EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF THE ETHANOLIC EXTRACT OF NARAVELIA ZEYLANICA LEAVES

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ABSTRACT

The objective of present study is to evaluate the immunomodulatory activity of the leaves of *Naravelia zeylanica*. Various extracts of the leaves of *Naravelia zeylanica* were evaluated for potential immunomodulatory activity, using the *in vitro* polymorphonuclear leucocyte (human neutrophils) function test. The ethanol extract was evaluated for immunomodulatory activity in *in vivo* studies, using rats as the animal model. The extracts were tested for hypersensitivity and hemagglutination reactions, using sheep red blood cells (SRBC) as the antigen. Distilled water served as a control in all the tests. The successive ethanol and water extracts exhibited a significant increase in the percentage phagocytosis versus the control. In the *in vivo* studies, the successive ethanol extract was found to exhibit a dose related increase in the hypersensitivity reaction, to the SRBC antigen, at concentrations of 200 and 300 mg/kg. It also resulted in a significant increase in the antibody titer value, to SRBC, at doses of 200 and 300 mg/kg in animal studies. The successive ethanol extract was found to stimulate cell mediated and antibody mediated immune responses in rats. It also enhanced the phagocytic function of the human neutrophils, *in vitro*.

1. INTRODUCTION

Life, disease and decay are inseparable. From his first awakening, man has sought to fight and control diseases. He turned to nature for inspiration and guidance (Sukh Dev et al., 1997). Herbs have been used as a source of drugs to combat diseases since time immemorial. The effectiveness, easy availability, low cost and non-toxic nature popularized herbal remedies (Mohd Ali, 1997).

Naravelia zeylanica (Ranunculaceae) is a climbing shrub with tuberous roots; wiry stem strong tendrils, leaves 3-foliate, opposite, terminal leaflet modified into a 3 branched tendril, leaflets ovate-lanceolate, serrate or crenate, prominently nerved; flowers yellow, fragrant, in axillary and terminal penicles, sepals downy, petals linear-clavate, elongate; fruits aggregate of achenes, ending in twisted feathery tales. The plant is available rich all around south India (Warrier, 1995; Raja Naika, 2008; Saldanha, 1984).

The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. In ayurveda the plant has been extensively used by native peoples as an astringent, bitter, antipruritic and anti-inflammatory. It is also useful in pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, cephalalgia, colic inflammation, wound healing & ulcer protection. The root and stem have a strong penetrating smell and is used to relieve malarial fever and headache. Root and stem paste is applied externally for psoriasis, itches and skin allergies. The traditional medicine practioners using the leaf and stem juices for treating intestinal worms, psoriasis & dermatitis (Harsha, 2003, Sivarajan, 1958 ; Anis, 2003; Saldanha, 1976).

A decoction of the fresh leavesof *Naravelia zeylanica*has been used by ayurvedic practitioners, in rural Maharashtra, to boost the immune system to fight a number of diseases. However, no phytochemical and pharmacological investigations of the fresh leaves have been conducted so far to substantiate this practice. The current study aimed at exploring the immunomodulatory potential of the fresh leaves of *Naravelia zeylanica*.

2. MATERIALS AND METHODS

Preparation of extracts

The fresh leaves of *Naravelia zeylanica* were collected from Udupi District during the month of September-October and authenticated By Prof Gopal Krishna Bhat, Poorna Prajna College Udupi, India. The fresh leaves were shade-dried, powdered and stored in airtight containers. The powder was subjected to successive Soxhlet extraction using solvents of varying polarity; petroleum ether, benzene, chloroform, acetone,

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ethanol and water. The solvent was removed under reduced pressure to obtain a total of six extracts, i.e, petroleum ether, benzene, chloroform, acetone, ethanol and water. The extracts were standardised with respect to their physico-chemical parameters such as consistency, pH and extractive value as prescribed in the Indian Pharmacopoeia. All the extracts were subjected to qualitative chemical tests to determine the nature of the phytoconstituents (Kokate, 1988). All the extracts were evaluated for immunomodulatory activity, using the *in vitro* polymorphonuclear (PMN) function test. An aqueous dispersion of the successive ethanol extract (SEE) was used for *in vivo* animal experiments. The vehicle (distilled water) served as the control.

High performance thin layer chromatography (HPTLC) study

HPTLC studies were carried out on the SME, using precoated silica gel G 60 F_{254} TLC plates as the stationary phase, and 1-propanol: water (7:2) as the mobile phase. The plates were spotted using the Camag Linomat applicator IV and the developed plates were scanned under UV - 254 nm and UV - 366 nm, using the Camag Scanner III.

Animals

Wistar rats (175-200 g) of either sex are procured from Indian Institute of Sciences. They are maintained under standard conditions (temperature 22 ± 2°C, relative humidity 60±5% and 12 h light/dark cycle)0. The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellet diet and water *ad libitum*. The Institutional Animal Ethics Committee approved the experimental protocol. All the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the "National Academy of Sciences" and published by the "National Institute of Health".

Antigen

Sheep red blood cells (SRBCs), collected in Alsevier's solution, washed in large volumes of sterile normal saline thrice and adjusted to a concentration of 5 X 10^9 cells per ml, were used for immunisation and challenge.

Polymorphonuclear leucocytes (PMN cells)

PMN cells, collected from normal healthy volunteers (18-22 years) with no evidence of bacterial, fungal or viral infection, were used in the study. This test protocol was approved by the Institutional Human Ethics Committee.

Reagents

The minimum essential medium (MEM) used for bioassay was procured from HiMedia Lab Pvt. Ltd. Ficoll Hypaque and bovine serum albumin were procured from Sigma Chemical Co. Candida albicans ATCC-10231, maintained on Sabourads agar HiMedia, was used as the test microorganism in the bioassay. All the solvents, reagents and chemicals used were of analytical grade.

In vitro phagocytosis test

All the extracts were evaluated for immunomodulatory activity, using the PMN function test. Peripheral venous blood, 10 ml, was collected from volunteers in a sterile heparinised tube. Neutrophils were isolated by Ficoll Hypaque density gradient sedimentation (Boyum, 1968). The RBC-PMN pellet was then subjected to dextran sedimentation. The supernatants, containing more than 90% of PMN cells, were collected and the cell density adjusted to 1X10^6 cells/ ml using MEM. *Candida albicans* (cell density adjusted to 1X10^6 cells/ml using MEM) was used as the test microorganism. The PMN cells (cell density adjusted to 1X10^6 cells/ml using MEM) were mixed with 1X10^6 cells/ml of *Candida albicans* and incubated at 37oC for one hour in 5% CO_2 atmosphere, in the presence of the test extracts. The control was the identical solution minus the test extracts. Cytosmears were prepared after incubation. The smear was fixed with methanol, stained with Giemsa and studied under 200 X 'oil immersion objective' to determine the phagocytic activity of PMN cells. Neutrophils (200 nos.) were scanned and the cells with ingested microorganisms were counted (Lehrer, 1969). The parameters evaluated were percentage phagocytosis (percentage of PMN cells involved in phagocytosis) and phagocytic index (ratio of number of *Candida albicans* engulfed to the total number of neutrophils).

In vivo tests - Acute toxicity study

The acute toxicity study for the SEE was conducted in rats as per the prescribed guidelines. Three animals of either sex were used. Their weights were recorded before beginning the study. They were administered a single bolus dose of the SEE (3000 mg/ kg) per orally and observed over 14 days for mortality and physical/ behavioural changes.

Hypersensitivity reaction which measures cellular immunity

Hypersensitivity reaction to SRBC was induced in rats, following the prescribed method (Doherty, 1981). The SEE (in doses of 100, 200 and 300 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats - three male and three female. The SEE was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunisation (i.e., Days -2, -1, 0, +1, +2). The rats were immunised by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad on day 0. The animals were challenged seven days later by injecting the same amount of SRBC into the left hind footpad. The thickness of the left hind footpad was measured with a micrometer at 4 h and 24 h after the challenge.

Hemagglutination reaction which measures the humoral immunity

The SEE (in doses of 100, 200 and 300 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats - three male and three female. The SEE was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunisation (i.e., Days -2, -1, 0, +1.+2).

The rats were immunised by injecting 0.5 ml of SRBCs intraperitoneally (i.p.) on the day of the immunisation. Blood samples were collected by retro-orbital puncture on the tenth day after the immunisation. Antibody levels were determined by the hemagglutination technique (Nelson, 1967). The antibody titer was determined by a two-fold serial dilution of one volume (200 µl) of serum and one volume (200 µl) of 0.1% bovine serum albumin (BSA) in saline. One volume (200 µl) of 0.1 % SRBCs in BSA in saline was added and the tubes were mixed thoroughly. They were allowed to settle at room temperature for about 60-90 min until the control tube showed a negative pattern (a small button formation). The value of the highest serum dilution showing visible hemagglutination was taken as the antibody titer.

Statistical analysis

The data was analysed using one-way analysis of variance (ANOVA), followed by Dunnett's test. $P < 0.05$ was considered significant.

3. RESULTS

Physico-chemical and phytochemical investigations

All the extracts were evaluated for physico-chemical parameters viz. consistency, colour, pH and extraction values. All the successive extracts had an acidic pH, except the water extract, which was alkaline. The water extract had the highest extractive value, indicating the presence of a high amount of water-soluble polar phytoconstituents in the fresh leaves. Phytochemical screening (chemical tests) of all the successive extracts was conducted to determine the presence of various phytoconstituents. These investigations revealed the presence of steroids and flavonoids in the petroleum ether, benzene and chloroform extracts. The acetone, ethanol and water extracts were found to contain flavonoids, phenolics, steroids, glycosides, carbohydrates and proteins (Table1.).

Table 1. Phytochemical evaluation of Naravelia Zeylanica leaves extracts

Phytoconstituents	Extracts of *Naravelia zeylanica* leaves					
	Petroleum ether	Benzene	Chloroform	Acetone	Ethanol	Water
Alkaloids	-	-	-	-	-	-
Carbohydrates	-	-	-	-	+	+
Flavanoids	+	+	+	+	+	+
Proteins/Amino acids	-	-	-	-	+	+
Steroids	+	+	+	+	+	+
Saponins	-	-	-	-	+	+
Phenolics/Tannins	-	-	-	+	+	+
Glycosides	-	-	-	+	+	+

(+) indicates presence, (-) indicates absence

HPTLC studies

The results of the HPTLC studies conducted on the successive ethanol extract indicated the presence of fluorescent phenolic compounds. The HPTLC fingerprint at 254 nm showed the presence of eight components, while the HPTLC chromatogram at 365 nm showed the presence of seven components.

Spraying the HPTLC plates with anisaldehyde-sulfuric acid reagent resulted in the formation of three green bands and one bluish violet band, indicating the presence of steroidal and triterpenoidal saponins in this extract.

Spraying the plates with Folin Ciocalteau reagent resulted in the formation of two blue bands, indicating the presence of phenolics with hydroquinone/ catechol nucleus (Harborne, 1988). As the pH of the SEE is around 5.0. These results indicate the presence of phenolic acids.

Pharmacological investigations

Acute toxicity study

The results of the acute toxicity study indicated that the LD_{100} of the SEE of Naravelia zeylanicawas more than 3000 mg/Kg body weight.

In vitro phagocytosis test :

All the extracts were evaluated at concentrations of 1.0, 2.0 and 3.0 mg/ml. The petroleum ether, benzene, chloroform and acetone extracts did not show any significant increase in the percentage phagocytosis versus the control. The SEE and water extracts exhibited a significant increase in percentage phagocytosis. The SEE showed significant activity at concentrations of 1.0 mg/ml (53%), 2.0 mg/ml (49%) and 3.0 mg/ml (46%) as compared to 31% in the control.

The water extract also exhibited a 55% phagocytosis at a concentration of 2.0 mg/ml as compared to 32% in the controlv(Table 2). As neutrophils form the first line of host defense by virtue of their ability to phagocytose invading microorganisms, they have a major role in modulating the immune function. The stimulation of neutrophils results in an increase in the immediate cellular immune response.

Hypersensitivity reaction

Per oral administration of the SEE (100, 200 and 300 mg/kg) for five days produced a dose related increase in early (4 h) and delayed (24 h) hypersensitivity reaction in rats. The 4 hour-reaction was found to be of higher magnitude than the 24 hour-reaction. These results indicate that the extract has a greater effect on the early hypersensitivity reaction and a less pronounced effect on the delayed hypersensitivity reaction.

Hemagglutination reaction

The antigen antibody reaction results in agglutination. The relative strength of an antibody titer is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The antibody titer is useful to measure the changes in the amount of the antibody in the course of an immune response.

Per oral administration of the SEE (100, 200 and 300 mg/kg) for five days produced a dose related increase in the antibody titer in rats(Table 3.).

Table 2. In vitro test of the successive extracts of Naravelia zeylanica leaves

Test extract	Concentration (mg/ml)	Percentage Phagocytosis (mean±SD)	Phagocytic Index (mean±SD)
Petroleum ether	Control	30±1.0	1.62±0.06
	1.0	33±1.15	1.74±0.15
	2.0	35±2.52	1.77±0.05
	3.0	32±2.0	1.76±0.11
Benzene	Control	29±3.21	1.66±0.02
	1.0	27±1.00	1.60±0.07
	2.0	30±1.00	1.61±0.05
	3.0	29±0.58	1.66±0.06
Chloroform	Control	35±1.15	1.98±0.02
	1.0	36±0.58	1.94±0.15
	2.0	34±1.00	2.01±0.08
	3.0	36±2.08	2.11±0.09
Acetone	Control	35±1.75	1.98±0.02
	1.0	36±2.40	1.97±0.01
	2.0	39±0.75	1.94±0.15
	3.0	36±2.78	1.94±0.06
Ethanol	Control	31±0.58	1.66±0.01
	1.0	53±2.51*	1.90±0.08*
	2.0	49±1.15*	1.88±0.05*
	3.0	46±361*	1.86±0.06*
One-way ANOVA	F	132.08	32.62
	df	3, 20	3, 20
	P	<0.05	<0.05
Water	Control	32±2.83	1.52±0.14
	1.0	34±4.24	1.62±0.20
	2.0	55±1.47*	1.90±0.12*
	3.0	30±2.71	1.56±0.09
One-way ANOVA	F	91.85	8.55
	Df	3,20	3,20
	P	<0.05	<0.05

Values are mean±SD, n=6 in each group. *P<0.05 when compared with respective control group (Dunnett's test)

Table 3. Hypersensitivity and hemeagglutination reactions of the successive ethanol extract of Naravelia zeylanica leaves

Successive ethanol extract (mg/kg, post oral)	Hypersensivity reactions#		Hemeagglutination antibody titer	
	4h (mean=SD)	24h(mean± SD)	Range	(mean±SD)
Control	0.14±0.12	0.03±0.34	128-256	213±66.1
100	0.66±0.21	0.37±0.35	128-1024	448±321.3
200	0.78±0.15*	0.50±0.23	256-2048	1067±570.5*
300	0.92±0.28*	0.76±0.28*	512-2048	1126±627.1*
One-way F	5.94	5.89		
ANOVA P	<0.05	<0.05		

Values are mean ± SD, n=6 in each group. Df=3, 20, *P<0.05 when compared to control group (Dunnett's test). # Inflammation after challenge. The differences in rat paw thickness before and after the antigen in min.

4. DISCUSSION

The present study demonstrates, for the first time, the immunostimulant potential of the SEE of *Naravelia zeylanica*. The results of the *in vitro* PMN function test showed a significant increase in the

percentage phagocytosis and phagocytic index for successive ethanol and water extracts. This indicates that these extracts enhance the phagocytic efficacy of the PMN cells by causing more engulfment of the Candida cells versus control, thereby stimulating a non-specific immune response. As the SEE showed promising immunostimulant activity in the *in vitro* test, it was taken up for *in vivo* animal studies. The results of *in vivo* animal studies showed an increase in the early and delayed hypersensitivity reaction to SRBC at doses of 200 mg/kg and 300 mg/kg. This indicated the stimulatory effect of SEE on chemotaxis dependent leucocyte migration. In the early hypersensitivity reaction, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and infiltration of PMN leucocytes. The early increase in vascular permeability as well as neutrophil influx has been ascribed to the complement C_{5a} fragment which is activated by this immune complex (Cochrane, 1974).

Antibody molecules which are secreted by plasma cells mediate the humoral immune response. The SEE showed an increase in the hemagglutination titer at doses of 200 mg/kg and 300 mg/kg in animal studies. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978).

The SEE probably stimulates lymphocyte proliferation, which in turn leads to production of cytokines that activate other immune cells such as B cells, antigen-presenting cells and other T cells. Studies such as the lymphocyte transformation test and cytokine studies are currently underway to understand the exact mechanism for the observedimmunostimulation.

The SEE of Naravelia zeylanicawas found to have a significant immunostimulant activity on both the specific and non-specific immune mechanisms. These results are encouraging enough to pursue bioactivity-guided fractionation of this extract and structure elucidation of the active phytoconstituents.

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