Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(1); 1-7

ISSN: 0975-4873

Research Article

Evaluation of the Immunomodulatory Activity of *Hoslundia opposita* Vahl (Lamiaceae) Leaf Extract

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Available Online:02nd December, 2015

ABSTRACT
The aerial parts of *Hoslundia opposita* are known to be used for treating many ailments including wounds and sores. The immunomodulatory properties of methanol extract and fractions of *Hoslundia opposita* leaf on Delayed Type Hypersensitivity reaction (DTHr), primary and secondary humoral response and *in vivo* leucocyte mobilization were evaluated. The methanol extract (ME) at 200, 400 and 800 mg/kg body weight produced significant ($P<0.05$) inhibition of DTH response in rat by 85, 75 and 85% respectively. The n-hexane, ethyl acetate and methanol fractions produced significant ($P<0.05$) inhibition of DTH response. Treatment of rats with single intraperitoneal injection of carrageenan after oral administration of extract and fractions resulted in an increase in leucocyte mobilization into the rat's peritoneal fluid which was significant ($p<0.05$). The total leucocytes counts (TLC) were higher in the extract and fractions-treated groups when compared to the control. However, a fall in primary and secondary antibody titre was observed with both ME and fractions suggesting that *H. opposita* may act through a cell mediated mechanism.

Keywords: *Hoslundia opposita*, immunomodulation, delayed type hypersensitivity, humoral response, leucocyte mobilization.

INTRODUCTION

Immunomodulation is a change in the body's immune system, caused by agents (immunomodulators) that activate or suppress its function. It is the adjustment of the immune response to a desired level, and involves immunostimulation, immunodepression, or induction of immunologic tolerance. Many proteins, amino acids, and natural compounds have shown a significant ability to regulate immune responses for example interferon-ɤ, steroids etc (Priyanka et al., 2012).

It has been observed that immunomodulatory substances from natural sources could play a role in disease prevention and treatment, especially with the increasing global emphasis on natural system and the campaign on "greening" of the society. So there has been a growing interest in identifying and characterizing natural compounds with immunomodulatory activity (Wang et al., 1991) ever since their possible uses in medicine have been suggested.

Hoslundia opposita Vahl (Figure 1) is an herbaceous perennial shrub belonging to the lamiaceae family. It is widely distributed in tropical and subtropical open lands of Africa (Morton, 1981). In Africa, various parts of *Hoslundia opposita* are popular remedies for gonorrhea, cystitis, cough, wounds, sores, conjunctivitis, epilepsy, chest pain, stomach disorders, mental disorders and also in management of snake bites (Ayensu and De Filipps, 1978; Watt and Breyer-Brandwijk, 1962).

It is also used to treat fever, liver disease, rheumatism and convulsion.

It is documented that methanol extract, methanol and ethyl acetate fraction of *H. opposita* stems have hepatoprotective effects against carbon tetrachloride and paracetamol induced liver damage (Akah and Odo, 2010). The crude extract of the entire plant have been found to exhibit strong antibacterial activity and volatile constituents have been identified as well as Jacarandic acid which was also isolated (Ogura et al., 1977; Takahashi et al., 1974). Extract from plant exhibited central nervous system depressant effect (Jide et al., 1999) and acaricidal effect against *Amblyomma variegatum* (Annan et al., 2011). It is also documented that leaf essential oil of *H. opposita* posses antidyslipidemic effect (Akolade et al., 2011). Pharmacological evaluation of the extract in rodents showed anti inflammatory, analgesic and antipyretic property (Oladije et al., 1998).

H. opposita leaf is used to treat boils in Nnobi Community of Idemili South Local Government Area of Anambra State, Nigeria. This study was aimed at investigating the immunomodulatory activity of leaves of *H. opposita.*

MATERIALS AND METHODS

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Table 1: Effect of ME on *in vivo* leucocyte mobilization in rats

Treatment	Dose (mg/kg)	TLC (cells/mm^3)	Differential Leucocyte Count (%) Neutrophils	Lymphocytes	Monocytes
ME	200	9900±1456.7* (83.3)	11.4±0.6 {8.1}	88.6±0.6 (1.4)	0.0±0.0
	400	7880±526.68 (45.9)	16.6±2.44 (33.9)	83.2±2.48 {4.8}	0.2±0.2
	800	7060±2322.8 (30.7)	15.6±3.46 (25.8)	84.4±3.46 {3.4}	0.0±0.0
Levamisole	2.5	6960±1034.7 (28.8)	14.4±3.26 (16.1)	85.6±3.26 {2.1}	0.0±0.0
Control	2.5 ml/kg	5400±1244.9	12.4±1.78	87.4±1.75	0.2±0.2

n=5; * P<0.05 compared to control LSD post-hoc

Value in parenthesis represent percentage increase (%) in TLC, neutrophils or lymphocytes as the case may be compared to control while values in curly bracket represent percentage decrease {%} in neutrophils or lymphocytes as the case may be compared to control.

Table 2: Effect of NHF, EF and MF on *in vivo* leucocyte mobilization in rats

Treatment	Dose (mg/kg)	TLC (cells/mm^3)	Differential Leucocyte Count (%) Neutrophils	Lymphocytes	Monocytes
NHF	200	21800±2219.9* (79.6)	9.2±1.59 (21.1)	90.8±1.59 {1.7}	0.0±0.0
	400	28400±3942.9* (133.9)	10.6±1.60 (39.5)	89.2±1.59 {3.5}	0.2±0.2
	800	29000±2717.3* (138.8)	9.4±3.08 (23.7)	90.6±3.08 {1.9}	0.0±0.0
EF	200	27400±1426.5* (125.7)	7.4±1.08 {2.6}	92.6±1.08 (0.22)	0.0±0.0
	400	30800±5096.5* (153.7)	11±3.36 (44.7)	89±3.36 {3.7}	0.0±0.0
	800	48900±641.87* (302.8)	6.4±1.17 {15.8}	93.4±1.21 (1.1)	0.2±0.2
MF	200	11400±322.49 {6.1}	10.6±2.16 (39.5)	89±2.12 {3.7}	0.4±0.4
	400	29400±965.40* (142.2)	8±0.71 (5.3)	91.2±1.07 {1.3}	0.8±0.8
	800	34700±694.26* (185.8)	13±2 (71.1)	87±2 {5.8}	0.0±0.0
Levamisole	2.5	12900±1184.9 (6.3)	12.2±2.82 (60.5)	87.4±2.82 {5.4}	0.4±0.4
Control	2.5 ml/kg	12140±2640	7.6±0.93	92.4±0.93	0.0±0.0

n=5 per group; *P <0.05 compared to control LSD Post hoc; Values in parenthesis represent percentage increase (%) in Total Leucocyte Count, Neutrophils or Lymphocytes as the case may be compared to control while values in curly bracket represent percentage decrease {%} in Total Leucocyte Count, Neutrophils or Lymphocytes as the case may be compared to control.

Drugs used were Levamisole (Reals Pharmaceutical Ltd, Nigeria), Dexamethasone (Pharmacare Ltd, Nigeria),.

Chemicals, Solvents and Reagents include analytical grades of methanol, n-hexane, ethyl acetate, Silica gel of size 60-120 mesh, carrageenan, Tween 80 (Sigma-Aldrich, Germany), distilled water, normal saline, Phosphate buffer saline, Ethylenediaminetetraacetic acid (EDTA).

Animals

Adult albino rats (100-150 g) of either sex were used. The animals were obtained from the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka. Animals were housed in steel cages within the facility under standard conditions and allowed free access to commercial pelletized rodent diet (Vital® feed Nigeria, Ltd) and water *ad libitum.*

Preparation and extraction of plant

Table 3: Effect of ME on delayed type hypersensitivity reaction in rats

Treatment	Dose (mg/kg)	Edema (ml)	Inhibition of edema (%)
ME	200	0.3±0.02*	85
	400	0.05±0.02*	75
	800	0.03±0.02*	85
Levamisole	2.5	0.13±0.02*	33
Control	-	0.2±0.3	-

n=5 per group; *$P < 0.05$ compared to control LSD Post hoc=

Table 4: Effect of NHF, EF, and MF on Delayed type hypersensitivity reaction in

Treatment	Dose (mg/kg)	Edema (ml)	Inhibition of edema(%)
NHF	200	0.163±0.03*	37.79
	400	0.113±0.02*	56.87
	800	0.05±0.00*	80.92
EF	200	0.21±0.03	19.85
	400	0.1±0.03*	61.83
	800	0.09±0.01	65.65
MF	200	0.11±0.02*	58.02
	400	0.09±0.03*	65.65
	800	0.082±0.03*	68.70
Levamisole	2.5	0.056±0.02	78.63
Control	-	0.262±0.04	-

n=5; *P <0.05 compared to control LSD Post hoc

Table 5: Effect of ME on hemagglutination antibody titre in rats

Treatment	Dose (mg/kg)	Hemagglutination Antibody Response	
		Primary	Secondary
ME	200	4±0.32	3±0.55*
	400	5.67±0.18*	4.67±0.48
	800	6.33±0.48*	6.0±0.63
Levamisole	2.5	5.67±0.18*	6.0±0.63
Control	2.5 ml/kg	4.33±0.48	6.33±0.85

n = 5 per group; * $p < 0.05$ compared to control LSD Post hoc

Fresh leaves of *Hoslundia opposita* Vahl were collected in September from bushes in Aregbe Obantoko in Abeokuta, Ogun State, Nigeria. The plant was identified and authenticated by O.A. Ugbogu, and O.S. Shasanya, taxonomists at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria and designated voucher specimen No. 109563. The leaves were cleaned, dried under shade for 3 days and pulverized to coarse powder using a milling machine. The leaf powder (4.608 kg) was extracted by cold maceration in methanol for 48 h and filtered. The residue was macerated again for 24 h to ensure exhaustive extraction. The residue was repeatedly washed with fresh solvent until filtrate became clear. The filtrate was concentrated using a rotary vacuum evaporator (40°C) under reduced pressure to obtain methanol extract (ME). The dry extract (174.538 g) was mixed with silica gel by triturating in a mortar to obtain a uniform mix. The mixture was air-dried at room temperature, packed into the glass column and was successively eluted with n-hexane, ethyl acetate and methanol in order of increasing polarity. The fractions were collected and concentrated in a rotary evaporator (40°C) under reduced pressure to obtain n-hexane (NHF), ethyl acetate (EF) and methanol (MF) fractions.

Carrageenan Induced Leucocyte Mobilization in Rats.

The rats of both sexes were randomly grouped (n=5). Groups 1, 2, and 3 received the extract ME (200, 400 or 800 mg/kg). Group 4 received levamisole (2.5 mg/kg) as reference standard and group 5 received 3% Tween 80 (2.5 ml/kg) as control. Administration was by the oral route according to their body weight. After one hour, each rat received intraperitoneal injection of 0.5 ml of 1% (w/v) carrageenan suspension in normal saline. Four hours later, the rats were sacrificed and the peritoneum washed with 5 ml of 5% solution of EDTA in Phosphate Buffered Saline (PBS) to recover the peritoneal fluid (Ribeiro et al., 1991). Total and differential leukocyte count (TLC) of the peritoneal fluid were performed.

This experimental protocol was carried out for HF, EF, and MF (200, 400 and 800 mg/kg).

Sheep Red Blood Cell (SRBC)-Induced Delayed Type Hypersensitivity Assay

Delayed type hypersensitivity was induced in rats using SRBCs as antigen (Sharma, 1994). Five groups (n=5) of rats were used for this test. The first, second and third groups received 200, 400 or 800 mg/kg of the extract respectively. Group 4 received 2.5 mg/kg of levamisole while group five received 2.5 ml/kg of vehicle (3% Tween 80) as negative control. All drug administration was done orally. One hour later, each animal received 0.1 ml of 40% sheep red blood cell (SRBC) suspension injected into the subplantar region of the right hind paw. The day was taken as day zero. Drug administration was continued once daily for seven days. On the 7^{th} day, the sizes of the left hind paw of the rats were measured by water displacement and the animals challenged by injecting 0.1 ml of 40% SRBC into the subplantar region of the left paw (Doherty, 1981). The volume of the left paws of each rat was measured 24 h after the challenge.

This experiment was also carried out for each of the fractions.

Hemagglutination Antibody Titre in rats

Five groups (n=5) of rats were used for this test. The first, second and third groups received 200, 400, 800 mg/kg of the extract respectively. Group 4 received 2.5 mg/kg of levamisole while group five received 2.5 ml/kg of vehicle (3% Tween 80) as negative control. All drug administration was done orally. One hour later, each animal received 0.1 ml of 40% sheep red blood cell (SRBC) suspension injected into the subplantar of the right hind paw. The day was taken as day zero. Drug administration was continued once daily for seven days.

Table 6: Effect of NHF, EF and MF on hemagglutination antibody response in rats

Treatment	Dose (mg/kg)	Hemagglutination Antibody Response	
		Primary	Secondary
NHF	200	3.75±0.73*	7.75±1.16*
	400	4.0±0.32	8.2±0.49
	800	4.0±0.32	9.5±0.22
EF	200	4.8±0.20	7.8±0.58*
	400	3.4±0.40*	8.0±0.63*
	800	4.0±0.32	9.5±0.22
MF	200	4.0±0.32	7.6±0.51*
	400	4.2±0.49	8.4±0.51
	800	3.8±0.58	8.4±0.68
Levamisole	2.5	3.6±0.87*	6.6±0.75*
Control	2.5	5.25±0.73	10±0.00

n = 5 per group; * p < 0.05 compared to control LSD Post hoc

On the 7th day, blood samples were collected from the retro-orbital plexus and serum separated to estimate primary hemagglutination antibody titre (Sharma et al., 1994).

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 highest dilution giving visible hemagglutination was taken as primary antibody titre.

The animals were challenged on day 7 by injecting 0.1 ml of 40% SRBC into the subplantar of the paw after collecting blood samples from the retro-orbital plexus. Drug administration was continued for the next seven days. On day fourteen, blood samples were collected for secondary antibody titre (Sharma et al., 1994).

Statistical analysis

Results were expressed as Mean ± standard error of mean (SEM). The results obtained were analyzed using One Way Anova in SPSS (version 16.0) and subjected to least significant difference (LSD) post-hoc test. Differences between means of treated and control groups were accepted significant at $P<0.05$

RESULTS

Effect of ME and fractions on in vivo leucocyte mobilization in rats

The ME elicited a significant and non-dose related increase in TLC. It increased neutrophils and elicited mild decrease in lymphocyte count (Table 1). The NHF, EF and MF elicited dose-dependent and significant increase in TLC. They also increased neutrophils but decreased lymphocytes with the exception of EF (200 and 800 mg) which elicited mild reduction in neutrophil count with mild increase in lymphocyte count (Table 2).

Effect of ME and fractions on Delayed type hypersensitivity reaction (DTHr) in rats

Methanol extract at 400 and 800 mg/kg elicited a significant ($P<0.05$) and non dose related inhibition of delayed type hypersensitivity response in rats but not 200 mg/kg ME (Table 3). The fractions (200, 400 and 800 mg/kg) elicited a significant ($p<0.05$) and dose related inhibition of DTHR (Table 4).

Effect of ME and fractions on hemagglutination antibody titre in rats

The ME elicited a dose dependent and significant ($p<0.05$) elevation of primary antibody titre compared to the control. The secondary antibody titre increased with increase in dose of MF; however, it was lower than the control (Table 5). Rats treated with the fractions produced a lower primary and secondary antibody titre than in the control group; however, antibody titre slightly increased with higher doses of the fractions (Table 6).

DISCUSSION

This experiment investigated the immunomodulatory effect of methanol extract and fractions of *H. opposita* leaves in rats. Methanol extract and fractions elicited increase in TLC and neutrophil count compared with the negative control. The differential count showed that neutrophils were the most mobilized leucocyte. The increase in neutrophil count compared to the control group may help in increasing the general resistance of the body (as PMNs are the primary cells which engulf and eliminate invading micro-organisms) against microbial infections via phagocytosis (Wheater and Stevens, 2002). It has been observed that the chemotactic movement of neutrophils towards the foreign body is the first and most important step in phagocytosis (Ganachari et al., 2004). Little percentage of monocytes was mobilized while eosinophils and basophils were absent. Monocytes float in the blood stream, enter tissue and turn into macrophages which clean up pus as part of the healing process. They share the phagocytic function of neutrophils and also present pieces of pathogens to T cells so the pathogens may be recognized again and killed.

The manifestation of DTHR induced by sheep red blood cell was inhibited by the ME and the fractions. Decrease in DTHR revealed the inhibitory effect of methanol extract and fractions on T lymphocytes required for the expression of the reaction (Vinothapooshan and Sundar, 2011). DTHR has been shown to be absolutely dependent on the presence of memory T cell (both CD4+ and CD8+) which also produce interferon-gamma (IFN-ɤ)-producing $CD4^+$ (THI) or $CD8^+$ (TCI) T cells (Biedermann et al., 2001; Allen, 1999; Furr, 1998). Also, in 1940, Chase and Landsteiner proved that DTHR was mediated by the cellular not the humoral arm of the immune system. It usually takes 24-72 h to develop and it involves activation of T-cells which results in the infiltration of monocytes and lymphocytes into the area of inflammation because the immune cells are also involved in mediating inflammatory responses. The observed inhibition of DTHR may be related to the anti-inflammatory property of this plant (Iwu and Igboko, 1982), as immune cells are also involved. This

inhibition can occur by immune deviation which entails steering T-cells towards an IL-4 producing TH2 or TC2 phenotypes (Biedermann et al., 2001). Levamisole acts by elevating cGMP levels in lymphocytes. Its imidazole ring seems to be one of the active moieties responsible for the functional increase of peripheral T-cell and macrophages (Alan et al., 2001). Thus levamisole should not inhibit DTHR.

Administration of ME caused a clear reduction in secondary antibody titre compared with the control but primary antibody titre at doses of 400 and 800 mg/kg were higher than the control. Primary and secondary antibody titres in the fraction treated rats were lower than the control. The secondary antibody titres of all animals except the ME treated rats were expectedly higher than the primary, since subsequent antigenic stimulation of primary-sensitized animals may result in high antibody production as there is now an expanded clone of cells with memory of the original antigen available to proliferate into mature plasma cells (Furr, 1998). This result was expected since very little monocytes were mobilized. Sasaki and others, (Sasaki et al, 1989), have observed that depletion of monocytes from peripheral blood mononuclear cells of patient with systemic lupus erythematosus resulted in a decreased antibody synthesis *in vitro* and addition of monocytes restored the response by lymphocytes (Sasaki et al, 1989). It is not immediately clear why the secondary titre in ME treated rats were lower than primary titre.

Humoral immunity is the aspect of immunity that is mediated by secreted antibodies. Antibody synthesis requires the cooperation of at least 3 major cell types; the macrophages, B lymphocytes and T lymphocytes (Benecerraf, 1978; Janeway et al., 2001) but the cells most ostensibly involved in antibody production are the B lymphocytes (Lauralee, 2004). These cells make antibodies if stimulated to do so, but they are incapable of stimulating themselves. The primary response consists mainly of immunoglobin M, whereas the secondary response consists mainly of immunoglobin G (Ratt et al., 2001). The decrease in antibody titre is consistent with the mild decrease in lymphocyte count produced by extract and fractions. This result may indicate that the methanol leaf extract and fractions of *H. opposita* have immunomodulatory effects on the cell mediated component of the immune system.

The results of this study reveal that the extract and fractions of *H. opposita* have immunomodulatory effect. These results suggest that *Hoslundia opposita* has effect on cell-mediated components of the immune system, although the immunomodulatory activity is yet to be associated with the specific constituents of the leaves.

CONCLUSION

The results of this study have established immunomodulatory activity of *Hoslundia opposita* extract and fractions and justified the claims made by herbalists about the use of its leaf to treat abscess, catarrh and wounds. Further studies are recommended to identify and isolate the exact constituent(s) responsible for the immunomodulatory effect and also establish the mechanisms of action.

ACKNOWLEDGEMENT

Authors are grateful to Chief Cornel A. Umeh (Okaaobuluzo Nnobi) for information on the ethnomedicinal use of the plant, and collection of samples used for pilot studies from the botanical garden of Late Chief Michael Umeh (Ezeifedi) at Nnobi. Thanks to Prof. John A. Umeh (Ezekwueme) for supply of some plant samples used in the pilot studies.

I am grateful to Dr. Jayeola of the University of Agriculture, Abeokuta and Brother Osasona Sola who were instrumental in collecting, cleaning and taking the plant to the Forestry Research Institute of Nigeria (FRIN) for voucher specimen number.

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