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Original Article

IMMUNOMODULATORY ACTIVITY OF NUTRACEUTICAL FORMULATION AND ITS POTENTIATION BY SELF-FORTIFICATION AND COW URINE DISTILLATE FORTIFICATION METHODS

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ABSTRACT

Objective: This study prepared, evaluated immunomodulatory activity of nutraceutical formulation and studied the effect of self-mortification and cow urine distillate fortification methods on the immunomodulatory potential of nutraceutical formulation.

Methods: Three types of nutraceutical formulations i.e. Nutraceutical formulation (NF), self fortitfied nutraceutical formulation (SFNF) and self fortitfied nutraceutical formulation fortified with cow urine distillate (SFNECUD) were prepared using fine powders of amla, apple, garlic, onion, wheat grass, papaya, turmeric and cow urine distillate by different methods. The immunomodulatory activity of nutraceutical formulations at a dose of 500 mg/kg was assessed by various immune function parameters like cell-mediated immunity (neutrophil adhesion, delayed type hypersensitivity (DTH) response and cyclophosphamide-induced neutropenia), humoral immunity (serum immunoglobulins level and haemoagglutination antibody titer), and phagocytic activity (carbon clearance and polymorphonuclear (PMN) cell activity).

Results: Oral administration of NF, SFNF and SFNFCUD showed significant ($p<0.01$) increase in adhesion of neutrophils, potentiation of the DTH reaction and attenuation of cyclophosphamide-induced neutropenia. A significant increase in serum immunoglobulin levels and production of circulating antibody titer in response to sheep red blood cells (SRBCs) was also observed. In addition, an increase in the phagocytic index in carbon clearance assay and an increase in the phagocytic activity of PMN cells was observed.

Conclusion: From the above results, it can be concluded that all three types of formulations showed significant immunostimulant activity. SFNF and SFNFCUD showed better immunomodulatory activity than NF suggesting the potentiation of immunomodulatory potential of NF activity by fortification methods.

Keywords: Nutraceutical formulation, Fortification, Cell mediated immunity, Humoral immunity and Phagocytic activity

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INTRODUCTION

Immunomodulation is an alteration of the immune system and interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulation which primarily implies stimulation of non-specific system, that is, granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors [1]. Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Hence, both immune-stimulating agents and immunosuppressing agents have their own standing and search for better agents exerting these activities is becoming the field of major interest all over the world [2].

Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are a major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system. Immunosuppression is a major drawback in the conventional therapy of cancer such as radiation and chemotherapy. Both this method have sever side effect such as nausea, vomiting, alopecia, mucosal ulceration etc. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles [3].

'Nutraceutical' is defined as a food or any part of food that gives a health benefit above and beyond providing simple nutrition. This generally accepted definition establishes that health benefit may include not only the prevention or treatment of disease but also a simple improvement in the body's immune system and performance. Further, it is also hypothesized that a combination of foods and/or multiple dietary agents may offer better therapeutic potential as compared to isolated compounds [4, 5].

Fortification refers to "the practice of deliberately increasing the content of essential components in a food irrespective of whether the nutrients were originally in the food before processing or not, so as to improve the quality of the food supply and to provide a public health benefit with minimal risk to health [6]. Therefore, the present study was designed to develop nutraceutical formulation with immunomodulatory potential and to study the effect of self-mortification and fortification with cow urine distillate methods on the immunomodulatory potential of prepared nutraceutical formulation.

MATERIALS AND METHODS

Chemicals and reagents

Drugs were procured; Levamisole (Khandelwal Pharmaceutical Ltd. Mumbai), Cyclophosphamide (Biochem Pharmaceutical, Mumbai), Colloidal carbon (Indian ink, camel India Pvt. Ltd.). All other reagents and chemicals were of analytical grade.

Nutraceuticals

All the nutraceuticals used in the preparation of formulations were of fine grade and collected from the local market.

Preparation of nutraceutical formulations

Nutraceutical Formulation (NF) was prepared by mixing fine dried powders of apple fruit, amla fruit, garlic bulbs, onion bulbs, papaya

leaves, turmeric rhizomes and wheat grass in equal quantity. Self fortitfied nutraceutical formulation (SFNF) was prepared by using fine dried powders of self-fortified amla, self-fortified papaya, self-fortified wheat grass along with apple, garlic, onion and turmeric powders. Self-fortification was done by deliberately fortifying the powder with their respective freshly prepared juice (100 g of powder fortified with 50 ml of juice) for three times before adding to the final formulation. Similarly, SFNFCUD was prepared by fortifying SFNF with cow urine distillate (100 g of formulation fortified with 50 ml of cow urine distillate each time).

Animals

Swiss albino mice of weighing 25-35 gm of either sex were used in the study and housed under good laboratory conditions. All the mice were fed with standard pellet diet (Nutrimix Std-1020, Nutrivet Laboratories, Pune) and water *ad libitum*. Animal experiments and handling were in accordance with CPCSEA guidelines, the prior permission for the study was obtained from "institutional animal ethical committee" (IAEC) (Registration no. 516/01/A/CPCSEA).

Experimental groups and treatment

The mice were divided into four groups consisting six animals in each.

Group I: Received sodium CMC (0.1%; p. o.) and served as vehicle control.

Group II: Received NF (500 mg/kg; p. o.)

Group III: Received SFNF (500 mg/kg; p. o.)

Group IV: Received SFNFCUD (500 mg/kg; p. o.)

Preparation of SRBC antigen

Fresh sheep blood was collected in Alsever's solution at the proportion of 1:1 ratio from local slaughter's house. The blood was centrifuged at 3000 rpm for 15 min and serum is removed. The blood cells were washed for 2-3 times with phosphate buffered saline to remove plasma. Then the number of SRBC was then adjusted to a required concentration. The prepared SRBC antigen is stored at 4 ºC [7].

Neutrophil adhesion test

After 14 d of treatment, blood samples were collected by puncturing the retro-orbital plexus into heparanized vials and analyzed for total leucocyte count (TLC) and differential leucocyte count (DLC) by fixing blood smears and staining with Field stain I and II Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample [8]. Percent neutrophil adhesion was calculated using fallowing formula.

$$\text{Neutrophil adhesion (\%)} = NIu - NIt \times 100/NIu$$

Where NI_u= Neutrophil index of untreated blood samples NI_t= Neutrophil index of treated blood sample.

Delayed-type hypersensitivity (DTH) response

In this method, mice were treated for 14 d. On 7thday of treatment, the mice were immunized with 0.1 ml of SRBCs at a concentration of $0.5x10^9$ cells/ml (20% SRBC) intraperitoneally (I. P). On 14thday the mice were challenged with 0.03 ml of 20% SRBCs in subplantar region of the right hind paw, and the same volume 0.03 ml of saline was injected to the left hind paw which serves as control foot [9]. Foot pad reaction was assessed after 24 h using vernier caliper in terms of increase in the thickness of footpad as a result of edema. The footpad reaction was expressed as the difference in the thickness (mm) between the right footpad injected with SRBC and the left footpad injected with normal saline. Cyclophosphamide-induced neutropenia in this method treatment was given for 14 d. On 14th day after 3 h of treatment, a neutropenic dose of cyclophosphamide (200 mg/kg, S. C.) was injected and this day was marked as the first day. Prior to injection blood samples were collected and the total leukocytes count and differential leukocytes count were estimated by using haemo analyser. Again on 3rdday after injection, the TLC and neutrophil counts (%) were estimated [10]. Effect on serum immunoglobulins All the groups were treated for 21 d. Six hours after the last dose of the drug, blood was collected and the serum was used for estimation of immunoglobulin levels using a method devised by Mullen *et al.*, 1975 [11]. Briefly, for each serum sample to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution was prepared. To each, 0.1 ml of serum was added from a pipette [12].

Hemagglutination antibody (HA) titer

All the animals were treated for 21 d. On 7th and 14th day of the study, mice from all the groups (i.e. group I to VIII) were immunized and challenged respectively, with SRBCs in normal saline (0.1 ml of 20% SRBCs) intraperitoneally. Blood was withdrawn on 14th and 21st day from retro-orbital plexus under mild ether anaesthesia from all antigenically sensitised and challenged mice respectively. Blood was centrifuged to obtain serum, normal saline was used as a diluent and the SRBCs count was adjusted to (0.1% of SRBCs). Each well of a microtiter plate was filled initially with 20 µl of saline and 20 µl of serum was mixed in the first well of microtiter plate. Subsequently, the 20 µl diluted serum was removed from first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were similarly carried out till the last well of the second row (24th well) so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 µl SRBC (0.1% of SRBCs) were added to each of these dilutions and the plates were incubated at 37 °C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titer. The antibody titers were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance. Antibody titer obtained on 14th day after immunization (on 7th day) and on 21st day after challenge (on 14th day) with SRBCs was considered as primary and secondary humoral immune response respectively [13, 14].

Carbon clearance test

The colloidal carbon ink or Indian ink was diluted with PBS (pH 7.4) to eight times before using at a dose of 10 µl/gm of body weight of mice. In this method, treatment was given for 14 d. After 3 h of last treatment, the mice of all groups were injected with indian ink at a dose of 10 µl/gm of body weight via the tail vein. Blood samples were collected by retro-orbital plexus method at the intervals of 0 and 15 min. Blood samples (25µl) was mixed with 3 ml of 0.1% sodium carbonate solution and absorbance was determined at 660 nm by using spectrophotometer [15].

The phagocytic index was calculated using the following formula

$$K = \log OD1 - \log OD2/t2 - t1$$

Where, OD_1 and OD_2 are the optical densities at time t_1 and t_2 respectively.

Phagocytic activity of polymorph nuclear (PMN) cells

In this method, treatment was given for 14 d and on 15thday blood sampling was performed and two drops of blood were collected on a clean glass slide and allowed to dry for 5 min at room temperature which permits to clot. The clot was gently removed by washing under running tap water, by leaving a thin layer of cells to adhere the glass slide. This layer of cells are known as PMN cells, which are covered by a layer of *Candida albicans* (yeast cells 10^6 cells/ml) and incubated for 1 h. at 37 °C. The slide was then stained with Giemsa stain and observed under a microscope for phagocytosis of PMN cells. Phagocytic activity was expressed as the average number of *Candida* per PMN [16].

Statistical analysis

The results were expressed as mean±SEM (standard error mean) and statistical analysis was carried out using one-way ANOVA followed by Dunnett's test. The P-value<0.05 is considered statistically significant.

RESULTS

Neutrophil adhesion test

In the current study, a significant increase in neutrophil adhesion at a dose of 500 mg/kg was observed with

nutraceutical formulation (NF). Further, a more significant increase in neutrophil adhesion was seen with SFNF and SFNFCUD. The % neutrophil adhesion in control group animals was 20.4, in NF treated group animals, it was 32.7 whilst for SFNF and SFNFCUD treated group animals, it was 40.4 and 50.1 respectively. The results were given in the table 1.

Table 1: Effect of neutraceutical formulations on neutrophil adhesion

Treatment	Neutrophil index		Neutrophil adhesion (%)
	UB	NFTB	
Vehicle control	312.5±14.1	248.7±7.8	20.4
NF (500 mg/kg)	402. 3±16.7	270.9±9.5	32.7*
SFNF (500 mg/kg)	421.7±17.8	251.3±13.2	40.4**
SFNFCUD (500 mg/kg)	481.4±13.4	239.8±12.1	50.1***

Values are mean±SEM, n=6 in each group. The P***<0.001, P**<0.01 and P*<0.05, are statistically significant.

Delayed-type hypersensitivity reaction

In delayed type hypersensitivity reaction model, treatment with nutraceutical formulation (NF) showed a significant increase in paw edema and percentage of inflammation. The SFNF and SFNFCUD showed more significant activity than NF when compared with control. The results were shown in the table 2.

Cyclophosphamide-induced neutropenia

Pretreatment with NF before cyclophosphamide administration showed a significant protection against cyclophosphamide-induced neutropenia when compared with the control group. Similarly, Pretreatment with SFNF and SFNFCUD showed more significant protection than NF when compared with control. The results were given in the table 2.

Table 2: Effect of nutraceutical formulation on cell-mediated immunity (CMI)

Treatment	DTH response	Cyclophosphamide-induced neutropenia	
	(Mean difference in paw edema(mm))	Reduction in TLC (%)	Reduction in neutrophil count (%)
Vehicle control	0.74±0.08	58.3	62.6
NF (500 mg/kg)	1.39±0. 11**	36.6**	41.7**
SFNF (500 mg/kg)	2.49±0.14 **	28.7***	30.6***
SFNFCUD (500 mg/kg)	2.82±0.09**	20.2***	24.6***

Values are mean±SEM, n=6 in each group. The P***<0.001 P**<0.01 and P*<0.05, are statistically significant.

Serum immunoglobulin level

Administration of NF showed a significant increase in the serum immunoglobulin levels when compared with the control. Further, SFNF and SFNFCUD treated animals showed a greater increase in serum immunoglobulins levels. The percentage of increase in serum immunoglobulins levels in NF treated mice was 39.4. Whereas in SFNF and SFNFCUD treated animals, it was 47.5 and 52.8 respectively. The results were illustrated in the table 3.

Hemagglutination antibody titer

Nutraceutical Formulation (NF) treated mice showed a significant rise in hemagglutination titer value of both primary and secondary immunity when compared with the control group. Similarly, SFNF treated mice showed more significant activity in both primary and secondary immunity when compared with control. The SFNFCUD showed even more significant activity than SFNF. The results were given in the table 3.

Table 3: Effect of nutraceutical formulations on humoral immunity

Treatment	Serum immunoglobulins level (Turbidity units)	Hemagglutination titer values	
		Primary immunity	Secondary immunity
Vehicle control	12.34±0.62	7.16±0.13	7.5±0.19
NF (500 mg/kg)	17. 2±2. 5*	7.98±1.03*	8.52±1.51*
SFNF (500 mg/kg)	23.546±1.52**	8.33±0.16*	9.33±0.16**
SFNF CUD (500 mg/kg)	26.16±1.05** *	9.16±0.13**	12.5±0.19**

Values are mean±SEM, n=6 in each group. The P***<0.001 P**<0.01 and P*<0.05, are statistically significant.

Table 4: Effect of nutraceutical formulations on phagocytic activity

Treatment	Phagocytic index	PMN cells activity (no. of candida/PMN)
Vehicle control (0.1% Sodium CMC)	0.0054±0.0013	3.36±0.05
NF	0.0101±0.002*	4.03±0.08*
SFNF (500 mg/kg)	0.0131±0.004**	4.15±0.06**
SFNFCUD (500 mg/kg)	0.0181±0.001***	4.7±0.052**

Values are mean±SEM, n=6 in each group. The P***<0.001 P**<0.01 and P*<0.05, are statistically significant.

Carbon clearance test

A significant increase in phagocytic activity was observed in NF, SFNF and SFNFCUD treated groups when compared with control group. The SFNF and SFNFCUD showed more significant activity than NF when compared with control. The results were given in the table 4.

Phagocytosis of PMN Cells

NF treatment showed a significant increase in phagocytic activity against *Candida albicans* when compared with the control group. The SFNF and SFNFCUD showed a more significant increase in phagocytic activity against *Candida albicans* when compared with the control group. The results were given in table 4.

DISCUSSION

Neutrophils circulate in the vasculature in a passive state and become more adhesive upon stimulation at sites of inflammation, while it was marginated to the vessel wall, subsequently by transmigration and phagocytosis [17]. In the present study, all three nutraceutical formulations when administered orally at 500 mg/kg body weight significantly increased the recruitment of neutrophils adhesion to nylon fibres which correlate to the process of margination of cells in blood vessels. In delayed type hypersensitivity (DTH) reaction test, the DTH response directly correlates with cell-mediated immunity (CMI). The mechanism behind this elevated DTH during the CMI responses could be due to sensitized T lymphocytes. During immunization when an antigen is recognized by the Th1 cells secretes cytokines which in turn increases the vascular permeability, vasodilation, activation of macrophages and also colony formation with class II MHC molecules. On subsequent challenging with the same antigen even in the trivial amount, the Th1 cells secrete cytokines, activate macrophages and other non-specific inflammatory mediators and produces swelling, redness etc [18, 19]. In our present investigation, DTH response was significantly increased by nutraceutical formulation (NF) and potentiated by both the fortification methods suggesting the stimulation of cell-mediated immunity.

Cyclophosphamide is an important anticancer drug which acts by causing alkylation of DNA. The main side effect of cyclophosphamide is bone marrow depression leads to neutropenia. The immune-stimulants significantly enhances the bone marrow functions and reducing the side effects of various anticancer drugs [10]. In the present study, the nutraceutical formulations exhibited significant protection against cyclophosphamide-induced neutropenia, suggesting that nutraceutical formulations may also have an effect on the haematopoietic system.

The estimation of serum immunoglobulin level is a direct measure to detect the humoral immunity. Immunoglobulins are the proteins which are produced by B-lymphocytes, these are also known as antibodies as they fight against antigens. Zinc sulphate causes precipitation of the immunoglobulin making the solution cloudy. The extent of turbidity indicates the immunoglobulin level in serum [11]. In our study, all the test formulations increased the serum immunoglobulin levels suggesting the immunostimulant potential of nutraceutical formulations.

Antibodies are the effectors of the humoral response when an animal is immunized with an antigen the antibodies are released in response to antigen. The released antibodies are of two types they are active and inactive antibodies. The active antibodies immediately interact with the antigen which is known as primary immunity. The inactive antibodies are called “Memory antibodies” which are released in large amounts when an animal is exposed with the same antigen even in trivial amounts, which is known as secondary immunity [20]. The high values of haemagglutinating antibody titer (primary and secondary) obtained in the case of nutraceutical formulations have indicated that immunostimulation was achieved through humoral immunity.

Phagocytosis is the mechanism of removal of microorganisms, foreign bodies etc. from the body. Macrophages play an important role in phagocytosis. The reticuloendothelial system is the best defined functionally by its ability to scavenge debris or other foreign matter and forms the first line of defence. The rate of removal of carbon particles, by the sessile intravascular phagocytes in the liver and spleen, from the bloodstream, is a measure of reticuloendothelial phagocytic activity. In carbon clearance test; nutraceutical formulations treated groups exhibited significantly high phagocytic index. This indicates stimulation of the reticuloendothelial system by nutraceutical formulations treatment [21, 22]. The polymorphonuclear phagocytes (which include neutrophils, basophils, and eosinophils) are of key importance in the containment of infection. Neutrophils, in particular, are specialized killer cells which destroy the microbial prey by releasing various cytokines [23]. In the present investigation, nutraceutical formulations treated groups showed significant engulfment of the *Candida albicans* per PMN cells suggesting the immunostimulant property of nutraceutical formulations. The order of immune-stimulant activity of nutraceutical formulations was NF> NFCUD> SFNFCUD.

CONCLUSION

From the results of this study, it can be concluded that the combination nutraceuticals like amla, apple, papaya, turmeric, wheatgrass, onion and garlic acted synergistically evidenced by stimulation of not only non-specific immune response but also stimulation of humoral immunity and cell-mediated immunity by nutraceutical formulation (NF) when administered orally at a dose level of 500 mg/kg. Further, SFNF and SFNFCUD treatment resulted in even more immunostimulant activity suggesting the potentiation of immunostimulant activity of nutraceutical formulation by the self-fortification process and cow urine distillate fortification. Further studies are also warranted for understanding the exact mechanisms responsible for the immunomodulatory potential of NF and its potentiation by self-mortification and cow urine distillate fortification process.

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CONFLICTS OF INTERESTS

All authors have none to declare

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