81782.12	18.09±109934[b]
Extract	1000	20.87±11223	16.82±98852[b]
Levamisole	7.5	34.98±87822.65	35.12±21345[a]
Control	-	29.23±34245	89.93±23424[c]

Mean±SEM with different superscript letters are significantly different ($p<0.05$)

Table 3: Effect of Methanolic extract of *Buchholzia coriacea* seeds on primary and secondary antibody response ($log_{10}2$) in mice

Treatment	Dose (mg/kg)	Primary antibody	Secondary antibody
Extract	250	3.20±1.36	3.20±1.36
Extract	500	3.20±0.49	8.00±3.58
Extract	1000	5.60±2.64	6.80±2.50
Levamisole	7.5	2.80±0.80	6.80±3.77
Control	-	1.20±0.49	1.60±0.40

Mean±SEM with different superscript letters are significantly different ($p<0.05$)

Where the superscript letters in this table

Table 4: Effect of *Buchholzia coriacea* seeds extract on total and differential leucocytes mobilization (cells/ml) in mice

	250(mg/kg)	500(mg/kg)	1000(mg/kg)	Levamisole	Control
TLC	1450.00±0.22	1430±367.29	1670±371.01	1110.00±167.63	950.00±184.3
NEUT	279.80±70.59	362.60±104.53	397.40±84.72	277.80±69.35	182.00±733.40
LYMPH	1149.40±369.63	1042.80±265.98	1303.80±100.52	826.40±103.85	733.40±144.79
BASO	5.80±5.80[a]	0.00±0.00[a]	0.00±0.00[a]	1.60±0.60[a]	16.60±3.49[b]
MONO	10.20±2.34[ab]	1.40±0.40[a]	17.80±4.91[b]	1.60±0.60[a]	18.00±3.27[b]
EOSIN	0.00±0.00	5.80±0.50	5.80±0.55	0.00±0.00	6.40±2.91

Mean±SEM with different superscript letters are significantly different ($p<0.05$)

DISCUSSION

The result of the acute toxicity tests showed that the extract has a very wide safety margin. This is an indication that the extract was well tolerated by mice. This was in agreement with earlier work done by Nweze *et al*. [13]. The delayed hypersensitivity reaction was inhibited by the extracts at dose of 250 and 500 mg/kg. The ability of the extract to inhibit the delayed hypersensitivity reaction in this experiment may be due to its anti-inflammatory properties. This reaction is mediated by T cells and monocytes/macrophages rather than by antibodies. Delayed hypersensitivity reaction is a major mechanism of defense against various intracellular pathogens including Mycobacterium, fungi and certain parasites and it occurs in transplant rejection and tumor immunity [30, 31]. The term "Delayed" is used to differentiate a secondary cellular response which appears 48-72hours after antigen exposure from an immediate hypersensitivity response which generally appears within 12 minutes of an antigen challenge. DTH requires specific recognition of the given antigen by activated T lymphocytes which subsequently proliferate and release cytokines [32]. These will in turn increase vascular permeability and activation, promoting increased phagocytic activity and increased concentration of lytic enzymes for effective killing [33, 34]. This could be responsible for the increase in paw size (Erythema) as seen in 1000mg/kg. The increase noticed at 1000 mg was indication that the extract may have both pro- and anti-inflammatory properties, which depends on the dose used. The ability of the extract to reduce the parasite level was in agreement with work done by Nweze *et al*. [13] on *T brucei* but at variance with report by Nweze *et al*. [35]using *T congolense*.

The ability of the extract to reduce the parasitaemia in this experiment could be attributed to prolonged administration and its immunomodulatroy affect.

The sheep RBCs antibody titer was elevated in all extract treated groups. Effect of enhancement of the antibody production in this experiment may be associated with effect of the extract on lymphoid organs. When the mice were sensitized with the sheep RBCs, SRBC antigen were taken up by macrophages and are processed. When a T lymphocyte sees the processed antigen on the B cell, the T cell stimulates the B cells to undergo repeated cell divisions, enlargement and differentiation to form a clone of antibody secreted by plasma cells [36]. Hence the antibody then binds to the antigen, making them easier to ingest by the white blood cells. Antibodies are glycoproteins belonging to the immunoglobulin superfamily, they are typically made of basic structural units each with two large heavy chains and two small light chains [37]. Antibodies can occur in two physical forms [38], a soluble form that is secreted from the cell and a membrane bound form that is attached to the surface of B cells and is referred to as the B cell receptor (BCR) and is only found on the surface of B cells and facilitates the activation of these cells and their subsequent differentiation into either antibody factories called the plasma cells or memory B cells that will survive in the body and remember that same antigen, so the B cells can respond faster upon exposure [39]. Soluble antibodies are released into the blood and tissue fluids as well as many secretions to continue to survey for invading microorganisms. This indicates enhanced responsiveness of macrophages, T and B lymphocytes involved in antibody synthesis [40]. The present study showed that, the extract increased the total leucocytes count of the perfusate when compared with the control group. Leucocytes migration is important for the transport of immunological information between different compartments of the immune system [41]. In the current study, the differential leucocytes mobilization, all the tested doses improved the neutrophil and lymphocyte count.This result may be duo to enhanced the immune system. Neutrophils are normally found in the blood stream. During the beginning phase of inflammation (Acute) particularly as a result of bacterial infection, environmental exposure and some cancers. Neutrophils are one of the first responders of inflammatory cells to migrate towards the site of inflammation [42]. They migrate through the blood vessels, then through interstitial tissue, following chemical signals such as interlukin – 8, C5a, etc in a process called Chemo taxis. Neutrophils are recruited to the site of injury within minutes following trauma and are the hallmark of acute inflammation [43] while, the basophil, monocyte and Eosinophil of the extract treated group showed decrease in number when compared with the control group. These cells are more sensitive to the effects of immunomodulatory cytokines and pro-inflammatory factors, which are likely to be in abundance in inflammatory conditions [44]. Basophils, eosinophils and Th2 lymphocytes are also recruited to the site of inflammation and are direct leukocyte target of IL-33, suggesting a prominent role for these cells of the innate immune system in the biology of IL-33 [45]. Basophil play a potentially supportive role in anti-parasite immune responses through their activation by immune serum and production of cytokines [46].

The result of the current study suggests that immunomodulation may be a key factor in anti trypanosomal activity of Methanolic extract of *Buchholzia coriacea* seeds.

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