



EFFECT OF DRIED WHOLE PLANT POWDER OF *LEPTADENIA RETICULATA* (RETZ.) WIGHT & ARN. AND *PLUCHEA LANCEOLATA* C.B. CLARKE ON PHAGOCYTOSIS AND RESPIRATORY BURST BY HUMAN POLYMORPHONUCLEAR NEUTROPHILS (*IN VITRO* STUDY)

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ABSTRACT

In the present study, the effects of hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke was explored on the respiratory burst in Polymorphonuclear Neutrophils (PMNs), as compared to a known stimulant PMA (Phorbol 12-myristate 13-acetate). Prior to the *in vitro* tests, the viability of the PMNs was assessed using the Trypan blue dye exclusion test. The formation of various Reactive Oxygen Species (ROS) was measured by performing *in vitro* assays viz. Phagocytosis of *Candida albicans*, Nitro blue Tetrazolium (NBT) assay and Nitric oxide (NO) assay. The studied concentration of hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke were 50, 100, 200, 400mg/ml. An increase in the respiratory burst at all the studied concentrations was observed in all the assays indicating its immunostimulating effect.

KEY WORDS: Immunomodulatory *Leptadenia reticulata* (Retz.) Wight & Arn. *Pluchea lanceolata* (DC.) C.B. Clarke. Phagocytosis respiratory burst human Polymorphonuclear Neutrophils



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INTRODUCTION

Immune system plays an important role in body and its function is to protect the body against foreign pathogens and infectious agents. In many diseases, the immune response of the body is impaired and has to be modulated. Immunomodulators modulate the immune response through stimulation or support. Immunostimulants are used in immunodeficiency disorders like AIDS, cancer. The immunosuppressing agents are used in the treatment of autoimmune disorders, organ transplantations, etc. The natural resistance of the body against infection can be enhanced by the use of herbal drugs¹. Several herbal preparations that can enhance body's immune system status are extensively being used in the indigenous system of medicines. There is an upsurge in the clinical usage of indigenous drugs as they are free from serious side effects². *Leptadenia reticulata* (Retz.) Wight & Arn. (family *Asclepiadaceae*) is rich source of biologically active cardiac³ and pregnane glycosides⁴ which are known to possess anti-tumour and anticancer^{5,6} activity. Previous chemical studies of the plant showed the presence of flavonoids, triterpenes and steroids^{7,8}. *Pluchea lanceolata* (DC.) C.B. Clarke (Asteraceae) commonly known as 'Rasana' is an important xerophytic medicinal herb. It is a succulent, erect plant, traditionally used for dyspepsia, bronchitis and rheumatoid arthritis^{9,10}. It is also used as antipyretic, bitter, laxative, and nervine tonic¹¹. All parts of the plant are extensively used in indigenous system of medicine. The plant contains high amounts of medicinally important secondary metabolites viz. quercetin, β -sitosterol, triterpenol, etc., which gives it anti-inflammatory and analgesic properties^{12,13}.

Both the plants as per ayurvedic literature are mentioned as 'Rasayana' and 'Vayasthapaniya', collectively meaning enhancing longevity of life by prevention of diseases. This also suggests that the plants possess immunomodulating effect¹⁴. Hence, the plants, *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke was evaluated for immunomodulating activity through PMN cells for the Phagocytic activity, NBT Assay and NO Assay. Generally in mammals, phagocytic cells such as PMN,

monocytes, dendritic cells, mast cells and macrophages produce a number of antimicrobial and cytotoxic substances to destroy phagocytosed microorganism. In the process of production of microbicidal compounds, the PMN cells follow oxygen dependent host defence mechanism (respiratory burst). During the respiratory burst, the activated PMN cells produce a number of reactive oxygen intermediates (ROIs) such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), hydroxyl radical (OH^\cdot) and numerous other reactive products. In the present study, the formation of various ROIs were measured by performing *in vitro* assays viz. Phagocytosis of *Candida albicans*, NBT assay and NO assay.

MATERIALS AND METHODS

Chemicals

Phorbol 12-myristate 13-acetate commonly referred to as PMA (Sigma-Aldrich) was used as a standard drug. Minimum Essential Media (MEM) (Himedia, India), Nitro blue Tetrazolium (NBT) (Himedia, India), Griess Reagent (Sigma-Aldrich), Histopaque-1077 and Histopaque-1119 were supplied by Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany), Griess Reagent (G4410) was supplied by Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany), *Roswell Park Memorial Institute medium (Gibco® RPMI 1640)* was supplied by Genex Life Sciences Pvt. Ltd., Methanol (AR grade), Potassium hydroxide (AR grade), and Dimethyl Sulphoxide (DMSO) (AR grade) were supplied by S.D. Fine-Chem Limited.

Instrumentation

The instruments used were Laminar flow (Klenzaid Contamination Control Pvt. Ltd.), Cyclo centrifuge (Remi Laboratory Instruments), Microscope (Lecia Microsystems with digital camera), Neubaur chamber haemocytometer (GmbH, Wertheim, Germany) and ELISA reader (Bio-Rad, India).

Plant material

Whole plant of *Leptadenia reticulata* (Retz.) Wight & Arn. was collected from Bhumel village, Dist- Nadiad, Gujarat. The plant material was authenticated from Agharkar Research Institute, Pune, India (Voucher No. WP-090). Whole plant of *Pluchea lanceolata* (DC.) C.B. Clarke was collected from Sonmukhi Nagar, Jodhpur. Its herbarium was authenticated from Botanical Survey of India, Pune, India (Certificate No. BSI/WRC/Tech./2012/79). The duplicate herbaria of both plants were prepared and are preserved in the host institute for future reference. Both the plant materials were washed with water to remove soil particles, dried in shade, finely powdered and then sieved through BSS mesh size 85 and stored in an airtight container at room temperature ($25 \pm 2^\circ \text{C}$).

Sample preparation

About 2.0 g of dried whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. was accurately weighed and transferred to a 100 mL stoppered conical flask. 75.0 mL of distilled water and ethanol (1:1 v/v) was added to it and the flask was sonicated in an ultrasonic bath for 15 minutes. The flask was then shaken at 50 rpm, on a conical flask shaker overnight, at room temperature ($25 \pm 2^\circ \text{C}$). The extract was filtered through Whatman filter paper no.1. The filtrate was collected in a beaker and then evaporated on a hot water bath. The final volume was then made up to 5mL with distilled water in a 5 mL volumetric flask. The extract obtained was then filtered through 0.45 μm nylon filters (Millipore) before analysis. Similar procedure was followed for preparation of hydroalcoholic extract of whole plant powder of *Pluchea lanceolata* (DC.) C.B. Clarke. Thus, the resultant solution of concentration 400mg/mL was prepared. This sample extract stock solution was diluted with distilled water in the ratio of 1:1, 1: 3, 1:7 (v/v) to obtain solutions of 200mg/mL, 100mg/mL and 50mg/mL respectively. All the concentrations of the extract solutions were used in the assay.

PROCEDURE

The protocol was submitted to the Ethics Committee for Academic Research Projects

(ECARP) of TN Medical College & BYL Nair Hospital, Mumbai Central, Mumbai-08, to carry out *in vitro* study using hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke. A written consent was obtained to carry out *in vitro* study on human subjects before initiation of the study. For evaluation of the plant extract on respiratory burst in PMN leukocyte cells, isolated cells from the blood of six normal and healthy volunteers (age group of 25 to 27) were used. Volunteers were screened for smoking habit, any psychological disorder, those suffering with major illness, having history of receiving treatment for any condition four weeks prior to enrolment were excluded from the study. On the day of study, 6.0 mL of human blood of each healthy volunteer was aseptically collected from the ante-cubital vein into a Tarson tube containing heparin. This blood was then added to 6.0 mL of Histopaque in Tarson tube gradually along the walls of the tube. The tubes were centrifuged at 2200 rpm for 30 minutes at room temperature ($18\text{-}26^\circ \text{C}$). After centrifugation, the middle layer containing PMN cells was isolated by aspiration and washed three times with Phosphate buffer solution. After final washing, the PMNs were resuspended in MEM.

PMNs viability test

A viability test using the Trypan Blue dye exclusion method was done before conducting assay to measure respiratory burst, in order to exclude those concentrations of the plant extract affecting neutrophil viability. PMNs were incubated for 1 hour along with different concentrations of the test drug (concentrations ranging from 50 to 400 mg/mL). All sets were run in triplicates. Only those concentrations of the plant extract showing a viability of 90% and more were selected for the actual assay.

Assays to measure respiratory burst**Preparation of *Candida albicans* suspension (1.0×10^6 cells/mL)**

Candida albicans culture (ATCC 10231) was taken for preparation of suspension. A loop full of *Candida albicans* culture maintained on Saboraud's Dextrose agar slant was inoculated in 20 mL of the broth. The broth

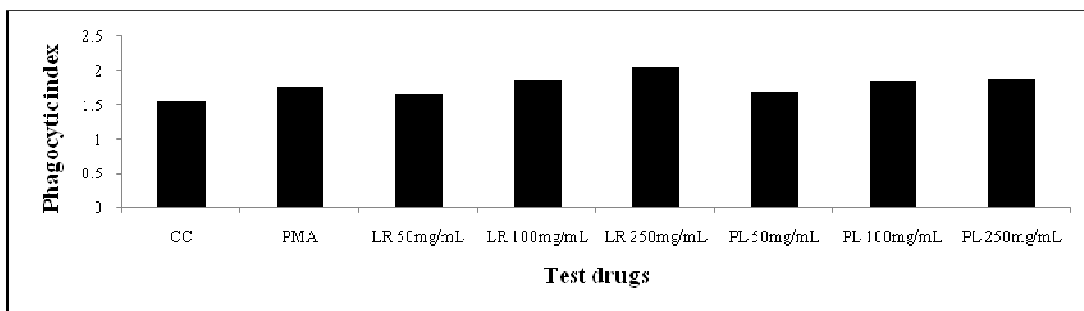
was incubated at 37°C for 18 hours. Using visible turbidity the growth of the cells were determined. The broth was centrifuged and the supernatant was discarded. The pellet obtained was washed two to three times using MEM and then was suspended in the same. Haemocytometer was used for the enumeration of the cells.

Method for determining Percent Phagocytosis and Phagocytic Index

Isolated PMNs from healthy human volunteers were used for the present study. 125µL of *Candida albicans* suspension (containing 1 X 10⁶ *Candida albicans* per mL), 245µL of Minimum Essential Medium and 5µL of Test drug/plant extract or PMA was added to 125µL of PMN (adjusted to 1 X 10⁶ PMN/mL)

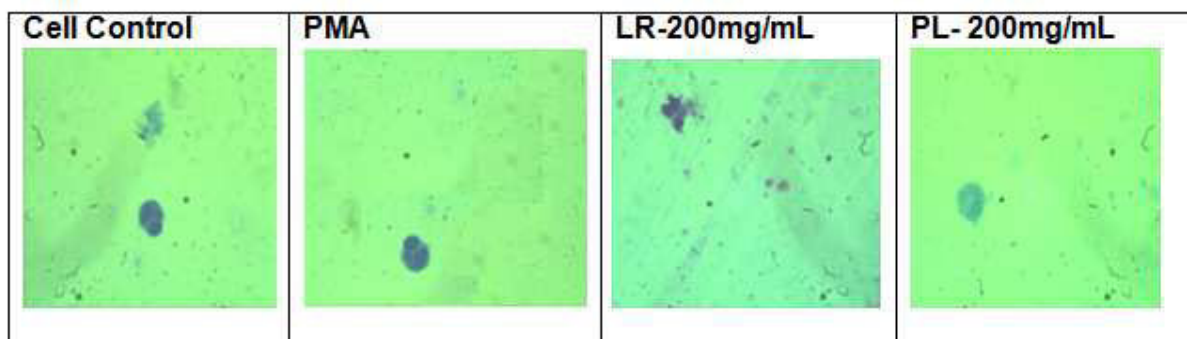
in a 1mL eppendorf tube. The eppendorf tubes containing the solutions were then incubated in a CO₂ chamber for 37°C and then centrifuged at 1500 rpm. A smear was prepared on a clear stain free slide from the pellet obtained in the eppendorf tubes containing the solutions after centrifugation. The slides were then fixed with 70% alcohol and stained with Giemsa stain. The slides were then observed under an inverted microscope using 100X magnification. Percent phagocytosis and phagocytic index were calculated. Graph of mean phagocytosis index for each concentration of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke was plotted and is represented in fig. 1.

Figure 1
Graph of mean phagocytic index observed for each concentration of *Leptadenia reticulata* (Retz.) Wight & Arn.(LR) and *Pluchea lanceolata* (DC.) C.B. Clarke (PL)



Representative slides of Phagocytosis exhibited by PMN is shown in figure 2. In each slide the dark spot indicates the *Candida albicans* engulfed by PMN cells.

Figure 2
Slides representing engulfment of *Candida albicans* by PMN cells



Nitro Blue Tetrazolium assay

Nitro blue tetrazolium (NBT) assay is a semi-quantitative assay that determines the

production of dependent increase in the formation of formazan crystals indicating the generation of superoxide anion (O²⁻) in

various phagocytic cells. To each well of the ELISA plate, 100 μ L of adjusted cells (1×10^6 cells) in MEM medium was added. The plate was incubated for 30 minutes in humid conditions for the adherence of the PMNs to the bottom of the well. Adherence of cells to the bottom of the wells of ELISA plate was confirmed by observing under inverted microscope. After the observation, the supernatant was discarded and replaced by fresh MEM. Test drug (plant sample) was added to the wells excluding cell control (CC) and standard control (PMA). 50 μ L of the 1:1 diluted NBT solution (stock: 10mg/mL) was added to the wells and the plate was incubated for two hrs in the laminar air flow unit in dark conditions since NBT is sensitive to light. After two hours of incubation, the supernatant was discarded and the cells were fixed with absolute methanol. The cells were washed twice with 70% methanol and air dried. Formazan crystals observed after the reaction were then dissolved in 2M KOH and DMSO. The contents of all the wells were horizontally shaken and the plate was read at 620nm in the ELISA reader. Graph of observed NBT assay readings for each concentration of *Leptadenia reticulata* (Retz.)

Wight & Arn. (LR) and *Pluchea lanceolata* (DC.) C.B. Clarke (PL) is shown in fig.3

Nitric oxide (NO) assay

Activated neutrophils produce toxic nitrogen intermediates like Nitric Oxide (NO), which plays a vital role in immunomodulation and intercellular signaling and can be estimated using the Griess reaction in NO assay. 100 μ L of PMN cells in RPMI medium (1×10^6 cells) were added to each well of the ELISA plate. The plate was incubated for 30 minutes in humid conditions for the adherence of the PMNs to the bottom of the well. Adherence of cells to the bottom of the wells of ELISA plate was confirmed by observing under inverted microscope. After the observation, the supernatant was discarded and replaced by fresh RPMI medium. Wells containing only PMNs, served Test drug (plant sample) and PMA was added to the wells excluding cell control and standard control wells. The plate was incubated for 1 hour in incubator at 37°C. After 1 hour, 100 μ L of Griess reagent was added to the wells (except cell control wells). The plate was then immediately read at 550nm on an ELISA plate reader.

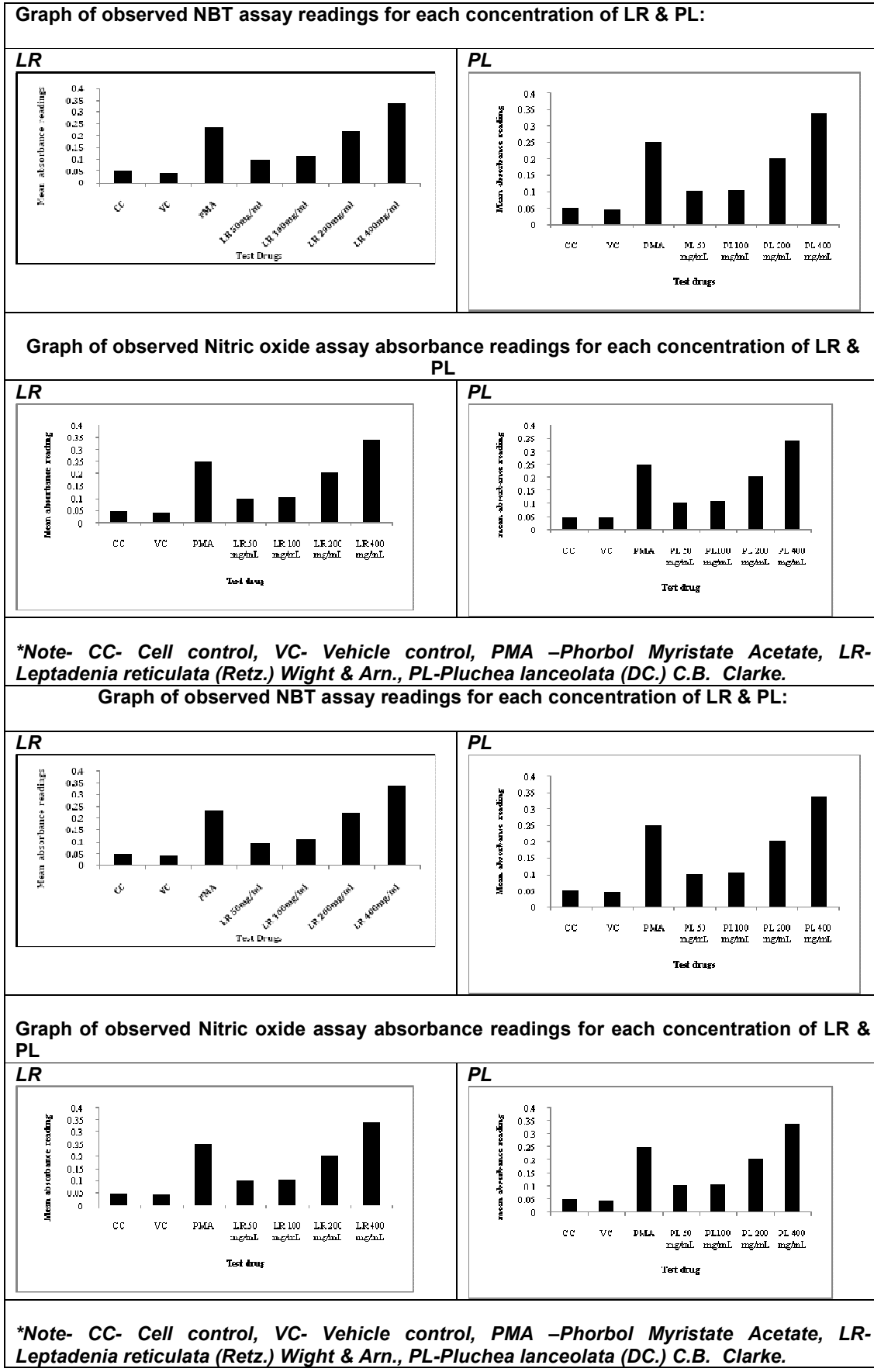


Figure 3

Table 1

Results:
The results for all the assays performed and the corresponding results for each concentration of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke is as shown in following table :

STUDY	TREATMENT PROVIDED									
	CC	PMA	LR				PL			
			50 mg/mL	100 mg/mL	200 mg/mL	400 mg/mL	50 mg/mL	100 mg/mL	200 mg/mL	400 mg/mL
% phagocytosis	35±19.85	46±10.54	45±20.95	51±10.94	60±18.27	N.O.	42±5.13	48±9.83	47±7.25	N.O.
Phagocytic index	1.56±0.07	1.76±0.09	1.66±0.19	1.85±0.15*	2.04±0.10***	N.O.	1.69±0.17	1.84±0.13*	1.86±0.17*	N.O.
Nitro blue Tetrazolium assay	0.051±0.036	0.246±0.062	0.098±0.016	0.115±0.074**	0.222±0.150	0.340±0.298	N.A.	N.A.	N.A.	N.A.
Nitro blue Tetrazolium assay	0.051±0.036	0.250±0.072**	N.A.	N.A.	N.A.	N.A.	0.130±0.023	0.107±0.051	0.204±0.107*	0.341±0.174** *
Nitric oxide assay	0.028±0.010	0.082±0.059	0.025±0.008	0.075±0.043	0.107±0.060	0.157±0.086** *	N.A.	N.A.	N.A.	N.A.
Nitric oxide assay	0.028±0.010	0.044±0.044	N.A.	N.A.	N.A.	N.A.	0.044±0.044	0.129±0.065	0.218±0.138*	0.299±0.196** *

* Note: N.O. – Not observed due to accumulation of aggregate at high concentration.
N.A. denotes not available, as test for the particular extract not evaluated on that ELISA plate batch.
CC-Cell Control, LR- *Leptadenia reticulata* (Retz.) Wight & Arn.,
PL- *Pluchea lanceolata* (C.B.) Clarke., PMA-PhorbolMyristate Acetate (Immunostimulant)
* - 0.1 <P, **- 0.01<P, ***- 0.001<P

DISCUSSION

In the present research work, immunomodulatory activity of hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke *in vitro*, has been evaluated. The phagocytosis activity was tested by Lehrer's method¹⁵ using *Candida albicans*. The test for superoxide formation was carried out by Nitro blue Tetrazolium salt reagent. Test for nitric oxide release was estimated by using Griess reagent. The tests were carried out on PMNs isolated from healthy human volunteers. Prior to the *in-vitro* tests, the viability of the PMNs was assessed using the Trypan blue dye exclusion test. The viability of the PMNs isolated from healthy human volunteer's blood was observed above 90%. Hence, the further investigating tests for evaluation of *in vitro* activity using PMNs were carried out. The hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke increased the phagocytic activity with the increase in concentration of the extracts from 50, 100 to 250mg/ml as compared to Phorbol Myristate Acetate (PMA), a known immunostimulant³. A comparative study of different doses of hydroalcoholic extract (50, 100, 200 and 400 mg/kg b.w.) of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke was done. In NBT assay, all the concentrations of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke ranging from 100 mg/mL to 200 mg/mL showed a concentration dependent increase in the formation of formazan crystals indicating superoxide generation as compared to the vehicle control. Maximum activity was observed at 400 mg/mL (fig.3). Similarly, in NO assay, all the concentrations of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke ranging from 100 mg/mL to 200 mg/mL showed a concentration dependent increase in the formation of nitric oxide as compared to the vehicle control.

Maximum activity was observed at 400 mg/mL. (fig.3). Hence, *in vitro* immunomodulatory activity of hydroalcoholic extract of dried whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke was studied using human PMNs, revealing the plant extracts have immunostimulant activity.

CONCLUSION

From the results obtained, it can be concluded that the hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke exhibited significant effect on phagocytosis by human neutrophils. The hydroalcoholic extract of whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke further showed to affect both superoxide formation (NBT assay) and NO release (NO assay). Thus, the dried whole plant powder of *Leptadenia reticulata* (Retz.) Wight & Arn. and *Pluchea lanceolata* (DC.) C.B. Clarke show immunomodulating effect and can be used in certain herbal formulations with immune enhancing activity. Further, isolation of active principle responsible for immunostimulant activity can be done.

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Conflict of interest: Conflict of interest declared none.

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