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Evaluation of immunomodulatory activity of *Balachaturbhadra Churna*- An *Ayurvedic* formulation

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Balachaturbhadra churna (BCBC) is an extensively used poly-herbal formulation of *Ayurveda* for pediatrics disorders. It is indicated for respiratory disorders, fever, diarrhoea, and vomiting. It is practiced as a potent immune enhancer in children. The present study was undertaken to evaluate immunomodulatory activity of BCBC to validate the classical claims of therapeutic efficacy made on it. The cell-mediated immune response was evaluated against triple antigen induced immunological paw edema in pre-sensitized rats. The humoral immune response against cyclophosphamide induced immunosuppression in sheep red blood cells (SRBC) pre-sensitized rats was evaluated by hemagglutination titer, ponderal changes, and histopathological studies. BCBC produced significant increase ($P<0.05$) in immunological paw edema in triple antigen pre-sensitized rats and significant increase ($P<0.05$) in hemagglutination titer value in humoral immune response compared to control and vehicle control groups. The drug had significant effect in reversal of cyclophosphamide induced immune suppression in thymus, spleen, and lymph nodes. The results indicate a significant effect of the BCBC on cell-mediated immune response and humoral immune response. The reversal of cyclophosphamide induced immune suppression of organs indicates cytoprotective activity of BCBC in rats.

Keywords: *Balachaturbhadra Churna*, Cell-mediated immunity, Cytoprotective activity, Hemagglutination titer, Humoral immunity.

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Introduction

Infectious diseases are now primarily considered immunological disorders while neoplastic diseases and several autoimmune diseases may be involved in an immunosuppressive state[1]. Suppressive and cytotoxic activity affecting the function of immune system has been reported by many of the synthetic and natural therapeutic agents. Among the synthetic substances, azathioprine and cyclophosphamide are alkylating agents and major drawback of this drug is myelo suppressive, which is undesirable. Immunomodulator of herbal origin in Indian system of medicines appears to be a better alternative to overcome the above problem[2]. Traditional and folklore medicines continue to play an important role in maintaining human health around the globe[3]. Many of the remedies and formulations in *Ayurveda* can be traced to texts that are hundreds or thousands of years old and are still in practice today. In *Ayurvedic* medical practice, generally medicinal plants are used in compound form[4]. The herbal drugs in compound forms characterized for polyvalent actions and interpreted as additives or in some cases, they synergistically produce the observed therapeutic effect[5].

Balachaturbhadra churna (BCBC) is one of the renowned formulations in *Ayurvedic* system of medicines particularly in *Kaumarabhritya* (pediatrics), which is indicated for respiratory disorders, fever, diarrhoea and vomiting in children and is also practiced as a potent immune enhancer in children. It is a powder formulation containing 4 drugs in equal proportion namely rhizome of *Musta* - *Cyperus rotundus* L. (Cyperaceae), fruit of *Pippali*- *Piper longum* L. (Piperaceae), root of *Ativisha* - *Aconitum*

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heterophyllum Wall. ex Royle (Ranunculaceae), and gall of *Karkatshringi- Pistacia integerrima* J. L. Stewart ex Brandis (Anacardiaceae)[6]. Various studies related to immunomodulatory activity of individual constituents *Musta*[7], *Ativisha*[8], and *Pippali*[9,10] of BCBC have been found during literature search. Also, immunomodulatory activity of drugs *Musta*, *Pippali*, and *Karkatashringi* as constituents in other *Ayurvedic* formulations have been reported earlier[11-13]. Till date, scientific report on the efficacy was not available, thus, present study investigated the immunomodulatory effect of BCBC with cell-mediated immune response against triple antigen and humoral antibody formation against cyclophosphamide induced immunosuppression in sheep red blood cells (SRBC) pre-sensitized rats.

Materials and Methods

Drug and chemicals

Rhizome of *C. rotundus,* fruit *P. longum*, root of *A. heterophyllum* and gall of *P. integerrima* were collected from the raw drug store of the Institute's Pharmacy. They were authenticated by Pharmacognosy laboratory of the Institution. Drugs were dried properly by shade drying, powdered by micro–pulverizer, and stored in an air–tight container. The BCBC was prepared by mixing the powder of these 4 ingredients in equal proportions[14]. Triple antigen (DPT) was procured from Serum Institute of India (Batch No- 026A200). Other chemicals used in the study were of analytical grade.

Experimental animals

Charle's foster strains of albino rats of either sex, weighing 180±20 g were used for the study. The animals were maintained under ideal husbandry conditions and reared under standard conditions of temperature, humidity, and were exposed to 12 h light and dark cycles. All the animals were exposed to the same environmental conditions and maintained on standard diet and water *ad libitum.* The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/10/2012/01) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSE), India.

Dose fixation

Animal dose of BCBC was fixed on the basis of human therapeutic dose mentioned in literatures[15,16]. Dose for the experimental study was calculated by extrapolating the human therapeutic dose of c*hurna* (7 g/day) to an animal dose on the basis of body surface area ratio by referring to the table of Paget and Barnes (1964)[17]. Thus the dose calculated was 630 mg/kg body weight of rat. The test drug suspension was prepared in honey with distilled water and administered orally in dose of 10 ml/kg body weight of rat.

Immunomodulatory activity

Cell-mediated immune response

The rats were randomly divided into 3 groups, each consisting of 6 rats. Group I was water control group that received distilled water (10 mL/kg, po); Group II was vehicle control (VC) group that received honey with distilled water (10 mL/kg, po); and Group III was BCBC treated group that received the test drug (630 mg/kg, po) with vehicle. Effect on cell-mediated immunity was evaluated using the procedure of Bhattacharya[18]. Initially, the rats were antigenically challenged by injecting the suspension of triple antigen with alum precipitates subcutaneously in the nape of the neck, in a dose of 0.5 mL/100 g body weight. The suspension of triple antigen with alum precipitate prepared by taking triple antigen- 1 mL, normal saline- 4 mL, and potash alum (10 % w/v)- 1 mL. pH of the above suspension was maintained between 5.6-6.8 using 10 % w/v sodium carbonate.

The administration of BCBC and vehicle was started on the day of sensitization and continued for the next 7 days. On 7th day, 1 h after administration of the test drug and vehicle, the rats were injected with 0.1 mL suspension of triple antigen with alum precipitates beneath plantar aponeurosis in the left hind paw. Initially and after 24, 48 h of injecting this alum adjuvant, the paw volume was measured with the help of a digital plethysmograph (IITC). Percentage increase in paw volume after alum adjuvant injection, which is the index of edema formation in comparison to initial value, was noted. The value of the test drug administered group was compared with control and vehicle control group to assess the cell-mediated immunity response of the drug.

Humoral immune response

The albino rats were divided into 4 different groups each consisting of 6 rats. Group I was kept as water control (WC) group and received distilled water (10 mL/kg, po); Group II was cyclophosphamide

(CPC) treated control group and received distilled water (10 mL/kg, po) and cyclophosphamide (80 mg/kg, po); Group III was vehicle control group (VC) and received honey with distilled water (10 mL/kg, po) and cyclophosphamide (80 mg/kg, po); and Group IV was BCBC treated group and received BCBC (630 mg/kg, p.o.) with vehicle and cyclophosphamide (80 mg/kg, po).

The study was carried out as described previously with some modifications[19]. Animals in all the above groups were maintained at normal nutritional status and the drug administration was continued for 11 consecutive days as per respective groups. On the 3rd day, sheep blood was collected in a sterilized bottle containing Elsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid, and 0.42 % sodium chloride) aseptically. The blood was thoroughly washed with sterile normal saline through repeated centrifugation until the supernatant fluid became colorless and was made to 30 % w/v suspension with normal saline. This sensitizing agent as SRBC suspension was injected subcutaneously in the dose of 0.5 mL/100 g of body weight to the rats. SRBC from the same animal was used for both sensitizing and to determine antibody titre.

Two doses of cyclophosphamide (80 mg/kg, po) were administered to produce immunosuppression. On the 4th day of drug administration, the first dose of cyclophosphamide was administered orally to Group II to Group IV and on the 6th day, once again, the same animals were administered with second dose of cyclophosphamide. On the 11th day, 1 h after drug administration, blood was collected by retro orbital puncturing under light anesthesia for hematological parameters. Serum was separated for evaluating hemagglutination antibody titre. All the animals were sacrificed and at the time of necropsy spleen, thymus, and lymph nodes were carefully dissected out. The relative weight of the organs were recorded and preserved for further histopathological studies.

Antibody titre

The serum separated from the blood and the complements were inactivated by incubating for 30 min at 56 °C in a serological water bath. Anti-body levels were determined by the hemagglutination technique[20]. The micro-titre plate was filled with 0.1 mL sterile normal saline and serial two fold dilutions of 0.1 mL of the serum in sterile saline solution were made in the micro-titre plate. About 0.1 mL of thrice saline washed 3 % SRBC was added to each well of the micro-titre plate. SRBC from the same animal was used for both, sensitization and to determine antibody titre. The plate was incubated overnight and examined for visual agglutination. The value of the highest serum dilution showing visible hemagglutination was taken as antibody titre and was converted to $\log_2$ values for easy comparison.

Statistical analysis

The data is expressed as mean±standard error of mean for 6 rats per experimental group. The data generated during the study was subjected to Student's t test for paired and unpaired data, to assess the statistical significance between the groups at $P < 0.05$.

Results

Effect on cell-mediated immune response

Triple antigen with alum adjuvant produced immunological paw edema in pre-sensitized rats (Table 1). BCBC treated group showed non-significant increase in immunological paw edema at 24 h (40.60 %) and 48 h (26.77 %) in comparison to control group. However, a statistically significant (P <0.05) increase in immunological paw edema was observed in BCBC treated group after 24 and 48 h, compared to vehicle control group.

Effect on humoral immune response

The effect of test formulation on anti-body formation against cyclophosphamide induced immunosuppression in SRBC pre-sensitized rats is shown in Table 2. Administration of cyclophosphamide severely decreased the antibody titre (P <0.001) compared to SRBC control group. BCBC significantly increased the anti-body formation in comparison to both cyclophosphamide control group (P <0.02) as well as vehicle control group (P <0.05).

Table 1—Effect of *Balachaturbhadra Churna* on immunological paw edema in triple antigen pre-sensitized albino rats

Groups	Dose (mg/kg)	% Increase in paw volume at different time intervals			
		After 24 h	% change	After 48 h	% change
Control	QS	41.79±6.76	-	36.31±7.66	-
VC	QS	33.33±5.71	20.25 ↓	27.02±7.18	25.57 ↓
BCBC	630.0	58.76±7.67*	40.60 ↑	46.03±3.04*	26.77 ↑

Values are Mean±SEM, ↑- Increase, ↓- Decrease, *P <0.05 when compared with vehicle control group

Effect on body weight

During experimental period of humoral immune activity, normal progressive weight gain in body weight was observed in control group (Table 3). In cyclophosphamide control group, statistically significant decrease in body weight was observed both in comparison to initial values and control group. Treatment with vehicle and BCBC failed to attenuate toxicant induced body weight loss.

Table 2—Effect of *Balachaturbhadra Churna* on antibody titer in cyclophosphamide induced immunosuppression in SRBC pre-sensitized rats

Groups	Dose (mg/kg)	Haemagglutination titer (log_2 value)	% Change
SRBC	-	4.505±0.237	-
CPC	80.0	1.802±0.563$^{\#}$	60.00 ↓
VC	QS	1.964±0.631	8.99 ↑
BCBC	630.0	4.020±0.340*$^{@}$	123.08 ↑

Value are Mean±SEM, ↑- Increase, ↓- Decrease, $^{\#}P$ <0.001 compared with SRBC control group, *P <0.02 compared with cyclophosphamide control group, $^{@}P$ <0.05 compared with vehicle control group, % change- Cyclophosphamide control group compared with SRBC control group and other groups compared with cyclophosphamide control group.

Table 3—Effect of *Balachaturbhadra Churna* on body weight of SRBC pre-sensitized albino rats

Groups	Dose (mg/kg)	Initial body weight (g)	Final body weight (g)	% change
SRBC	-	174.33±4.77	181.67±4.94*	3.58±1.24 ↑
CPC	80.0	162.00±5.83	148.00±4.89*	8.49±2.43 ↓
VC	QS	158.67±4.12	143.67±7.24*	9.64±3.14 ↓
BCBC	630.0	159.60±5.49	138.00±5.83$^{\#}$	13.60±1.11 ↓

Values are Mean±SEM, ↑- Increase, ↓- Decrease, *P <0.05, $^{\#}P$ <0.01 when compared with initial body weight (Paired 't' test)

Effect on spleen, thymus, and lymph nodes

Data pertaining to the effect of the test formulation on the relative weight of organs with cyclophosphamide induced immunosuppression showed that administration of cyclophosphamide did not affect the relative weight of spleen but non-significantly increased the relative weight of thymus in comparison to control group (Table 4). Treatment with the vehicle and test formulation did not affect the relative weight of these organs to significant extent in comparison to cyclophosphamide control group.

Histopathological study

Histopathological study (Plate 1-3) of spleen, thymus, and lymph nodes from SRBC control group

Table 4—Effect of *Balachaturbhadra Churna* on relative weight of spleen and thymus in cyclophosphamide induced immunosuppression in SRBC pre-sensitized rats

Groups	Dose (mg/kg)	Spleen (mg/100mg)	% Change	Thymus (mg/100mg)	% Change
SRBC	-	241.99±09.88	-	168.00±8.41	-
CPC	80.0	243.90±18.17	0.78 ↑	187.97±4.62	11.88↑
VC	QS	280.29±41.64	14.91 ↑	191.02±18.23	1.62 ↑
BCBC	630.0	240.41±21.11	1.43 ↓	185.79±12.89	1.15 ↓

Value are Mean±SEM, ↑- Increase, ↓- Decrease, % change- Cyclophosphamide control group compared with SRBC control group and other groups compared with cyclophosphamide control group.

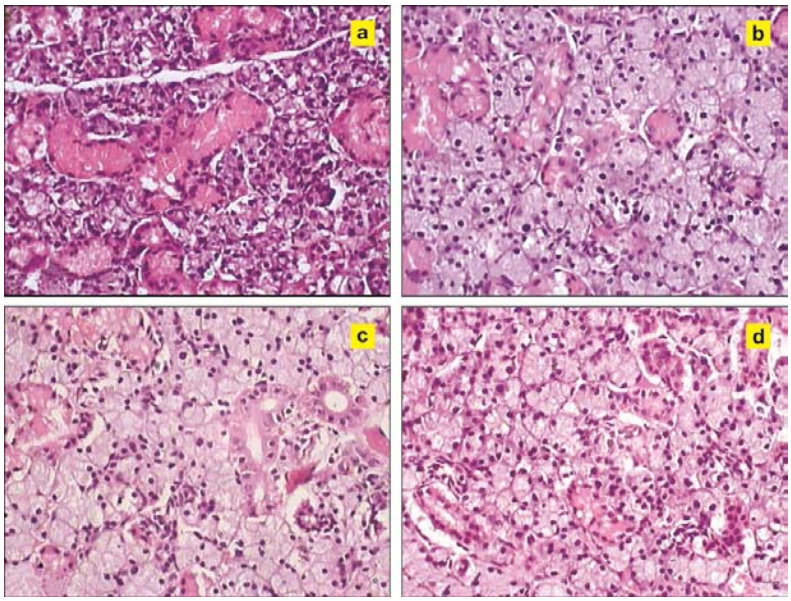

Plate 1—Photomicrographs of sections of thymus taken at 400X magnification. a) Normal cytoarchitecture (Control group), b) Decrease in cellularity and lymphocytolysis (VC), c) Decrease in cellularity and lymphocytolysis (CC), and d) Mild decrease in cellularity (BCBC)

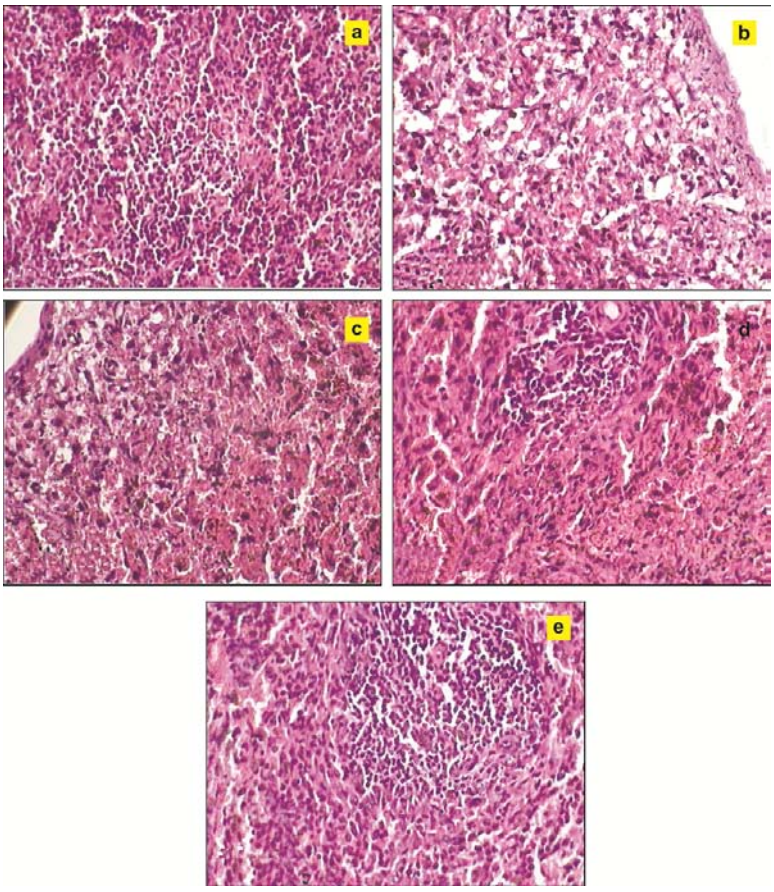

Plate 2—Photomicrographs of sections of spleen taken at 400X magnification. a) Normal cytoarchitecture (Control group), b) Peripheral lymphocytolysis and lymphoid cell depletion (VC), c-d) Decrease in white pulp, peripheral lymphocytolysis, lymphoid cell depletion and fibrosis (CC), and e) Almost normal cytoarchitecture (BCBC)

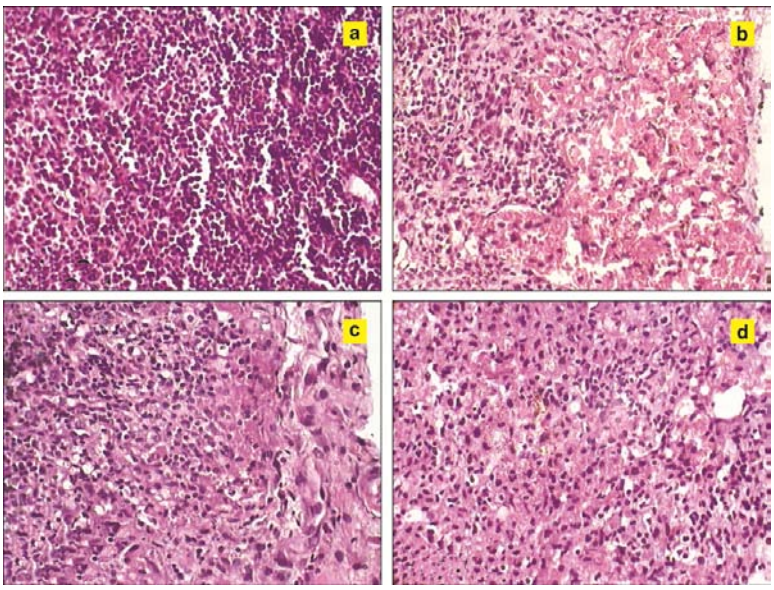

Plate 3—Photomicrographs of sections of lymph node taken at 400X magnification. a) Normal cyto-architecture (Control group), b) peripheral lymphocytolysis, lymphoid cell depletion and fibrosis (VC), c) peripheral lymphocytolysis, moderate lymphoid cell depletion and fibrosis (CC), and d) moderate decrease in cellularity (BCBC)

exhibited normal cyto-architecture. The features of cytotoxicity, in thymus section like decrease in cellularity and lymphocytolysis; in spleen section as decrease in white pulp, peripheral lymphocytolysis, lymphoid cell depletion and fibrosis; and in lymph node section as peripheral lymphocytolysis, moderate lymphoid cell depletion and fibrosis were evident in the group treated with cyclophosphamide in comparison to normal control group. Sections from vehicle control exhibited similar degenerative changes observed in cyclophosphamide control group. Treatment with the BCBC remarkably reversed the cyclophosphamide induced pathological changes in spleen, thymus, and lymph node sections.

Discussion

BCBC is a popular *Ayurvedic* immune enhancer in pediatrics practice. The C*hurna* was evaluated for immunomodulatory activity to revalidate the classical claims for its therapeutic efficacy. Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. They may selectively activate either cell-mediated or humoral immunity by stimulating either TH1 or TH2 type cell response, respectively. Cell-mediated immunity is a part of the process of graft rejection, tumor immunity, and many intracellular infections or to microorganisms that cause chronic diseases. During delayed type hypersensitivity (DTH) responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive inflammatory reaction[21].

In the present study, marked increase in immunological paw edema was observed in BCBC treated group after 24 and 48 h. Data obtained indicated that BCBC produced an increase in the immunological paw edema indicating the cell-mediated immune response of drug against triple antigen in rats. BCBC influenced T-cell activity significantly, which in turn increased vascular permeability, induced vasodilation, macrophage accumulation and activation, and finally resulted in increase in paw volume that promotes phagocytic activity and increase the concentration of lytic enzymes for more effective killing. Therefore, an increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of BCBC on T cells.

Cyclophosphamide, a cytotoxic bi-functional alkylating agent belongs to the class of nitrogen mustard. It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, and other benign diseases[22]. One of the major problems attendant with cyclophosphamide administration is the cytotoxic effect produced on the blood cell forming tissue especially bone marrow leading to suppression of immune response. In the present study, the test formulation was tested in immune suppressed SRBC pre-sensitized animals. The humoral immunity involves interaction of B-cell with the antigen and their subsequent proliferation and differentiation into antibody secreting plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by crosslinking to form clusters that are more readily ingested by phagocytic cell. Humoral anti-body response is mediated by anti-body produced by B-lymphocytes. Hemagglutination antibody titre is a primary method for studying the humoral response[23]. Cyclophosphamide showed significant inhibition in antibody titre response, while adjuvant with BCBC significantly increased the antibody titre against cyclophosphamide induced immunosuppression in SRBC pre-sensitized rats. The observed immune potentiation in the form of reversal of anti-body formation suppression caused by the toxicant is likely to involve a major role for specific cytokines. CD4+ T cells mainly contribute to the regulation of antigen specific immune reactions, mainly by playing a role in the recognition of antigens and consequent production of cytokines.

Reduction in body weight of cyclophosphamide treated rats after 10 days of exposure is indicative of impending toxicity. Loss of body weight is frequently the first indicator of the onset of an adverse effect. The dose, at which body weight loss is 10 % or more, is considered to be a toxic dose, irrespective of whether or not it is accompanied by any other changes. The cytotoxicity in the gut may interfere with the absorption of nutrients. The toxicant induced decrease in the body weight was not attenuated to significant extent indicating mild effect of the BCBC on this parameter as well as on relative weight of the thymus or spleen in comparison to cyclophosphamide control group.

Cyclophosphamide group demonstrated severe pathological changes in all the 3 organs in comparison

to the normal control groups. The white pulp (lymphatic tissue) of spleen form sheath around the arteries. The stroma is a network of reticular fibers and phagocytic reticular cells or fixed macrophages. As in all lymphatic tissue, the meshes of the frame work are filled with free lymphocytes of various size, distributed to form diffuse and nodular lymphatic tissue that vary continuously and reflect the reaction of lymphatic tissue to various generalized stimuli[24]. Cyclophosphamide treated group produced lymphocytolysis, caused decrease in white pulp and cellularity. Fibrosis suggests the loss of normal function to some extent. BCBC showed increase in cellularity in white pulp, which may be due to the increase in lymphatic tissue and free lymphocytes in spleen, in comparison to cyclophosphamide control group. The increase in cellularity in the thymus and lymph node in comparison to cyclophosphamide control group is indicative of reversal of cyclophosphamide induced toxic cellular changes in these organs.

Previous studies have suggested the role of *Piper longum* and piperine as bioavailability enhancer[25] and in production of high titre antibody response in mice[26]. Further, piperine reported reversing of cyclophosphamide-induced chromosomal aberrations in rats[27]. The alkaloid atisine is an important constituent of *A. heterophyllum*, which acts as antiperodic, aphrodisiac, and tonic. Due to the presence of benzyl ester and OH-groups in its molecular structure, aconitine has definite action on central nervous system, cardio vascular system and respiratory system[28]. *Cyperus rotundus* has reported to induce significantly higher inflammatory response and have cytokines mediated immunomodulatory properties[29]. Further formulation containing *C. rotundus* as the main ingredient, exert their immunomodulatory effect via cytokine expression and can attenuate the immunosuppression induced by cyclophosphamide[30]. Honey as an adjuvant in the present study is well known for immune-patenting[31]. Combination of reputed herbal drugs in compound formulation as BCBC along with adjuvant characterized for polyvalent actions and interpreted as synergistically produce the observed immuno modulatory effect. Thus, present study validates the traditional use of BCBC in *Ayurvedic* system of medicine. However, further studies in other experimental models give probable mode of action of BCBC.

Conclusion

From the present study, it is concluded that *Balachaturbhadra Churna* produces a significant increase in cell mediated immune response against triple antigen induced immunological paw edema in rats. It produces significant enhancement of humoral immunity in cyclophosphamide induced immunosuppression in SRBC pre-sensitized rats. It also has cytoprotective activity in the organs like thymus, spleen, and lymph nodes against cyclophosphamide induced cellular changes in rats.

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