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ORIGINAL ARTICLE **OPEN ACCESS**

Immune Potentiating effect of Aqueous aerial parts extract of *Nerium indicum* Mill. (Apocynaceae) in mice.

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ABSTRACT
Nerium indicum Mill belongs to Apocynaceae family which is 5th largest medicinal plants family. The present study evaluates phytoconstituents and immunomodulatory activity. Aqueous extract of aerial parts of Nerium indicum Mill. was evaluated for immune parameters in Swiss albino mice. Standard methods of Phagocytosis (Invivo), DTH, HA Titre, neutrophil adhesion (NA) test and Cyclophosphamide induced myelosuppression and Mice Lethality assay were used. Sheep red blood cells were used as antigen and Levamisole and Cyclophosphamide used as positive and negative controls respectively. Statistical analysis was performed using GraphPad Software, Inc. The extract showed improved immune parameters when compared to Cyclophosphamide. Restoration of immune cells and platelets were dose related. Comparative data for the following studies were analyzed; DTH response as footpad thickness (26.9%), HAT (7th day and 14th day), % NA. Immune cells (TLC, Lymphocytes, Neutrophils and Monocytes). Relative organ body mass index. The results validates that aqueous extract of aerial parts of N. indicum possess immunostimulatory potential and play role in adjuvant therapy.
Keywords: *Apocynaceae, Phagocytosis assay, Phytoconstituents, DTH, HA Titer, Myelosuppression.*

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INTRODUCTION

Immunity is the defense accomplishment of living the system. Adaption to the stressed and variegated internal and external atmosphere requires active role of immune machinery. Owning, Building and maintaining strong immune system is the key to lead disease free healthy life. It is said that "Nature is the best healer". Remedy for all sort of illness is surmounted within Mother Nature. Aboriginals of the remotest places, disconnected from the central facilities survived and surviving even today by their skill of utilizing natural sources [1]. India is the ancient and most cherished practitioner of Natural medicines and surgery. It includes the names of Lord Dhanvantri, Ashwani Kumars, Atreya, Agnivesa, Sushruta, Charaka and Vagabhatt. Aboriginals, Saints, Vaids and Villagers since years have learned, applied and spread the doctrine of the art of healing from nature around them. This tribal and folkloric knowledge of traditional systems of medicine has now taken the trend of Reverse Pharmacology, an alternative to develop modern medicine. Importance of Plants and their secondary metabolites are being recognized globally for their pharmaceutical, neutraceutical and other livelihoods. Herbal drugs form an inseparable part of the allopathic drug as an adjuvant for management of various disorders [2].

More often oncopatients are immunosupressed. Immunotherapy work well for immunosensitive tumors. The effect of the therapy depends on the achieved immune harmony of immunomodulators. Patient's right access to immunotherapy abide the technical viewpoint of the dose ratio, duration (pre-surgical treatment or during, chemotherapy, after chemotherapy or post surgical), route (local/systemic), type of drug, type of patient selected and benefit to risk ratio.

Immunotherapy should be if introduced at the initial stage in immunecompetent patients along with other methods of treatment with known substances for safety and effectiveness. It is important that over 50% of these patients are taking food supplements, and other substances to enhance their immune system.

Plants consumed in our daily routine are best immunomodulators [3-5]. Phytoconstituents of plants are the foremost source of immunomodulators like, flavonoids, polysaccharides, lactones, alkaloids, diterpenoids and glycosides [6]. Immunity is the safeguard against stressful environment. Scientific

evidences claim that lost immunity is the reason for development of neoplasia, tuberculosis and other infections. Immunomodulators are needed to compensate immune balance especially in the difficult struggle with cancer [7, 8]. Some of the well known immunomodulators are Corticosteroids, cyclosporine, azathioprine, cyclophosphamide, antibodies (ALG-antilymphocyte globulin), monoclonal anti-T cell antibodies eg Muromonab (OKT-3), Rh_0 (D) immune globulin, Thymosin, Dipyridamole, Levamisole, Isoprinosine, Melatonin, vaccine and sera, Vitamin D, Cytokines (IFNs, CSFs, TNFs and interleukins ILs) and Others [9, 10].
Apocynaceae family is the fifth valuable medicinal plant family in Angiosperms [11-16]. Apocynaceae family plants has vast traditional uses and potential role in cancer remediation [17- 23]. Results of various studies and trials have shown that *Nerium indicum* is very effective immunomodulator and hence it has become remedy for many diseases like cancer, diabetes, hypertension, malaria and infections etc. This research article evaluates the immunomodulatory potential of *Nerium indicum* aerial part extracts in water (N3). The selection of the plant is random based on an exhaustive literature review.

MATERIAL AND METHODS

Collection and authentication of plant

Fresh parts of *Nerium indicum* mill.(Figure 1) were collected in the month of February-March, from Jhansi region of Bundelkhand (Figure 1). The Plant was identified and authenticated by Dr. Mudailiya, taxonomist National Vrkshayuveda Research Institute (NVARI), Jhansi, UP India with accession no. 24381for future reference.

Fig. 1: Fresh parts of *Nerium indicum* mill.

Extraction

Soxhlet extraction of powdered drug was performed in distilled water. The extracts were, concentrated to dryness in Rotary evaporator and dried in Lyophilizer and stored in deep freezer for future use.

Drug and Chemicals

Levamisole as positive control and Cyclophosphamide as negative control (Yarrow chemicals, Mumbai), Leishman's Stain (Span Diagnostics Ltd., Surat), WBC Diluting Fluid (S D Fine-Chem. Ltd., Mumbai). The other chemicals used are of analytical and reagent grade from Qualigens and Hi-Media Laboratories Pvt. Ltd. (Mumbai, India).

Animals

Animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Bundelkhand University [Project no. BU/Pharm/IAEC/a/16/15]. Adult healthy female and male Swiss Albino mice, of similar physical constitution in terms of age and body weight (6-8 week, 25-35g) were procured and housed in the Institutional Animal facility. Guidelines of CPCSEA (Regd. No. 716/02/a/CPCSEA) and IAEC were strictly followed for the care and use of animals. All animals were provided sanitized paddy husk bedding and fed with nutrient rich diet and water *ad libitum*. Mice were segregated in polypropylene lab cages in a room conditioned with a12h light: dark cycle, temperature at 20 ± 2°C and relative humidity 54% ± 12%. The animals were tamed and caressed for one or two weeks before use.

Acute toxicity studies [24, 25].

Acute toxicity of test plant extracts were performed according to OECD 425, acute toxic class method. Swiss albino female mice (20-30g) were fasted for 6 hrs prior to dosing. Toxicity was checked at the initial dose 2500 mg/kg b.wt. Each single dose (1ml) was administered in three mice by oral gavage feeding needles for mice. Any change in the behavior, neurological profile and autonomic profile keenly watched at 24, 48 and 72 hrs.

Immunomodulatory activity

Treatment schedule (Table 2)

Grouping of animals done according to doses and duration of treatment followed from standard protocol with modification as described in Table 2 [26, 27]. Animals were divided into groups according to the set of experiments. Group I received normal saline. Groups II received Cyclophosphamide (CYP, 180 mg/kg, p.o). Group III received standard drug Levamisole (7.2 mg/kg). Groups IV to IX were given the aqueous extract of *Nerium indicum* (N3) at the dose of 250, 600 mg/kg BW orally. Cyclophosphamide was given 2 days before immunization and Levamisole given at the start of treatment schedule.

Prep of SRBC suspension for immunization and Antigenic Challenge:

Sheep blood was collected from external jugular vein of sheep under the supervision of Dr.Devendra Kumar (veterinary medical officer, Government Veterinary College, Itawah, UP, India). It was mixed with Alsever's solution in 1:1 proportion stored in a refrigerator. The Blood was washed three times with buffer saline (pH 7.4) centrifuged at 3000 × g for 10 min on each occasion. With the help of haemocytometer the density of RBCs counted to desired concentration ($0.5x10^9$ cells/1ml and 0.5ml/100g) for immunization and challenge respectively [28, 30].

Cellular immunity/ Delayed type of hypersensitivity reaction

The mice were immunized with 1ml of SRBC ($0.5x10^9$, i.p.) 6 days after extract dosing, it considered as day 0 of immunization. After seven days a challenging dose of 20µl of SRBC (10^8 cells, s.c./i.d.) was made into the subplantar surface of right hind foot pad. Paw volume measured at 0, 24, 48 and 72 hrs using a Plethysmometer. The average increase in mercury level in each group was recorded [31].

Experimental results were expressed as percentage footpad swelling according to the formula:

% footpad swelling = [(right hind paw vol in mm) - (left hind paw volume in mm) / mean thickness of uninjected left hind paw vol in mm] x 100

Measurement of antibody titre by haemagglunation reaction (HA Titre)

The mice were given the treatment as before for 14 days. On 6th day immunization of each mice done with 0.4ml of SRBC ($0.5x10^9$ cells/mice, i.p.), considered as day zero. On 7th and 14th day of immunization the blood was withdrawn for HA titer determination before giving antigenic dose of SRBC. The blood was collected into the centrifuge vials and centrifuged for separating the serum. Two fold serial dilution of serum using buffer saline performed in microtitration plates. To each well 25µl of SRBC's (1x 10^8 cells, 1%w/v) was added. The plates were incubated at 37^0 c for 2 hours. The highest dilution producing haemagglutination is ranked 1, and minimum rank (1:2) is the HA titre ($2^{\wedge rank}$ units/µL [32, 33].

Neutrophil adhesion (NA) test

Blood was collected from retro orbital plexus of the animals in EDTA containing tubes on 20th day after HA titre determination. Total Leukocytes (TLC) and Differential Leukocytes counted (DLC). Afterwards the blood samples were introduced with 80 mg of nylon fiber in each vial and incubated at 37°C for 15 min and again TLC and DLC determined. Count of fiber bound neutrophil was taken by dispensing the fibers in fresh 1 ml saline. The results are given as the percentage of neutrophils adhering to the fibers [34].

The product of TLC and % Neutrophils gives neutrophil index (NI) of the blood.

% NA = [(NI of untreated – NI of treated)/ NI of untreated] x100

Cyclophosphamide induced Myelosuppression

Grouping of animals done according to previous experiment. Group I normal control received normal saline till 10 days, group II received normal saline and Cyclophosphamide (180 mg/kg BW, p.o.), this is considered as day 0. Other groups continued receiving treatment for 14days. Blood samples were obtained by capillary tube puncture of the retro-orbital plexus on a day before and 24 hours interval for 3 days. Manual determination of TLC and DLC done [35, 36].

Phagocytic activity

The mice were weighed and divided into five groups. Group I received normal saline. Animals of group II, III, IV and V received extracts of *Nerium indicum* at the dose of 250 and 650 mg/kg body weight for seven days. On 8th day, i.v injection of 0.5 ml of Indian ink (1%w/v) four times diluted in saline was given through tail vein. Afterwards, 20µl of blood samples were withdrawn and mixed with 4 ml of 0.1%w/v sodium carbonate. Absorbance of this mixture was measured at 670 nm at 0 and 15 minutes time interval. At the end three mice from each group were sacrificed and their liver and spleen removed [37, 38]. Calculation performed as:

Carbon clearance index (k) = $\log(OD_0) - \log(OD_t) / t2-t1$

$t½ = 0.693/k$; where OD_0 is the OD at 0 min and OD_t is the OD at t min

Phagocytic index (α) = $(W_B.\ k^{1/3}) / (W_L + W_S)$

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Statistical analysis
All values are expressed as Mean ± SEM/SD. The analysis was performed using GraphPad software Inc. (Instat), version 3.10, copyright 1999-2009, 32 bit for Windows. The p- value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results of the study are tabulated in Table 1 to Table 4.
Extraction
Qualitative tests revealed the presence of varied phytoconstituents like alkaloids, flavonoids, glycosides, phenols, saponins, tannins, anthraquinone, proteins and Sugar (Table 1).

Acute toxicity studies.
The extract was found to be safe at the dose of 2500mg/kg p.o. Dose of 600 mg/kg and 250 mg/kg were selected as high and low doses, respectively.

DTH Response
Edema produced by the challenge dose of SRBC antigen is was enormous at the first 24 hrs. Increasing time reduced the edema possibly due to the immune enhancement by extract and interaction of antigen-antibody due SRBC immunization. Percentage change in paw volume measured by Plethymometre (Table 3).
Humoral immunity is suppressed by cyclophosphamide (CYP) and so DTH reaction persisted for long in Group II (CYP treated). Standard drug Levamisole had effects between N3-250 and N3-600. Higher dose of Aqueous *Nerium indicum* (N3) showed prominent edema subsiding role.

HAT titer
Humoral immunity was measured by HA Titre of blood obtained on 7th (primary humoral response) and 14th day (secondary humoral response) (Table 3). Cyclohosphamide do not decrease 14th day titer. HA titres were seen increasing in standard and extract treated groups. The order is as follows:

Neutrophil adhesion
Neutrophil adhesion defines the emmigration and adhesion of neutrophils to the injured site for healing. The results (Table 3) showed that percent reduction of neutrophil adhesion was higher in N3-600 when compared to normal and N3-250 group animals.

Phagocytic index/ carbon clearance
Phagocytosis is the process of antigen recognition and eating away of particles by mononuclear macrophages. Functionality of reticuloendothelial system (RES) can be assessed through this method. Cyclosphosphamide suppressed phagocytosis (Table 3). Significant increase in carbon clearance (k) and phagocytic index (α) were seen in extracts treated animals.

Cyclophosphamide induced myelosuppression
Cyclophosphamide group showed an absolute drop of TLC and DLC. Decrease in neutrophil count was found in all groups when compared to normal. However, treatment with standard (levamisole) and extract treated showed significant restoration of reduced cells (Table 4).

Mice organ weight index
Three animals from each group were sacrificed by deep ether anesthesia at the end of the experiment. Determination of body weight before the start of experiment and before sacrifice was taken. Calculation body and organ mass ratio was performed as: organ weight (mg)/body wt (per 100g) (Table 4).

Discussion and Conclusion
The homeostatic balance is maintained by the stimulation or suppression of immune cells and it keeps the body in normal healthy condition. One of oncology goals should be the recovery of the immune balance. Immunotherapy has to be started at an early stage to support other methods of treatment with known substances [39]. The models and protocols used here are widely applicable among researcher [40]
Cyclophosphamide reduced hematological parameters, cell mediated immune responses and macrophage production. Levamisole and Nerium extracts restored the normal immunity affected by cyclophosphamide and SRBC antigens. *Nerium indicum* increased percentage neutrophil count in mice. Thus supporting the claims for immunomodulatory principles of respective plant described in various literatures.These effects may be due to toxic phytoconstituents like; alkaloids, cardiac glycosides, tannins, terpenoids, irridoids, secoirridoids and flavonoids [41-44].
It is concluded that aerial part extract of *Nerium indicum* possess immunostimulant activity. Further it is suggested to screen the same activities of isolated bioactive phytoconstituents from this extract. Mechanistic view of the activities can be preceded further.

Table 1: Qualitative phytoconstituent Analysis

S.NO.	Test	*Nerium indicum*		
Tests	Extracts	N1	N2	N3
Glycosides-cardiac/anthraquinone	Borntrager's test	++	++	+/-
Saponins	Foam test	-	-	++
Oils and fat	Spot test	+++	++	+
Phlobatannins/ Chalcones	HCl test/spot test	-	-	-
Flavonoids	$AlCl_3$ test/ alkaline reagent test/lead acetate test	+++	+++	+++
Tannins/Phenolic compound	$FeCl_3$ test/ Lead acetate test	+++	+++	+++
Alkaloids	wagner's test/mayer's test/hagers/dragendorffs	++++	++++	++++
Protein/Amino acids	ninhydrin/ xanthoproteic	++	++	++
Steroids	salkowski's test/ Liebermann buchard	-	-	-
Phytosterols	sulphuric acid test	+	-	-
Carbohydrates/ sugar	molish's test/ Benedict's test	+	-	+
Coumarins	fluorescens test	+	-	-

Table 2: Treatment protocol (total duration 30 days)

group n=6	Treatment (mg/kg bwt), Route	treatment (mg/kg bwt, oral gavage)	immunization and challenge	parameters studied
I	Normal saline, p.o.			all parameters
II	Normal saline + CYP200, i.p.	CYP had been given three days before immunization.		DTH, HAT, PLT, TLC, DLC, organ body index,
III	Leva 50mg/kg, p.o.	Given a day before CYP.		DTH, HAT, NA
IV	250 N3, oral gavage	Extract given once daily for 28 days.		
V	600 N3, oral gavage	Extract given once daily for 28 days.		
VI	250 N3 +CYP (as above)	Day of CYP injection is considered as day 0. Immune cells counted on after 3 days.		TLC, DLC, organ body index
VII	600 N3 +CYP (as above)			

Table 3. Immune response of various groups

GROUP	TREATMENT	Carbon clearance rate	Phagocytic index	HAT(primary and secondary humoral response)		% NA	DTH (% change in thickness of mice paw in mm		
				7th day	14th day		24 hrs	48 hrs	72 hrs
Normal	Normal saline	0.006684 ± 0.0006263^{a}	3.658 ±0.1146^{a}	1.957± 0.06731	2.007± 0.1003	10.977± 2.34	58.847± 1.778	52.56 ±0.664	51.048 ±0.596
LEVA	Levamisol 7.2 mg/kg	0.02781± 0.003001^{abc}	5.622 ±0.1936^{abc}	5.569± 0.1289	7.425± 0.5077	-	51.663± 1.191	40.63 ±1.984	33.789 ±1.194
CYP	Cyclophosp-hamide 180mg/kg	-	-	1.405± 0.1003	0.7024± 0.1003	-	54.800 ± 3.082	55.85± 4.597	49.997 ±2.925
N3	250 mg/kg	0.01802 ±0.001698 ^{abc}	4.509 ±0.1481^{abc}	2.759± 0.05017	3.311± 0.1555	38.729 ± 1.292	51.779± 4.192	42.198 ±2.467	35.772 ±4.059
N3	600 mg/kg	0.04230 ± 0.002808 ^{ab}	6.555 ±0.1469^{ab}	4.415± 0.2539	5.619± 0.2655	50.943 ± 4.669	34.761± 2.014	31.109 ±2.427	23.331 ±0.8226
F value			68.243	11.858	13.116	43.55	24.962	153.1	98.986

All values are Mean ± SEM. Data analyzed using One-way Analysis of Variance (ANOVA) .The P value of < a=0.0001***,p<b=0.01**, p<c=0.05*, $P>0.05^{ns}$ is considered significant. Variation among column means is significantly greater than expected by chance. Tukey Kramer Multiple Comparisons post hoc test applied. If the value of q is greater than 3.958 then the P value is less than 0.05. ANOVA assumption of Gaussian distributions of sampled data was tested using Kolmogorov and Smirnov method

Table 4. Cyclophosphamide induced immunosuppression and relative organ index

Group	Treatment (n=6)	TLC	Lympho	Neutro	Mono	Eosino	Platelets (x10^3/ml)	spleen index	thymus index
Normal	Saline	5298.17 ±471.39	3579.3 ±435.55	1159 ±312.391	233 ±43.456	57.83 ±15.664	933.167 ±74.32	0.452 ±0.043	0.187 ±0.013
CYP	Saline + CYP200mg/kg	62.964 ±7.098	61.474 ±8.309	56.345 ±16.981	60.825 ±6.997	95.352 ±2.73	683.167 ±74.32	0.285 ±0.005	0.066 ±0.001
N3-CYP	N3-250mg/kg +CYP200mg/kg	35.297 ±5.160	30.51 ±6.002	27.153 ±12.78	36.81 ±15.898	76.60 ±6.31	640.67 ±49.062	0.446 ±0.08	0.135 ±0.009
N3-CYP	N3-600mg/kg + CYP200mg/kg	14.097 ±4.46	10.763 ±3.06	7.660 ±3.367	8.0595 ±3.54	64.99 ±15.535	869.3 ±45.298	0.665 ±0.032	0.215 ±0.009
F value	F = 43.455 =(MS treatment/MS residual)								
q value	Normal vs N3-250 , 6.316 ** P<0.01 and Normal vs N3-600, 9.096 ** P<0.01								

All values are Mean ± SD. Data analyzed using One-way Analysis of Variance (ANOVA) .The P value of < a=0.0001***,p<b=0.01**, p<c=0.05*, P>0.05^{ns} is considered significant. Dunnett Multiple Comparisons Test. Control column: Normal. If the value of q is greater than 2.440 then the P value is less than 0.05. ANOVA assumption of identical SDs were tested using Bartlett statistic (corrected) = 6.911 and P value is 0.0316.

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CONFLICT OF INTEREST

The authors declare none conflicts of interest.

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