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RESEARCH ARTICLE

Immunomodulatory activities of Punarnavine, an alkaloid from *Boerhaavia diffusa*

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Abstract

The effect of Punarnavine on the immune system was studied using Balb/c mice. Intraperitoneal administration of Punarnavine (40 mg/kg body weight) was found to enhance the total WBC count on 6th day. Bone marrow cellularity and number of α-esterase positive cells were also increased by the administration of Punarnavine. Treatment of Punarnavine along with the antigen, sheep red blood cells (SRBC), produced an enhancement in the circulating antibody titer and the number of plaque forming cells (PFC) in the spleen. Maximum number of PFC was obtained on the 6th day. Punarnavine also showed enhanced proliferation of splenocytes, thymocytes and bone marrow cells both in the presence and absence of specific mitogens *in vitro* and *in vivo*. More over administration of Punarnavine significantly reduced the LPS induced elevated levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in mice. These results indicate the immunomodulatory activity of Punarnavine.

Keywords: *Punarnavine; immunomodulation; LPS; α-esterase; proinflammatory cytokines*

Introduction

Immune system is a complex organization of white blood cells, antibodies, and blood factors that protects the body from foreign invaders, while simultaneously maintaining self tolerance.[1] The basic function of immune system is to protect against foreign pathogens and infectious agents. This is achieved either through innate or natural immunological mechanisms which essentially serve as a short term first line defense or through elaborate adaptive mechanisms which are highly specific, complex, and marked by diversity and memory. In both types of immunity, cells and molecules play important roles.

The mammalian immune system is composed of many cell types like B cells, T cells, NK cells, neutrophils, basophils, eosinophils, macrophages, mast cells, and mediators like cytokines that interact with non-immune cell types and each other in complex and dynamic networks to ensure protection against foreign pathogens. When tissue homoeostasis is perturbed, sentinel macrophages and mast cells immediately release soluble mediators, such as proinflammatory cytokines like TNF, and interleukins.[2]

Cytokines are elaborated by different types of cells, may act in autocrine, paracrine or endocrine manner, stimulate or regulate the growth and functions of nearby cells through specific receptors, and are highly potent as they are effective in picomolar quantities. They are truly language of communication between cells of the immune system. Some of them produce inflammation and some are chemotactic (chemokines) and these may facilitate innate immune reactions.

Due to wide range of effector mechanism possessed by various groups of immune cells and its ability to exert effects with exquisite specificity, immune system provide a good target in cancer therapy. Involvement of the host immune system in the control of cancer progression has been suspected but remained inconclusive for many years. Innate immunity, which according to the immune surveillance theory is responsible for early detection and elimination of malignant cells,[3] may be inefficient in patients who develop malignancy. Evidence is convincing that individuals who are older, who have been on immuno-suppressive medications over prolonged periods of time, or have underlying immune abnormalities,

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such as an autoimmune disease or a chronic infection (e.g., AIDS) are particularly at risk of malignancy.[4,5]

Immunomodulators are substances, which modify the activity of the immune system. They can enhance or inhibit immunological responsiveness of an organism by interfering with its regulatory mechanisms. This may be antigen independent and may directly induce production of mediators and effector molecules by the immunocompetent cells. This type of antigen independent immunity is thus distinct from one achieved by conventional immunization or by passive immunization using antibodies.[6] Immunomodulators can regulate the cytokine production such as tumor necrosis factor, interleukins and interferons and these cytokines may, in turn activate T-cells or NK cells.

Use of plants and plant products as immunomodulators is still in a developing stage. There are few plants reported with known immunomodulatory activity. *Viscum album* a semiparasitic plant has shown to stimulate both humoral as well as cell mediated immune response.[7] Similarly, an extract from the plant *Withania somnifera* has shown to stimulate the immune system[8] and reduce leukocytopenia during chemotherapy.[9] and radiation therapy[10] and inhibit urotoxicity induced by chemotherapeutic drug cyclophosphamide.[11]

It has been reported that *Piper longum*, an immunopotentiating plant, enhances the total bone marrow cells.[31] *Tinospora cordifolia* which is widely used in Indian system of medicine has been reported for its immunomodulatory and antitumor activities.[13] Curcumin, which is present in the plant *Curcuma longa,* has shown to stimulate the immune system in animals.[14] It has also been reported to reduce the leukocytopenia in radiation[15] and chemotherapeutic drug treated animals.[16]

Punarnavine ($C_{17}H_{22}N_2O$ mp 236–237°C), is an alkaloid present in the plant *Boerhaavia diffusa* Linn. (family-Nyctagenaceae).[17–19] It is also considered as the active principle in the plant extract.[20] We have already reported the antimetastatic potential of Punarnavine[21] which can also activate cell mediated immune responses in B16F-10 metastatic melanoma-bearing mice.[22] *B. diffusa* (Punarnava) has attracted lot of attention due to its prevalent uses in Ayurvedic system of medicine. It is widely used in jaundice, hepatitis, edema, oligurea, anemia, inflammations, eye diseases, etc. Pharmacologists and clinicians have investigated "Punarnava" for all these activities and the findings support the existing clinical uses.[23, 24]

Materials and methods

Animals

BALB/c mice (4–6 weeks; 20–25 g body weight) were taken from the breeding section, Amala Cancer Research Centre, Thrissur. The animals were kept in air-controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed according to the rules and regulations of the Animal Ethical Committee, Govenent of India.

Chemicals

RPMI-1640 medium containing 10% FCS, streptomycin (100 μg/ml) and penicillin (100 U/ml) from Hi-Media, Mumbai, India. Para-Rosaniline and α-napthyl acetate were obtained from Loba Chemie, Mumbai. Harri's hematoxylin was purchased from Glaxo, Mumbai, India. Radioactive (^{3}H)-thymidine was purchased from the Board of Radiation and Isotope Technology, Mumbai, India. All other chemicals used were of analytical reagent grade.

Drug preparation

The alkaloid Punaranavine was isolated from the plant *Boerhaavia dffusa* Linn. as per the protocol.[17] Authenticated *B.diffusa*. Linn. was obtained from the Amala Ayurvedic Centre Pharmacy, Thrissur, India, and a voucher specimen is kept in herbarium (Ref. No. ACRC/C/3/2001). Eight kilograms of dried and finely powdered, *Boerhaavia diffusa* Linn. was extracted with boiling ethyl alcohol.

The extract was concentrated to 20% of its volume, and boerhaavic acid was filtered off. The filtrate was concentrated to dryness leaving 998 grams of sticky material. This was extracted with hot water and concentrated to yield Potassium nitrate. It was filtered and the filtrate was made ammoniacal and extracted repeatedly with chloroform. The chloroform extract was evaporated and the residue from evaporation was macerated with diethyl ether. It was then evaporated and gave amorphous Punarnavine. A further amount of Punarnavine was obtained from the sticky material, remaining from the water extraction by extraction with dilute hydrochloric acid.

Amorphous Punarnavine was then crystallized from a small volume of ethyl alcohol and was purified again by recrystalization. The overall yield of Punarnavine was 0.01%. The melting point of crystallized Punarnavine is 236°C. The isolated compound gave positive results for the alkaloid Punarnavine giving a green color with $FeCl_3$, a greenish yellow color with concentrated H_2SO_4, red color with HNO_3, no color with HCl, a black precipitate with KI_3, a blue color followed by a blue precipitate with phosphomolybdic acid, and a brown precipitate with Dragendorff's reagent.

Drug administration

The dosage of Punarnavine was determined based on the toxicity study and was dissolved in double distilled water

to the desired concentration (40 mg/kg body weight) and administered intra-peritonially.

Determination of the effect of Punarnavine on hematological parameters and body weight

BALB/c mice (6 animals/group) (male; 4–6 weeks old; 20–25 g body weight) were divided into two groups. Group 1 was treated as control. Group 2 was treated with Punarnavine (40 mg/kg body weight) for 5 days. Blood was collected from caudal vein and parameters such as body weight, total WBC count (hemocytometer), differential count (Leishman's stain), and hemoglobin level (Cyanmethemoglobin) were recorded prior to the Punarnavine administration and continued every third day for 30 days after the administration of the Punarnavine.

Determination of the effect of Punarnavine on the organ weights

Body weights of the animals grouped above were recorded before sacrifice at 24 h after the last dose of the drug and weight of the lymphoid organs such as spleen and thymus were recorded and expressed as relative organ weights.

Determination of the effect of Punarnavine on the bone marrow cellularity and α-esterase activity

The femurs from above experimental animals were taken, the bone marrow cells collected and made into single cell suspension and the cell number was determined using hemocytometer.[25] Bone marrow cells from the above preparation was smeared on clean glass slides and stained with Harri's hematoxylin to determine the non-specific α-esterase activity by the azodye coupling method.[26]

Determination of the effect of Punarnavine on the circulating antibody titer

Two groups (6 animals/group) of BALB/c mice (male; 4–6 weeks old; 20–25 g body weight) were used in this study. Group I – Normal animals were immunized with SRBC (0.1 ml, 20%). Group II – animals were treated with Punarnavine (40 mg/kg bodyweight) for 5 consecutive days and with the 5th dose SRBC was administered. Blood was collected from the caudal vein every third day after drug administration and continued for a period of 30 days. Serum was separated, heat inactivated at 56°C for 30 minutes and used for the estimation of antibody titer[27] using SRBC as antigen.

Determination of the effect of Punarnavine on the antibody producing cells

BALB/c mice were grouped and with the 5th dose of the Punarnavine (40 mg/kg bodyweight) administration SRBC was administered. The animals were sacrificed on different days starting from the third day after immunization up to the 9th day, spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's Plaque assay.[28]

Determination of effect of Punarnavine on the blastogenesis of spleen, thymus and bone marrow

In vivo

BALB/c mice (male; 4–6 weeks old; 20–25 g body weight) were divided into two groups (6 animals/group). Group I animals were kept as normal control and group II animals were treated with Punarnavine (40 mg/kg bodyweight) for 5 consecutive days. Animals were sacrificed 24 hr after last dose of Punarnavine administration and spleen, thymus, and bone marrow were collected aseptically.

Splenocyte blastogenesis assay

Spleen was processed aseptically into single cell suspension and 5×10^4 cells were cultured in 96-well round bottomed titer plate in the presence and absence of various mitogens such as PHA-2.5 μg/ml, Con A-10 μg/ml, PWM-10 μg/ml, and LPS-10 μg/ml in a humidified 5% CO_2 atmosphere at 37°C and incubated for 48 hr. Cells were labeled with 1 μCi of ^{3}H-thymidine and further incubated for 18 hr. After incubation, DNA was precipitated using 10% ice cold perchloric acid and pellets were dissolved in 0.5 ml of 6N NaOH and transferred to 10 ml scintillation fluid and radioactivity was measured using a Rack Beta fluid scintillation counter.

Thymocyte blastogenesis assays

Thymus cells were processed aseptically into single cell suspension and 5×10^4 cells were cultured in 96-well round bottomed titer plate in the presence and absence of various mitogens such as PHA (2.5 μg/ml), Con A (10 μg/ml) and PWM (10 μg/ml). The rate of proliferation was checked as described earlier.

Bone marrow blastogenesis assays

Bone marrow cells from the femurs (5×10^4 cells/well) were cultured in the presence and absence of various mitogens such as PHA (2.5 μg/ml), Con A (10 μg/ml), PWM (10 μg/ml), and LPS (10 μg/ml), and rate of proliferation were checked as described earlier.

In vitro

Determination of lymphocyte blastogenesis (splenocytes, thymocytes and bone marrow) in the in vitro system

Spleen, thymus and bone marrow were taken from a normal BALB/c mice and a separate single cell suspension

for each tissue were prepared and resuspended in RPMI-1640 medium containing 10% FCS, streptomycin (100 μg/ml) and penicillin (100 U/ml). The cells (5×10^4/well) were incubated with various concentrations of Punarnavine (1, 5 and 10 μg/ml) with and without mitogens in a final volume of 200 μl of medium in 96-well titer plate under humidified atmosphere containing 5% CO_2 at 37°C for 48 hr. The concentrations of the mitogens added were LPS-10 μg/ml; Con A- 10 μg/ml; PHA-2.5 μg/ml and PWM-10 μg/ml. After 48 hr of incubation, cells were labeled by adding [^{3}H]-thymidine (1 μCi) to each well and incubated further for 18 hr under the same conditions.

The cultures were centrifuged at 1500 rpm for 10 min. Supernatant was discarded and the pellets were dissolved in 0.5 ml of NaOH (6N) and incubated at 37°C for 2 hr. The contents were transferred to 5 ml dioxin-based scintillation fluid and kept for 12 hr in the dark. Radioactivity was measured as counts per minute (cpm) in Rack beta liquid scintillation counter (LKB 1209).

Determination of effect of Punarnavine on IL-1β, TNF-α, and IL-6 production in LPS induced animals

BALB/c mice (male; 4–6 weeks old; 20–25 g body weight) were divided into 3 groups (4 animals/group). Group I animals were kept as normal and Group II animals kept as LPS treated control. Group III animals were treated with Punarnavine (40 mg/kg bodyweight) for 5 consecutive days. Two hr after the 5th dose of Punarnavine, group II and III animals were treated with a single dose of LPS (250 μg/animal). After 6 hr, the mice were sacrificed, blood was collected, and serum separated and used for cytokine analysis using specific quantitative sandwich ELISA kits for mouse TNF-α, IL-1β and IL-6 purchased from Pierce Biotechnology, Rockford, IL, USA.

Statistical analysis

Values were expressed as mean ± S.D. The statistical analysis was done by Dunnett's multiple comparison tests using Graphpad InStat software.

Results

Effect of Punarnavine on the hematological parameters and body weight

Administration of Punarnavine increased the total WBC count in Balb/c mice (Figure 1A). In the animals treated with Punarnavine the maximum WBC count was 15078.75 ± 334.7 cells/mm^3 obtained on 6th day. Prior to the Punarnavine treatment the total WBC count was only 6952.5 ± 274.7 cells/mm^3. The untreated control animals maintained the normal total WBC count during the experimental time.

Hemoglobin content was moderately increased by the administration of Punarnavine. There was no appreciable change in the differential count (Data not shown). The body weight of animals also increased to 28.26 ± 2.43 g on 30th day but it was only 24.86 ± 1.77 g before the starting of experiment. For normal animals the bodyweight was increased only to 26.9 ± 2.1 g on 30th day from 24.9 ± 1.87 g on starting day of experiment (Figure 1B).

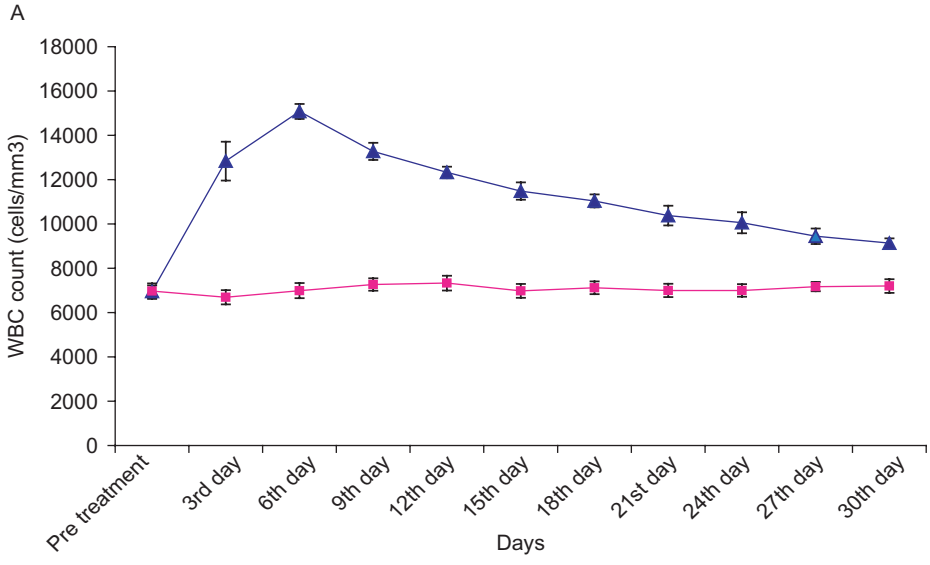

Figure 1A. Effect of Punarnavine on total WBC count. BALB/c mice (6 animals/group) (male; 4–6 weeks old; 20–25 g body weight) were divided into two groups. Group 1 was treated as control. Group 2 was treated with Punarnavine (40 mg/kg bodyweight) for 5 days. Blood was collected and total WBC count (haemocytometer) was recorded prior to the Punarnavine administration and continued every third day for 30 days after the administration of the Punarnavine.

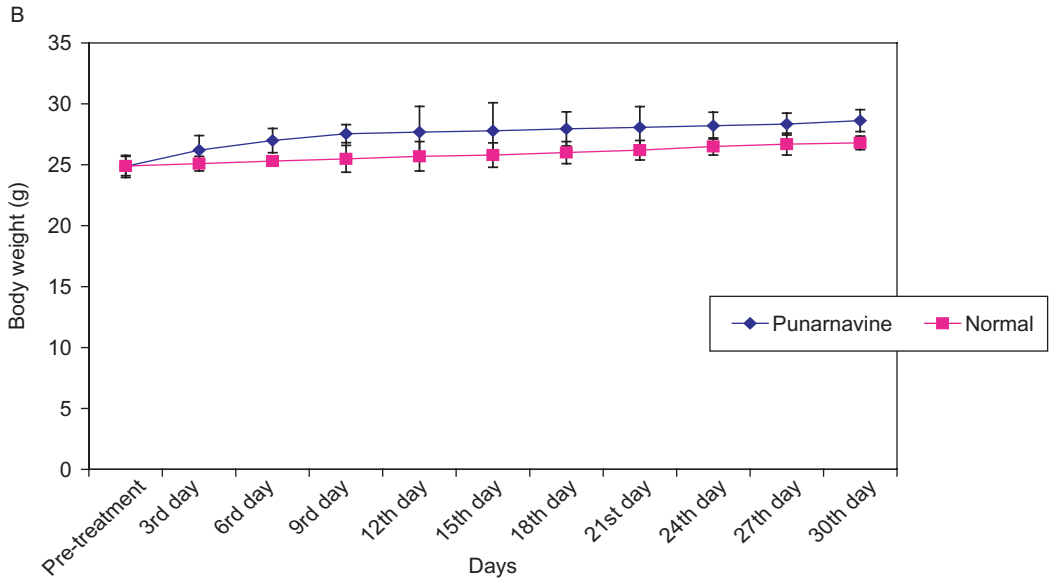

Figure 1B. Effect of Punarnavine on body weight. BALB/c mice (6 animals/group) (male; 4–6 weeks old; 20–25 g body weight) were divided into two groups. Group 1 was treated as control. Group 2 were treated with Punarnavine (40 mg/kg bodyweight) for 5 days. Blood was collected from caudal vein and body weight was recorded prior to the Punarnavine administration and continued every third day for 30 days after the administration of the Punarnavine.

Effect of Punarnavine on relative organ weights of lymphoid organs thymus and spleen

Table 1 shows the effect of Punarnavine on the relative organ weights of thymus and spleen. The relative weight of both thymus and spleen were increased in Punarnavine treated group of animals comparing the untreated control showing statistical significance ($p < 0.001$).

Effect of Punarnavine on the bone marrow cellularity and α-esterase positive cells

The effect of Punarnavine on bone marrow cellularity and α-esterase positive cells is given in Table 2. Punarnavine treated group of animals showed a significant ($p < 0.001$) increase in bone marrow cell number ($29.4 \pm 0.84 \times 10^6$ cells/femur) compared to normal animals ($15.7 \pm 0.3 \times 10^6$ cells/femur). The number of α-esterase positive cells was also increased significantly ($p < 0.001$) in Punarnavine treated animals (1191.7 ± 8.33 cells/4000 cells) compared to the untreated control (837.7 ± 8.14 cells/4000 cells).

Effect of Punarnavine on production of specific antibody

The aim of this experiment was to check the specific immune response using sheep red blood cells (SRBC) as the antigen. There was a significant increase in the production of specific antibody in animals treated with the Punarnavine (Figure 2). The maximum antibody titer of 1024 was observed in the Punarnavine treated animals on 9th day after antigen administration and it was remained as 1024 until 15th day.

Table 1. Effect of Punarnavine on the relative lymphoid organ weight (g/100 g body weight).

Treatment	Spleen	Thymus
Control	0.377 ± 0.031	0.106 ± 0.018
Punarnavine	0.507 ± 0.182*	0.175 ± 0.026*

BALB/c mice (6 Nos /Group) were treated with five consecutive doses of Punarnavine (40 mg/kg bodyweight (i.p). Animals were sacrificed after 24 h of last dose and organ weights were taken. Values are mean ± SD. The statistical analysis was done by using Dunnett's multiple comparison test.
*$P < 0.001$.

Table 2. Effect of Punarnavine on bone marrow cellularity and α-esterase activity.

	Bone marrow cellularity (cells/femur)	α-esterase activity (no.s/4000 cells)
Untreated control	$15.7 \pm 0.3 \times 10^6$	837.7 ± 8.14
Punarnavine treated	$29.4 \pm 0.84 \times 10^{6*}$	1191.7 ± 8.33*

BALB/c mice (6 Nos/Group) were treated with five consecutive doses of Punarnavine (40 mg/kg bodyweight (i.p). Bone marrow cells were collected from femur and made into single cell suspension. The bone marrow cellularity was determined using hemocytometer. The α-esterase positive cells were determined by the azodye coupling method. Values are mean ± SD. The statistical analysis was done by using Dunnett's multiple comparison test.
*$P < 0.001$.

Effect of Punarnavine on plaque forming cells (PFC) in spleen

The effect of Punarnavine on the number of plaque forming cells is shown in Figure 3. The maximum number of

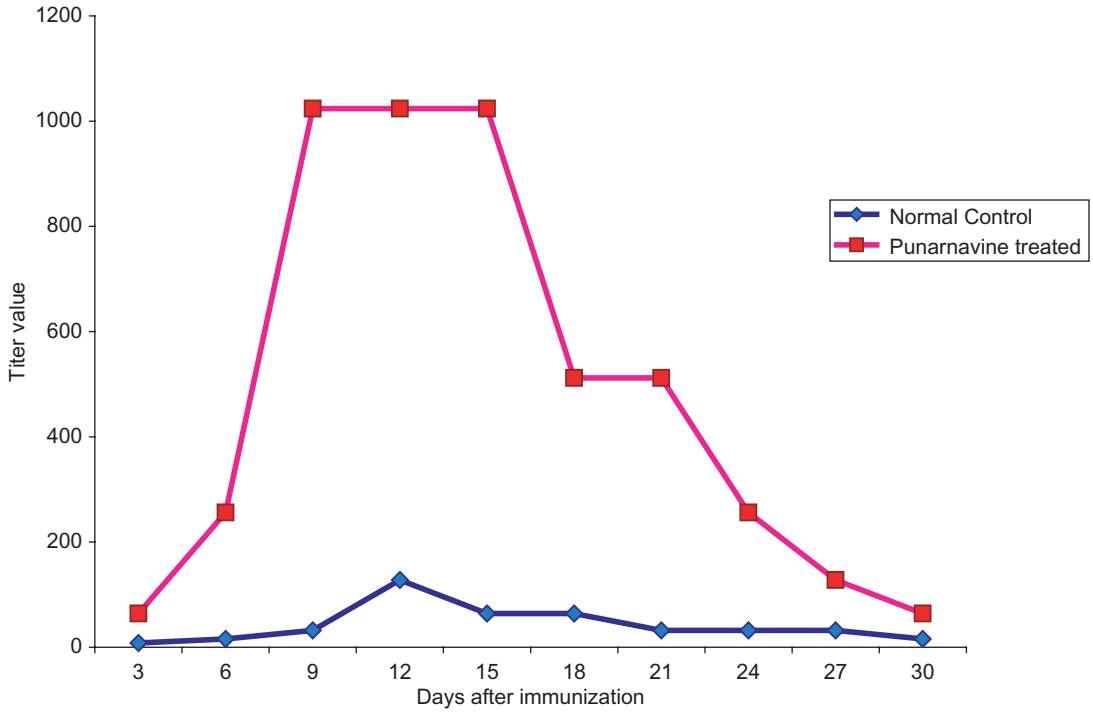

Figure 2. Effect of Punarnavine on the Circulating Antibody Titer. Two groups (6 animals/group) of BALB/c mice (male; 4–6 weeks old; 20–25 g body weight) were used in this study. Group I – Normal animals were immunized with SRBC (0.1 ml, 20%). Group II – animals were treated with Punarnavine (40 mg/kg bodyweight) for 5 consecutive days and with the 5th dose SRBC was administered. Blood was collected every third day after drug administration and continued for a period of 30 days. Serum was separated, heat inactivated, and used for the estimation of antibody titer.[27] using SRBC as antigen.

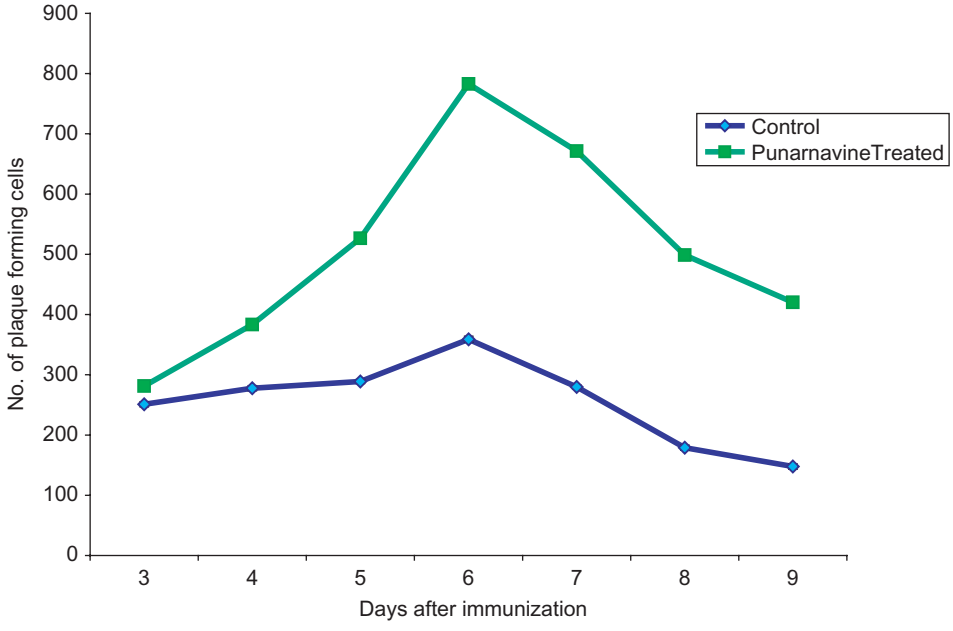

Figure 3. Effect of Punarnavine on the Antibody Producing Cells. BALB/c mice (male; 4–6 weeks old; 20–25 g body weight) were divided into two groups (6 animals/ group) Group I : Normal animals immunized with SRBC (0.1 ml, 20%) Group II animals were treated with Punarnavine (40 mg/kg bodyweight) for 5 consecutive days. Along with the 5th dose of the drug SRBC was administered. The animals were sacrificed on different days starting from the third day after immunization up to the 9th day, spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's Plaque assay.[32]

Table 3. Effect of Punarnavine on splenocytes proliferaton *in vivo*.

	Rate of proliferation (CPM)				
	without mitogens	Con A	PHA	PWM	LPS
Splenocytes (untreated control)	1701 ± 20	4790 ± 76	3738 ± 56	4027 ± 93	4065 ± 49
Punarnavine treated	5093 ± 20*	5975 ± 56*	5196 ± 84*	5377 ± 47*	6011 ± 54*

PMA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen, LPS = lipopolysaccharide.
Treated animals received Punarnavine (40 mg/Kg body weight, i.p) for 5 consecutive days. Spleen cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.001$ compared with specific mitogen control.

plaque forming cells observed in Punarnavine treated animals was 783 ± 3 on the 6th day after immunization. But the number of PFC was only 359 ± 5 in control animals.

Effect of Punarnavine on spleen, bone marrow and thymus blastogenesis in vivo

Spleen blastogenesis

The effect of Punarnavine on blastogenesis of spleen cells is given in Table 3. Significant ($p < 0.001$) enhancement in proliferation of spleen cells in the Punarnavine-treated group (5093 ± 20 counts per minute-cpm) was observed compared with the normal group (1701 ± 20 cpm). There was an enhancement in the rate of proliferation, when the spleen cells were incubated with mitogens along with Punarnavine from that of mitogen alone. In the LPS treated group alone the maximum count was only 4065 ± 49 cpm, but in the Punarnavine treated group along with LPS it was 6011 ± 54 cpm. Administration of Punarnavine also significantly ($p < 0.001$) enhanced the mitogenic potential of PWM (4027 ± 93 cpm), PHA (5196 ± 84 cpm), and con A (5975 ± 56 cpm) on spleen cell proliferation.

Thymocyte blastogenesis

The effect of Punarnavine on thymocyte proliferation is presented in Table 4. Thymocyte blastogenesis observed after stimulation by the mitogens such as PHA (4163 ± 132 cpm), Con A (4828 ± 96 cpm) and PWM (4405 ± 34 cpm) was significantly ($p < 0.01$) increased to 4713 ± 465 cpm, 5025 ± 45 cpm and 4767 ± 63 cpm, respectively in Punarnavine treated animals. The enhancement in the proliferation of thymus cells was observed in Punarnavine treated animals (2278 ± 54 cpm) compared with normal group without mitogen (1558 ± 64 cpm).

Bone marrow blastogenesis

The effect of Punarnavine on bone marrow proliferation is given in Table 5. Administration of Punarnavine significantly ($p < 0.001$) enhanced bone marrow proliferation. Significant enhancement in proliferation of bone marrow cells in the Punarnavine-treated group (3340 ± 113 counts per minute-cpm) was observed compared with the normal group (1164 ± 46 cpm).

Table 4. Effect of Punarnavine on thymocytes proliferaton *in vivo*.

	Rate of proliferation (CPM)			
	without mitogens	Con A	PHA	PWM
Thymocytes (untreated Control)	1558 ± 64	4828 ± 96	4163 ± 132	4405 ± 34
Punarnavine treated	2278 ± 54*	5025 ± 45*	4713 ± 46*	4767 ± 63*

PMA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen. Treated animals received Punarnavine (40 mg/Kg body weight, i.p) for 5 consecutive days. Thymocytes were cultured in the presence and absence of various mitogens and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.01$ compared with specific mitogen control.

There was also an enhancement in the rate of proliferation, when the bone marrow cells were incubated with mitogens along with Punarnavine from that of mitogen alone. Mitogenic activity of PHA (5081 ± 29 cpm) and LPS (5679 ± 33 cpm) on bone marrow proliferation was significantly($p < 0.001$) increased in bone marrow of Punarnavine treated animals compared with bone marrow of normal animals as well as bone marrow treated with PHA (3201 ± 38 cpm) and LPS (3304 ± 76 cpm) alone. Treatment with Punarnavine also enhanced the bone marrow proliferation by the mitogens PWM (4199 ± 64 cpm) and Con A (3674 ± 58 cpm).

Effect of Punarnavine on blastogenesis in vitro

Effect of Punarnavine on splenocyte blastogenesis is given in Table 6. Punarnavine demonstrated the proliferative effect on splenocytes in a dose dependent manner. At the concentration of 10 μg/ml, Punarnavine brought about strong mitogenic transformation of quiescent splenic lymphocytes to their blastic nature, resulting in the increased uptake of [^{3}H] thymidine. These results were comparable to commonly used mitogens such as LPS, Con A, PHA and PWM. Addition of LPS showed significant ($p < 0.001$) enhancement in the proliferation (6506 ± 42 cpm) of the Punarnavine treated splenocytes. The normal untreated splenocytes showed [^{3}H] thymidine uptake of 1701 ± 20 cpm. We found that, there was an enhancement in the rate of

Table 5. Effect of Punarnavine on proliferaton of bone marrow cells *in vivo*.

	Rate of proliferation (CPM)				
	without mitogens	Con A	PHA	PWM	LPS
Bone marrow (untreated control)	1164 ± 46	1597 ± 49	3201 ± 38	2099 ± 47	3304 ± 76
Punarnavine treated	3340 ± 113*	3674 ± 58*	5081 ± 29*	4199 ± 64*	5679 ± 33*

PHA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen, LPS = lipopolysaccharide.
Treated animals received Punarnavine (40 mg/Kg body weight, i.p) for 5 consecutive days. Bone marrow cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.001$ compared with specific mitogen control.

Table 6. Effect of Punarnavine on splenocytes proliferation *in vitro*.

		Rate of proliferation (CPM)		
		Punarnavine		
Spleen	Control	1 μg/ml	5 μg/ml	10 μg/ml
Without mitogens	1701 ± 20	4001 ± 63*	4072 ± 46*	4295 ± 45*
+Con A	4790 ± 76	5564 ± 76*	5650 ± 56*	6006 ± 179*
+PHA	3738 ± 56	4796 ± 55*	5061 ± 47*	5097 ± 57*
+LPS	4065 ± 49	5973 ± 40*	6252 ± 58*	6506 ± 42*
+PWM	4027 ± 93	5116 ± 21*	5366 ± 27*	5453 ± 38*

PHA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen, LPS = lipopolysaccharide.
Spleen cells were cultured in the presence and absence of various mitogens and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.001$ compared with specific mitogen control.

Table 7. Effect of Punarnavine on Thymocytes proliferation *in vitro*.

	Rate of proliferation (CPM)			
		Punarnavine		
Thymus	Control	1 μg/ml	5 μg/ml	10 μg/ml
Without mitogens	1558 ± 64	1673 ± 90*	2481 ± 89**	3256 ± 76**
+Con A	4828 ± 96	4960 ± 103*	5190 ± 17**	5381 ± 58**
+PHA	4163 ± 132	4314 ± 25*	4731 ± 46**	5109 ± 92**
+PWM	4405 ± 34	4626 ± 64**	4828 ± 97**	5280 ± 45**

PHA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen, LPS = lipopolysaccharide.
Thymus cells were cultured in the presence and absence of various mitogens and Punarnavine and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.05$ compared with specific mitogen control.
**$p < 0.001$.

proliferation, when the splenocytes were incubated with mitogens along with Punarnavine from that of mitogen alone.

Incubation of thymocytes along with Punarnavine (10 μg/ml) significantly enhanced ($p < 0.001$) their proliferation from 1558 ± 64 to 3256 ± 76 cpm. The rate of proliferation, was enhanced when the thymocytes were incubated with mitogens along with Punarnavine from that of mitogen alone. Punarnavine (10 μg/ml) along with mitogens resulting in higher [^{3}H] thymidine uptake showing 5381 ± 58 cpm with Con A, 5109 ± 92 cpm with PHA and 5280 ± 45 cpm with PWM. (Table 7).

The effect of Punarnavine on bone marrow proliferation is given in Table 8. At a concentration of 10 μg/ml, Punarnavine significantly enhanced the bone marrow proliferation (3609 ± 80 cpm), compared to the untreated control (1164 ± 46 cpm). There was an enhancement in the rate of proliferation, when the bone marrow cells were incubated with mitogens along with Punarnavine from that of mitogen alone. Punarnavine (10 μg/ml) along with mitogens resulting in higher [^{3}H]- thymidine uptake showing 5967 ± 52 cpm with LPS, 3972 ± 45 cpm with Con A, 5321 ± 43 cpm with PHA, and 4618 ± 41 cpm with PWM.

Effect of Punarnavine on proinflammatory cytokine production in LPS treated animals

Punarnavine could significantly inhibit the production of TNF-α, IL-1β, and IL-6 in LPS treated

Table 8. Effect of Punarnavine on bone marrow proliferation *in vitro*.

	Rate of proliferation (CPM)			
		Punarnavine		
Bone marrow	Control	1 μg/ml	5 μg/ml	10 μg/ml
Without mitogens	1164 ± 43.4	2931 ± 39*	3360 ± 87*	3609 ± 80*
+Con A	1596.7 ± 29.9	3110 ± 32*	3652 ± 48*	3972 ± 45*
+PHA	3201 ± 37	4810 ± 39*	5068 ± 42*	5322 ± 43*
+LPS	3304 ± 76	5316 ± 48*	5705 ± 68*	5967 ± 52*
+PWM	2099 ± 47	3877 ± 63*	4168 ± 66*	4618 ± 41*

PHA = phytohemagglutinin, CON A = concanavalin A, PWM = Poke Weed Mitogen, LPS = lipopolysaccharide.
Bone marrow cells were cultured in the presence and absence of various mitogens and Punarnavine and rate of proliferation was checked by ^{3}H-thymidine incorporation assay. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.001$ compared with normal.

Table 9. Effect of Punarnavine on proinflammatory cytokine production in LPS treated animals (pg/ml).

Treatment	TNFα	IL-1β	IL-6
Control	24.1 ± 0.9	18.12 ± 3.5	36.8 ± 5.6
LPS	646.53 ± 23.99	99.54 ± 2.08	335.09 ± 27.09
LPS + Punarnavine	131.99 ± 17.39*	37.66 ± 1.54*	111.43 ± 6.19*

BALB/c mice were divided into 6 groups (n = 6/group). Group I animals were kept as normal and Group II animals kept as control. Group III animals were treated with Punarnavine (1 mg/dose/animal/day, i.p) for 5 consecutive days. Two hrs after the 5th dose of Punarnavine, the group II and III animals were treated with a single dose of LPS (250 μg/animal). After 6 hrs, the mice were sacrificed ad determined serum TNF-α, IL-1β and IL-6 levels. The statistical analysis was done by using Dunnett's multiple comparison test.
*$p < 0.001$ compared with LPS treated control.

animals (Table 9). The normal levels of TNF-α, IL-1β, and IL-6 (24.1 ± 0.9, 18.12 ± 3.5 and 36.8 ± 5.6 pg/ml respectively) were increased by the treatment with LPS (646.53 ± 23.99, 99.54 ± 2.08 and 335.09 ± 27.09 pg/ml, respectively) which was then significantly ($p < 0.001$) reduced to 131.99 ± 17.39, 37.66 ± 1.54, and 111.43 ± 6.19 pg/ml, respectively, by the administration of Punarnavine.

Discussion

Immunity is a part of complex system of defense reactions of a body. Immunoregulation is a complex balance between regulatory and effector cells and any imbalance in the immunological mechanism may lead to pathogenesis.[29] Modulation of the immune system by cytostatic agents is emerging as a major area in pharmacology, especially in cases where undesired immunosuppression is the result of therapy. Immunomodulators are materials which can modify the body's defense mechanism either by enhancing or by controlling immune response. Both innate and adaptive immunity depends upon the activity of leukocytes of blood.

The common hemopoietic stem cells of bone marrow are the source of major cell types involved in the immune system. Bone marrow also provides microenvironment for the antigen independent differentiation of B-cells. The present study shows significant enhancement in the total WBC count and bone marrow cellularity. All the stem cells possess nonspecific esterase activity. But the differentiated monocytes have α-specific esterase activity. During α-esterase staining α-naphthyl acetate is hydrolyzed to α-naphthol by the esterase present in monocytes. Sodium nitrate present in the stain act as the coupler. The enzyme-substrate complex formed is stained using hematoxyline and hence can be observed. They can be identified by the red-brown azo dye reaction product that is developed by the simultaneous coupling of hexazonium pararosaniline to α-naphthol.[30]

The increment in the number of bone marrow cells and differentiating stem cells with α-esterase activity in Punarnavine treated animals also shows the effect of Punarnavine on enhancing the immunological response. The stimulated proliferation of bone marrow cells by Punarnavine administration with and without the presence of mitogens *in vitro* as well as *in vivo* clearly demonstrate the immonostimulatory effect of Punarnavine on stem cells.

Adaptive immune response, which depend on lymphocytes provide life-long immunity. The increased relative organ weights of lymphoid organs like thymus and spleen in Punarnavine treated animals compared to control indicated the activated immune response by lymphocytes. Radioactive (^{3}H)-thymidine incorporation assay clearly show a significantly enhanced proliferation of both thymocytes and splenocytes in the presence and absence of specific mitogens *in vitro* and *in vivo*. The significant enhancement of antibody producing cells in the spleen of Punarnavine administered animals compared untreated control (PFC) is supporting the above data showing activated humoral immune response. The circulating antibody titer also shows a significant enhancement in Punarnavine treated animals compared with untreated control. This shows stimulated production of anti-SRBC antibody after Punarnavine treatment. This increased titer remained for several days indicating that

there is a sustained immunological activity even after the treatment.

The inflammatory response in the body is mediated by the proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). IL-1 is a cytokine with diverse immunologic, physiologic, and hematopoietic effects and stimulates the production of other cytokines such as IL-6[31] and TNF-α.[32] Through induction of other cytokines, including TNF-α, a cascade of biologic events are affected by Interleukin-1. IL-1 also has a number of effects on the hematopoietic system, inducing the stimulation of bone marrow stromal cells to produce IL-6.[33, 34]

Here in our study, the administration of Punarnavine could inhibit the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in normal Balb/c mice. LPS is a component of the bacterial cell wall which stimulates the inflammatory response by activating the production of proinflammatory cytokines. The reduced levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in LPS induced Balb/c mice after administration of Punarnavine show reduced inflammatory response.

Conclusion

Immunomodulation is the regulation of immune responses by stimulating them to prevent infectious diseases or by suppressing them in the undesired conditions. In the present study we found that Punarnavine is a potent immunomodulator as it stimulated immune system with enhanced stem cell proliferation, stem cell differentiation and antibody formation and suppressing the proinflammatory cytokines in Balb/c mice.

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