Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 11s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

Evaluation of Immunomodulatory Activity of Milk-Based Nutraceutical Formulations Containing Alcoholic Extracts of Nyctanthes arbor-tristis, Asparagus racemosus, Withania somnifera, Berberis aristata, and Zingiber officinale in Mice

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Cite this paper as: Ms. Leena Chavan, Dr. Chhaya Gadgoli, (2025) Evaluation of Immunomodulatory Activity of Milk-Based Nutraceutical Formulations Containing Alcoholic Extracts of Nyctanthes arbor-tristis, Asparagus racemosus, Withania somnifera, Berberis aristata, and Zingiber officinale in Mice. *Journal of Neonatal Surgery*, 14 (11s), 950-969.

ABSTRACT

This study investigates the immunomodulatory potential of three milk-based nutraceutical formulations containing extracts from *Nyctanthes arbor-tristis, Zingiber officinale, and Withania somnifera* (F1), *Nyctanthes arbor-tristis, Zingiber officinale, and Asparagus racemosus* (F2), and *Nyctanthes arbor-tristis, Zingiber officinale, and Berberis aristata* (F3) in comparison to the standard immunosuppressant drug, cyclophosphamide.

The formulations were developed using a spray drying technique to improve the stability, absorption, and delivery of the active plant constituents. This approach is intended to enhance the shelf life and ensure the product can be used conveniently at any time, without compromising its efficacy. These formulations were evaluated for immunomodulatory activity cyclophosphamide-induced myelosuppression in mice.

Our findings demonstrated a marked enhancement in immune responses in groups treated with each of the individual formulations compared to the control group, the standard treatment group (negative control), and the group treated with a combination of the formulation and cyclophosphamide. These results suggest that the plant extract-loaded nanoparticles within the milk-based formulations hold promising potential as effective immunomodulatory agents.

Keywords: *Immunomodulation, nanoparticles, Plant extracts, Spray drying, animal study, nutraceuticals*

1. INTRODUCTION

The growing field of nutraceuticals has attracted considerable attention for its potential to leverage the health benefits of natural sources, particularly medicinal herbs. Among these, *Nyctanthes arbor-tristis* (Parijat), *Withania somnifera* (Ashwagandha), *Zingiber officinale* (Ginger), *Asparagus racemosus* (Shatavari), and *Berberis aristata* (Daruharidra) are notable for their diverse health advantages, making them promising candidates for innovative nutraceutical formulations [1,2]. In an era where holistic well-being is increasingly prioritized, combining these herbs into a nutraceutical preparation holds great potential for enhancing human health.

Immunostimulation, the enhancement of the immune system's ability to defend the body against pathogens, has emerged as a critical aspect of preventive healthcare. The immune system is fundamental in maintaining overall health by protecting against infections and diseases [3, 4]. Strengthening the immune response is a key strategy for improving health and resilience, making immunostimulant agents invaluable in the quest for better health outcomes.

The orange-colored tubular calyx of the flowers of *N arbor-tristis*, commonly known as Parijat, is recognized for its ability to enhance both humoral and cell-mediated immunity. It is a potential substitute for saffron stigma due to the presence of crocin, an apocarotenoid [6]. The roots and rhizomes of *W somnifera* (Ashwagandha) are known for their adaptogenic properties and their role in regulating immune responses and reducing inflammation. It has demonstrated significant stress

reduction, increased physical endurance, and various protective effects against conditions such as gastric ulcer, hepatotoxicity, and hypertension [5]. The roots and rhizomes of *Z officinale* (Ginger) possess potent anti-inflammatory and immunostimulant properties, contributing to improved immune function. Historically, it has been employed to treat ailments such as colds, nausea, arthritis, migraines, and hypertension [7, 8]. The roots of *A racemosus*, commonly known as Shatavari, are known to boost immune health and overall vitality, recognized for their antioxidant, anti-aging, and immune-boosting activities [9]. The roots of *B aristata* (Daruharidra) contain berberine, a compound that has shown significant immunostimulant and anti-inflammatory effects. It demonstrates antimicrobial efficacy against various microorganisms, making it useful in treating acute diarrhea and central nervous system problems [10, 11].

Given the proven immunostimulant properties of these medicinal plants, their incorporation into a nutraceutical formulation represents a rational approach to enhance immune function. The current study aims to explore the immunostimulant potential of a novel nutraceutical formulation containing alcoholic extracts of *N arbor-tristis*, *W somnifera*, *Z officinale*, *A racemosus*, and *B aristata*. By utilizing these extracts in a blend, the formulation is designed to potentiate immune responses and offer a holistic means of supporting human health.

The primary objective of this research is to comprehensively evaluate the immunostimulant effects of a novel nutraceutical formulation containing alcoholic/hydroalcoholic extracts of the orange-colored tubular calyx of *N. arbor-tristis*, roots or rhizomes of *A. racemosus, W. somnifera, B. aristata, and Z. officinale*. The study aims to evaluate the immunostimulant potential of the nutraceutical formulations containing alcoholic extracts of the above plants.

2. MATERIAL AND METHODS

Plant Materials:

- ***Nyctanthes arbor-tristis* (Parijat):** Fresh flowers were collected from local area of Dombivli, Maharashtra, India, in the early morning. The calyx was manually separated from the petals, washed with water and then dried under shade for its use in the study.
- ***Asparagus racemosus, Withania somnifera, Berberis aristata, Zingiber officinale*:** The root powders of *Asparagus racemosus, Withania somnifera*, and *Berberis aristata*, along with rhizomes of *Zingiber officinale*, were procured from Yucca Enterprises, Mumbai, India.

Authentication of the plant materials was carried out by Dr. Harshal Pandit at Guru Nanak Khalsa College, Matunga, Mumbai. The voucher specimens of namely lc p 07232794, lc p 07232805, lc p 07232856, lc p 07232827, lc p 07232895 of *N. arbor-tristis*, *Z. officinale*, *B aristata*, *A. racemosus* and *W. somnifera* respectively, are deposited in the herbarium.

3. CHEMICALS AND SOLVENTS

Toned milk with fat content of 3.0% to 3.5% (Amul brand) was utilized for preparation of formulations. The other chemicals and solvents from Loba Chemie, Mumbai, India of AR grade were utilized in the present study.

A. Extraction

***N arbor-tristis*:** The dried powder of tubular calyx (100 g) was extracted by macerating with ethanol (500 ml) in dark for 6 -8 hours at room temperature [13]. The ethanolic extract thus obtained was evaporated to syrupy consistency in rotary flash evaporator and dried under vacuum oven at temperature not exceeding 45 ^{0}C and the yield of the extract is found to be 8 gm. The extract was standardized for the content of crocin [12].

Similarly the dried powder of roots or rhizomes of *Z officinale* (200 g) was extracted with ethanol (1000 ml) using maceration process at room temperature in dark for 7-8 days and the extract thus obtained was concentrated under vacuum to yield 15 g of extract [13].

The dried root powders of rhizomes of *W somnifera* (200g) and *B. aristata* (150g) were defatted separately with Petroleum ether (60-80) using Soxhlet apparatus, followed by extraction of the defatted dried powders with alcohol using Soxhlet Extractor for 8 hours. The extracts were then concentrated in Rotary Flash Evaporator till syrupy mass and dried under vacuum to remove the solvent. The yields of alcoholic extracts of *W somnifera* and *B. aristata* are found to be 12 g and 10g respectively [14, 15].

The dried root or rhizome powder of *A racemosus* (250 g) was extracted with alcohol using Soxhlet extractor. The resulting ethanolic extract was concentrated into a syrupy mass using a rotary flash evaporator, and the solvent was subsequently removed to dryness at a controlled temperature of 50°C with the yield of the extract being 18 g [16]**.**

B. Standardization of Extracts

The alcoholic extract of *N. arbortristis*, was standardized for the content of Crocin, using UV spectroscopy. The alcoholic extracts of *Z. offianale* and *B. aristata* were standardized for the content of Zingerone and Berberine respectively using and HPTLC (High Performance Thin Layer Chromatography). The extracts of *W. somnifera*, and *A. racemosus* were standardized

for total content of steroids. The extract of *Z. officianale* was also standardized for the total phenol content [17, 18, 19].

B.1 Determination of content of Crocin, Zingerone and Berberine

Crocin was isolated from the ethanolic extract of *N. arbor-tristis* by column chromatographic technique as described by Sujatha *et al* [20]. Berberine was isolated by the procedure described by Chand *et al* [21]. Reference standard of Zingerone was procured from Yucca Enterprises, Mumbai.

Preparation of Calibration Curves

The standard solutions of Crocin (1000 ppm), Zingerone (200 ppm) and Berberine (150 ppm) were prepared in ethanol. The standard solutions of these markers were diluted to get 50 ppm solutions which served as working standards.

The UV spectroscopy and HPTLC conditions described in Table 1 were utilized for the preparation of calibration curves for the marker compounds

Table 1: UV spectroscopy and HPTLC Analytical Conditions for the Determination of Crocin, Zingerone, and Berberine

Parameters	Crocin	Parameters	Zingerone	Berberine
Solvent	Ethanol	Mobile phase	Toluene: Ethyl acetate (8:2)	n-propanol: formic acid: water (9: 0.9: 0.1
Detecting wavelength	430 nm	Stationary phase	Silica gel GF-254 plates	Silica gel GF-254 plates
		Detecting wavelength	320 nm	350 nm
		Bandwidth	8 mm	8 mm
		Derivatizing agent	Vanillin-sulfuric acid	Dragendorff's reagent

Preparation of sample

I) Weighed accurately about 100 mg of alcoholic extract of *B.aristata* and dissolved in 10ml of 5%v/v aqueous HCl solution. The solution was basified with 0.5ml of 10% w/v aqueous NaOH solution and extracted using a separating funnel three times with 10 ml of chloroform each time. Pooled all the chloroform extracts and evaporated to dryness. The residue was dissolved in 5 ml methanol and volume was made to 10 ml with methanol (Stock I). From this 1 ml aliquot was taken and again diluted in 10ml of methanol (Stock II). Again from stock II, 1 ml aliquot was taken and again diluted in 10ml of methanol (Stock III) and further utilized for determination of content of berberine using HPTLC method [22].

II) In case of alcoholic extract of *N.arbor-tristis* 100 mg of the dried ethanolic extract was accurately weighed and dissolved in 100 ml of ethanol to get concentration of 1000 ppm (stock I). 1 ml of the stock solution I was diluted to 10 ml with ethanol to get a concentration of 100 ppm (stock II).The solution was further diluted appropriately to get the absorbance value in range of standard curve. The content of Crocin was calculated by extrapolating the standard curve [20].

III) In case of alcoholic extract of *Z. officianale*, 50 mg of the extract dissolved in 100 ml of ethanol to achieve the stock solution of 500 ppm. 10 and 15 µl of the solution were loaded on HPTLC plates for the analysis. The conditions of HPTLC analysis described in Table 1 were utilized for the analysis of the extracts [23].

B2. Determination of Total Steroidal Content Using UV Spectroscopy

1. **Preparation of Standard Solutions:**

A cholesterol standard curve was used to determine steroidal content. The stock solution was prepared by dissolving 400 mg of cholesterol in 100 ml of isopropanol (4000 ppm). Various concentrations of 10, 25, 50, 100, 200, 300, and 1000 ppm were prepared from the stock solution using isopropanol. The standard solution (1 ml) was transferred into a 10 ml volumetric flask, followed by the addition of 3 ml glacial acetic acid, 0.3 ml ferric chloride solution (2.5 g ferric chloride in 100 ml phosphoric acid), and 3 ml concentrated sulfuric acid. Absorbance was recorded at 560 nm using a UV spectrophotometer (Shimadzu 1900) [24].

Preparation of Sample Solutions:

The alcoholic extracts of *W somnifera* and *A racemosus* (500 mg each) were separately placed in a beaker and mixed with 20 ml of concentrated hydrochloric acid, followed by heating for 30 minutes. After cooling, each mixture was transferred to a separating funnel and extracted with diethyl ether, repeating the extraction until the ether layer became colourless. The pooled ether layers were then evaporated to obtain a residue, which was dissolved in 10 ml of isopropanol, (solution 1). From solution 1, 1 ml aliquot was pipetted out and diluted to a final volume in a 10 ml volumetric flask. Then, 3 ml of glacial acetic acid, 0.3 ml of ferric chloride solution, and 3 ml of concentrated sulfuric acid were added. The absorbance of the resulting solution was measured at 560 nm using an ELICO UV SL159 spectrophotometer against a blank. The total steroidal content was then calculated by referencing the cholesterol standard curve.

B3. Determination of Total Phenolic Content Using UV Spectroscopy

1. **Preparation of Standard Solutions:**

A standard gallic acid solution was prepared by dissolving 100 mg of gallic acid in 100 ml of distilled water to create a 1000 ppm stock solution. From this stock, various dilutions (0.1, 0.5, 1.0, 2.5, and 5.0 mg/ml) were prepared in distilled water for use in colour development. For each dilution, 100 µl was mixed with 0.5 ml of distilled water and 0.1 ml of Folin-Ciocalteu reagent, and the mixture was allowed to stand for 6 minutes. Subsequently, 1 ml of 7% w/v aqueous sodium carbonate solution and 0.5 ml of distilled water were added to each sample. The absorbance was measured at 650 nm, and a calibration curve was constructed using the obtained values. [25].

Preparation of Sample Solutions:

The alcoholic extracts of *Z officinale*, *W somnifera* and *A racemosus*, *B.aristata* and *N.arbor-tristis* (250 mg) were diluted with 10 ml of distilled water in a volumetric flask. From this solution, a 1 ml aliquot was pipetted and further diluted to 25 ml in another volumetric flask. To the diluted solution, 1 ml of Folin-Ciocalteu reagent and 10 ml of 7% w/v aqueous sodium carbonate solution were added, and the final volume was adjusted to 25 ml with distilled water. The solution was allowed to stand for 6 minutes to develop a blue color. Absorbance was measured at 650 nm using an ELICO UV SL159 spectrophotometer against a blank. The total phenolic content was then calculated with reference to a gallic acid standard curve.

Formulation of Milk Blend

Three milk blend formulations in the form of powder containing combinations of alcoholic extracts of Z. officinale**,** N. arbor-tristis**,** W. somnifera**,** B. aristata, and A. racemosus as described in Table 2 were prepared using spray drying process.

Table 2 Formulation Composition Table

Ingredients	**N1**	**N2**	**N3**
N. arbor-tristis Extract	20 g	20 g	20 g
A. racemosus Extract	-	20 g	-
W. somnifera Extract	20 g	-	-
B. aristata Extract	-	-	20 g
Z. officinale Extract	10 g	10 g	10 g
Milk powder	40 g	40 g	40 g
Preservatives	0.1 g	0.1 g	0.1 g
Sugar	9.9 g	9.9 g	9.9 g
Flavouring Agent	q.s.	q.s.	q.s.
Total Weight	100 g	100 g	100g

The toned milk with fat content between 3.0 to 3.5% was utilized for the formulation. The 100 ml of milk was mixed with 20 g of extracts as stated in the formula (Table 2) and spray dried using LSD 48 with the operating conditions of 150°C inlet temperature and 80°C outlet temperature**.**

C1. Spray Drying Process: The alcoholic extracts weighing 10 gm each of Z. officinale**,** N. arbor-tristis**,** W. somnifera**,** B. aristata**,** and A. racemosus**,** along with other excipients, were mixed with 100 ml of milk. The mixture was diluted to 200 ml and spray dried using LSD-48 mini spray dryer with the operating conditions of 100 to 120°C inlet temperature**,** 118 Nm^3/hr

air flow rate**,** and 150 ml/hr feed flow rate [26]**.**

D. Evaluation of Powdered Milk Blend formulations

The spray-dried milk blend powder of the three formulations was evaluated for parameters like color, taste, moisture content (Aulton's Pharmaceutics: The Design and Manufacture of Medicines), % Compressibility Index, Hausner's Ratio, pH **(**1% solution in water) [27, 28].

D1. Determination of Marker Compounds in Milk Blend Powder Formulations

Sample Preparation for determination of Content of Crocin from spray dried powder:

For determination of content of crocin in the powder blend, 200 mg of the formulation was mixed with 5 ml of ethanol and diluted to 10 ml and filtered (stock solution A). 1 ml of stock solution A was diluted to 10 ml with ethanol (stock solution B), and 1 ml of stock solution B was further diluted to 10 ml to produce the sample solution for analysis. The absorbance of stock solution C was measured at 430 nm using an ELICO UV SL159 Spectrophotometer.

Sample Preparation for determination of Content of Berberine from spray dried powder:

HPTLC Method: To prepare the sample, 100 mg of the spray-dried powder was weighed accurately and heated with 10 ml of methanol for 10 minutes to extract berberine. The mixture was then filtered to remove any insoluble residue. The analysis was carried out using precoated silica gel GF-254 plates (10x10 cm). Solutions containing berberine at concentrations of 1000 ng and 1250 ng from the stock solution were applied to the plates. The mobile phase used for separation was composed of n-propanol, formic acid, and water in a 9:0.1:0.9 ratio. The plates were developed in this mobile phase until the solvent front reached a suitable height. After drying, the plates were observed under UV light at 350 nm, and spots were visualized with Dragendorff's reagent, which enhanced spot visibility.

Sample Preparation for determination of Content of Zingerone from spray dried powder:

HPTLC Method: 100 mg of the spray-dried powder was accurately weighed and heated with 10 ml of ethanol. The mixture was then filtered, and the volume was adjusted to 10 ml with ethanol to prepare the stock solution. For analysis, precoated silica gel GF-254 plates (10x10 cm) were used. The stock solution was applied to the plates, which were then developed in a mobile phase of chloroform and methanol in a 9:1 ratio. After development, the plates were dried and sprayed with vanillin-sulfuric acid reagent to enhance visibility. Detection of zingerone was carried out under UV light at 320 nm.

Sample Preparation for determination of Content of Steroid from spray dried powder:

500 mg of spray-dried milk powder was mixed with 10 ml ethanol, filtered, and the filtrate was evaporated to obtain a residue. The residue was treated with 20 ml concentrated HCl and heated for 30 minutes, then washed 3–5 times with diethyl ether (DEE) until the DEE layer became colorless. The DEE layers were combined, evaporated, and the residue was dissolved in 10 ml isopropanol to prepare the stock solution. Sequential dilutions were made to prepare solutions A and B. From Solution B, 1 ml was mixed with glacial acetic acid, ferric chloride, and sulfuric acid. Absorbance was measured at 560 nm, and total steroidal content was calculated using a cholesterol standard curve

Sample Preparation for determination of Content of phenol from spray dried powder:

500 mg of spray-dried milk powder was mixed with 10 ml ethanol, filtered, and the filtrate was evaporated to obtain a residue. The residue was dissolved in 10 ml distilled water to prepare Solution A. From Solution A, 1 ml was pipetted into a 10 ml volumetric flask and diluted to 10 ml with distilled water to prepare Solution B. Then, 1 ml of Solution B was transferred to a 25 ml volumetric flask, followed by the addition of 1 ml Folin-Ciocalteu reagent and 10 ml of 7% sodium carbonate solution, and the volume was adjusted to 25 ml with distilled water. The solution was allowed to stand for 6 minutes to develop a blue color. Absorbance was measured at 650 nm using the ELICO UV SL159 Spectrophotometer against a blank.

D2. Analysis of the Product

The product was analyzed for various parameters that are involved in the analysis of food product or nutraceutical product. The evaluation included visual inspection for appearance, quantification of total fat, protein, and carbohydrate content, as well as determination of energy and sugar levels. Heavy metal concentrations (lead, cadmium, mercury, and arsenic) were meticulously assessed to ensure compliance with safety standards. Additionally, microbiological quality was gauged through total bacterial count, while the formulations' nutritional profile was further characterized by measuring calcium content. These analyses collectively provide a comprehensive understanding of the nutraceutical formulations' composition, safety, and nutritional attributes.

D3. Evaluation of Pharmacological Activity

Immunomodulatory activity of the three formulations was evaluated using cyclophosphamide induced immunosuppression in mice model. The protocols were approved by Institutional animal ethics Committee (IAEC) with protocol number 704/PO/Re/S/02/CPCSEA.

Experimental Animals: Swiss albino mice (18-25 grams) from an inbred colony were procured from the National Toxicology Centre, Pune. They were housed in groups of 5-6 under standard laboratory conditions (ambient temperature of 22 ± 2 ºC, 60% relative humidity, 12-hour light/dark cycle) with free access to pelleted diet and water. A period of acclimatization preceded all experimental procedures [29, 30].

Acute Toxicity Study: A suspension of the three formulations was prepared by accurately weighing 4 g of each formulation and was suspended in distilled water to achieve a concentration of 40 mg/ml. The suspension was thoroughly mixed by sonication to ensure uniform dispersion. Three animals were initially administered the prepared suspension via oral gavage at a dose of 2 ml per 100 g body weight, following OECD 431 guidelines. The animals were then observed for any signs of toxicity, such as behavioural changes, and based on the results, dose gradation was carried out.

Evaluation of Immunomodulatory Activity

The animals were divided into four major groups: Control, Toxicant, Formulation, and Test, with each group consisting of six animals per subgroup. The treatment received by different groups is as follows

A mixture was prepared by dispersing 1 g of the formulation in 2 ml of distilled water. The mixture was thoroughly mixed and then diluted to 10 ml with water to achieve a 1:10 dilution. The final preparation was administered to animals based on their body weight, with a dose of 2 ml of the diluted formulation per 100 g body weight.

Preparation of Cyclophosphamide Solution: 100 mg of Cyclophosphamide added in 10 ml sterile distilled water (10 mg/ml). The 1 ml of this solution further dissolved in sterile distilled water, to achieve volume of 10 ml with sterile distilled water.

Preparation of SRBC Suspension: Fresh Sheep Red Blood Cells (SRBC) were collected and washed with sterile saline solution to remove plasma and cellular debris. The SRBC were centrifuged at 1000-1500 rpm for 5-10 minutes, and the supernatant was discarded. The pellet was re-suspended in sterile saline, and the washing process was repeated 2-3 times to ensure the thorough cleaning of the cells. Finally, the washed SRBC were re-suspended in sterile saline to achieve a 20% w/v concentration, which corresponds to 1×10^8 cells per milliliter. This 20% SRBC solution was used for the administration to animals in the control, toxicant, formulation, and test groups as per the study protocol.

1. **Control Group:** Received 0.9% w/v saline solution (i.p.) on day 0 followed by 0.1 ml of 20% SRBC (1×10^8 cells) i.p.
2. **Toxicant Group:** For the toxicant group, Cyclophosphamide 30 mg/kg b. w. .i.p. was administered on day 0, followed by 0.1 ml of 20% SRBC i.p..
3. **Formulation Groups:** Each of the three formulations was administered orally to three different groups of animals at a dose of 30 mg/kg b.w. p.o. (0.6 ml) once daily for 7 days. On day 0, 0.1 ml of 20% SRBC i.p. was administered.
4. **Test Groups:** Three different formulations were given once daily for seven days, along with Cyclophosphamide at 30 mg/kg body weight via i.p. route on day 0, followed by 0.1 ml of 20% SRBC through i.p. route on day 0.

Evaluation of Immunomodulatory Parameters

On day 7 blood was withdrawn from each animal through heart puncture, and the blood was evaluated for Humoral response through Hemagglutinating Antibody (HA) Titre. The cell mediated response was assessed through evaluating Delayed type of hypersensitivity which included measurement of footpad swelling or erythema. The nonspecific responses included Carbon clearance test, leucocyte count and lymphoid organ weights [31].

Procedure for obtaining serum from blood - Blood was collected into plain tubes without anticoagulant and allowed to clot at room temperature for 20–30 minutes. The tubes were centrifuged at 3000 rpm for 10–15 minutes, and the clear serum was carefully separated using a pipette. The serum was stored at 4°C for immediate use or frozen at -20°C for later analysis.[42]

Haemagglutination Antibody Titre Assay:

Serum samples were collected and two-fold serial dilutions were prepared in 96-well U-bottomed microtitre plates containing 100 µl of phosphate-buffered saline (PBS). Euthanasia has been carried out for organ collection or histopathological studies.

A 50 µl suspension of 1% SRBC was added to each well, gently shaken, and incubated at room temperature for 2 hours. Plates were visually examined for agglutination, and the highest dilution causing visible agglutination was recorded as the antibody titre.

Haematological Studies:

Blood samples (1-1.5 ml) were collected by heart puncture, and haematological parameters including total RBC, WBC, platelet, and hemoglobin (Hb) count were analyzed using a haematology analyzer.

Differential WBC counts were also performed to assess the immune response.

Thymus Weight Measurement:

The thymus gland was excised immediately after euthanasia, and its weight was recorded in milligrams. The relative weight was calculated using the formula:

Relative Weight = (Weight of Thymus (mg) / Weight of Animal (g)) ×100

Data Analysis: The results are presented as mean ± S.D. The data were statistically analysed using one-way ANOVA followed by Tukey-Kramer multiple comparisons test using INTA software. The values indicating difference at $P<0.05$ are considered to be statistically significant

Results and Discussion:

A. Extraction

The extracts from various medicinal plants exhibited distinct characteristics. *N arbor-tristis* yielded an orange-colored extract with a pungent smell, standardized for crocin content. *A racemosus* produced a brownish extract with an earthy smell and pungent taste, standardized for total steroidal content. The greenish-brown extract of *W somnifera* had an earthy smell and bitter taste, also standardized for steroidal content. *Z officinale* resulted in a dark brown extract with a pungent smell and spicy taste, analyzed for phenolic content. The *B aristata* extract was yellow-brown with an earthy smell and bitter taste, standardized for berberine content using HPTLC. TLC and HPTLC analysis confirmed the accuracy of the Rf values, matching well with standard literature.

B. Standardisation of Extracts

- The extracts were standardized based on characteristic marker compounds. Crocin was isolated from the extract of *N arbor-tristis*, with a content of 12.62%w/w in the alcoholic extract of the tubular calyx. Berberine, isolated from *B aristata*, was quantified at 4.48% w/w in the alcoholic extract of the roots or rhizomes. The zingerone content in the alcoholic extract of *Z officinale* was found to be 7.25% w/w.
- The alcoholic extracts of the roots or rhizomes of *A racemosus* and *W somnifera* contained steroid glycosides, specifically Shatavarin I, II, III, IV, and Withanolides, respectively. The total steroid content in the extracts was estimated to be 25.78% w/w for *A. racemosus* and 18.09% w/w for *W. somnifera*.
- In the alcoholic extract of *Z. officinale*, the active constituents, including Gingerols, Shagols, and Gingerone, contributed to a total phenolic content of 0.8% w/w, determined using the Folin-Ciocalteu method.

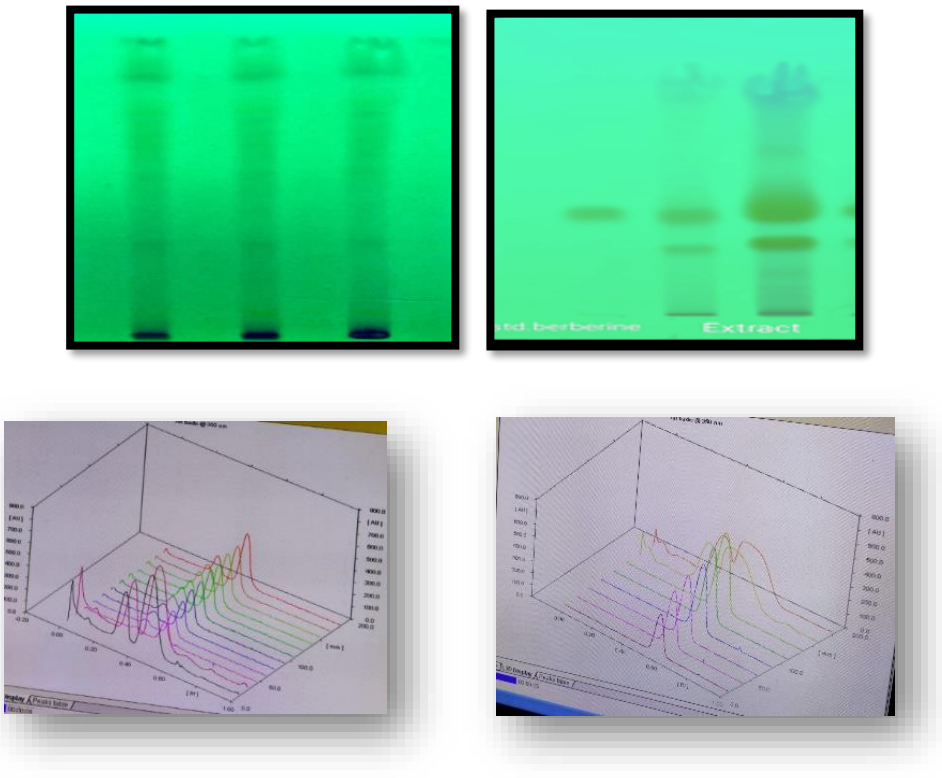

Figure 1-Chromatogram of Berbeine and Zingerone

Formulation development

Three milk blend formulations (N1, N2, and N3) were developed in powder form using a spray drying process, as detailed in Table 2. The formulations incorporated varying combinations of alcoholic extracts of *Z officinale*, *N arbor-tristis*, *W somnifera*, *B aristata*, and *A racemosus*, along with milk powder, sugar, preservatives, and flavoring agents.

Table -3 Composition of Herbal Extracts and Ingredients in Nutraceutical Formulation

Ingredients	**N1**	**N2**	**N3**
Parijat Extract (g equivalent to 12.65 % of crocin)	**20 g**	**20 g**	**20 g**
Shatavari Extract (g equivalent to 25.78 %w/w steroids content)	**-**	**20 g**	**-**
Ashwagandha Extract (g equivalent to 18.09% w/w steroids content)	**20 g**	**-**	**-**
Daruharidra Extract (g equivalent to 4.48% w/w Berberine)	**-**	**-**	**20 g**
Ginger Extract (equivalent to 0.8% w/w phenol content)	**10 g**	**10 g**	**10 g**
Milk powder	**40 g**	**40 g**	**40 g**
Preservatives	**0.1 g**	**0.1 g**	**0.1 g**
Sugar	**9.9 g**	**9.9 g**	**9.9 g**
Flavoring Agent	**q.s.**	**q.s.**	**q.s.**
Total weight	**100 g**	**100 g**	**100g**

The extracts (20 g per formulation) were mixed with 100 ml of toned milk (3.0-3.5% fat), diluted to 200 ml, and spray dried using LSD-48 at 150°C inlet and 80°C outlet temperatures. The process yielded fine, uniform powders with good flowability and reconstitution properties.

C. Evaluation of Milk blend Powder Formulations

The milk blend powder formulations were prepared by blending the standardized extracts of N arbor-tristis**,** A racemosus, *B aristata*, W somnifera, and Z officinale with milk using spray drying technique.

The formulation being in a powder form was free flowing, and had proper taste and appearance; it was evaluated for the physical characteristics. The results of the physical evaluation of the formulations is presented in Table no. 4

The moisture content of 1-2.2% w/w is a critical parameter for spray-dried products, as higher levels can promote degradation and lead to lump formation. The flow properties of the powder formulations, assessed by the Hausner's ratio and compressibility index, are crucial for packaging efficiency. The data presented in Table 4 demonstrate that the powder blend exhibits favourable flow characteristics, with both the compressibility index and Hausner's ratio falling within acceptable ranges, indicating good flowability suitable for free-flowing powders.

Table 4. Evaluation of spray dried optimised product

Sr. No.	**Parameters**	**N1**	**N2**	**N3**
1	Colour	Yellow	Yellow	Yellow
2	Taste	Sweet	Sweet	Sweet
3	Moisture Content (%w/w)	1.1 ± 0.2	2.1+0.2	2.2± 0.1

4	%Compressibility Index	20 ±0.1	7.6±0.1	7.6±0.1
5	Hausner's Ratio	1.25 + 0.1	1.08±0.1	1.08±0.1
6	pH	Neutral	Neutral	Neutral

D.1 Determination of marker compound

The content analysis of active compounds in individual plant extract powders and formulations is presented in Table 5. The results indicate that each plant extract contains specific bioactive components, which contribute to the overall therapeutic potential of the formulations.

Each formulation was assessed for the presence and concentration of key marker compounds, including crocin, zingerone, berberine, steroidal compounds, and phenolic content. The analysis revealed distinct profiles for each formulation based on the extraction methods employed and the composition of the herbal extracts used.

Crocin was extracted primarily using ethanol, resulting in significant concentrations in both F1 and F2, with values of 5.43% and 5.29% w/w, respectively. This demonstrates the effectiveness of the extraction method for capturing crocin, a compound associated with various therapeutic effects. Formulation F3 showed a comparable concentration of 5.00% w/w, indicating that crocin extraction was consistent across all formulations.

Zingerone exhibited varying concentrations, with F2 achieving the highest level at 1.85% w/w, indicating a favorable extraction from the herbal source. In contrast, F1 and F3 contained lower amounts of zingerone at 1.59% and 0.76% w/w, respectively. This variability suggests differences in the extraction efficiency and the potential therapeutic properties related to zingerone in the formulations.

Berberine was identified solely in formulation F3, quantified at 2.18% w/w.

Steroidal content showed marked differences, with F1 (containing *W* somnifera extract) 10.45% and F2 (containing *A* racemosus extract) exhibiting a higher concentration of 18.14% w/w. This indicates that F2 was more effective in extracting steroidal compounds as it is having *A* racemosus extract, which are known for their potential immunomodulatory effects.

Phenolic content was analyzed as well, revealing a higher concentration in F2 at 4.68% w/w compared to F1's 3.98% w/w, while F3 had a lower concentration of 1.94% w/w.

Table 5. Quantification of marker compounds in milk blend powder formulations by HPTLC

Marker Compound	Amount of Extract Added (g)	Expected Content (% w/w)	F1 Content (% w/w	F2 Content (% w/w)	F3 Content (% w/w)
Crocin	20	12.65	5.43	5.29	5
Zingerone	10	2.1	1.59	1.85	0.76
Berberine	20	4.48	-	-	2.18
Steroidal Content	20	18.09 (Ashwagandha) 25.78 (Shatavari)	10.45	18.14	-
Phenolic Content	10	-	3.98	4.68	1.94

The lower concentration of marker compounds in the final formulations is likely due to losses during the extraction and drying process, as well as dilution when extracts are mixed with excipients. Additionally, interactions between the active compounds and other ingredients may further reduce the final marker content in the formulations.

D.2 Analysis of the Product

The nutraceutical formulation was analyzed for nutritional composition, safety, and regulatory compliance, revealing key insights into macronutrients, contaminants, and adherence to standards. The results of the same are as below.

Table 6. Nutrition Report of formulation containing *N* arbor-tristis Extract (g equivalent to 12.65 % of crocin) *A* racemosus Extract (g equivalent to 25.78 %w/w steroids content) *W* somnifera Extract (g equivalent to 18.09% w/w steroids content) *B aristata* Extract (g equivalent to 4.48% w/w Berberine) *Z* officinale Extract (equivalent to 0.8% w/w phenol content).

Sr. no.	Name of Test	Result 100g
1	Appearance	Yellow coloured powder
2	Total fat	0.25 g
3	Protein	5.22 g
4	Carbohydrate	77.10 g
5	Energy	374.23 kcal
6	Sugar	9.0 %
7	Lead	N.D. <0.1
8	Cadmium	N.D. <0.1
9	Mercury	N.D. <0.05
10	Arsenic	N.D. <0.05
11	Total bacterial count	N.D.
12	Calcium content	28.11 mg

N.D. - Not Detected

Table 7. Microbiological analysis

Sr. no.	Microorganism	Result
1	*Escherichia coli.*	Absent
2	*Staphylococcus aureus*	Absent
3	*Salmonella typhi*	Absent
4	*Pseudomonas aeruginosa*	Absent

The nutraceutical product demonstrates a strong nutritional profile, with 28.11 mg of calcium supporting bone health and 374.23 kcal per 100 g providing a significant energy source. It contains 5.22 g of protein, 77.10 g of carbohydrates, and 0.25 g of fat, offering balanced nutrition. The product is safe, free from harmful bacteria like *E. coli* and *Salmonella*, and adheres to microbiological safety standards. Its sugar content (9%) is moderate, aligning with dietary guidelines, while the absence of heavy metals further ensures consumer safety. These results confirm the product's nutritional value, safety, and compliance with quality standards.

The nutraceutical product demonstrated a calcium content of 28.11 mg, supporting bone health, and an energy content of 374.23 kcal per 100 g, making it a robust source of energy. It exhibited a well-balanced nutritional profile with 5.22 g of protein, 77.10 g of carbohydrates, and minimal fat (0.25 g). The product's sugar content was 9%, adhering to dietary guidelines. Importantly, it met microbiological safety standards, with no detectable heavy metals or harmful bacterial contaminants like E. coli or Salmonella, ensuring its safety and quality.

D.3 Animal study

1**. Assessment of Humoral Immune Response by Haem agglutinating Antibody (H.A) Titre.**

The study results show that Haemagglutinating Antibody (H.A.) titers were significantly ($P < 0.05$) higher in the treatment groups (Treatment group and Formulation + Cyclophosphamide) compared to the negative control group, which received Cyclophosphamide (30 mg/kg b.w.) on day 0 along with 20% Sheep RBCs (SRBC) [33]. The treatment group (N2) exhibited the highest antibody titer of 90, while the negative control group had titers below 40, indicating a suppressed immune response. This enhancement is attributed to the immunostimulatory effects of compounds like steroid from *A racemosus* and *W somnifera*, which stimulate B-cell activity and enhance humoral immunity [34], [36]. Cyclophosphamide, an immunosuppressive agent, inhibits DNA synthesis in rapidly dividing immune cells, leading to reduced B-cell and T-cell activity, thus impairing antibody production [36].

The groups treated with the formulation plus Cyclophosphamide (N1, N2) exhibited reduced H.A. titers (around 60), suggesting partial counteraction of Cyclophosphamide's immunosuppressive effects. Cyclophosphamide-induced

immunosuppression depletes B-cells and impairs antibody production [35]. Notably, the partial restoration of antibody titers in these groups suggests that the bioactive compounds in the formulation may mitigate some of the drug's suppressive effects. Previous studies have shown that *W somnifera* offers protection against Cyclophosphamide-induced immunosuppression, likely through antioxidant and anti-inflammatory mechanisms that help maintain immune cell function [37]. This highlights the potential of these extracts to support immune function even in the presence of immunosuppressive treatments.

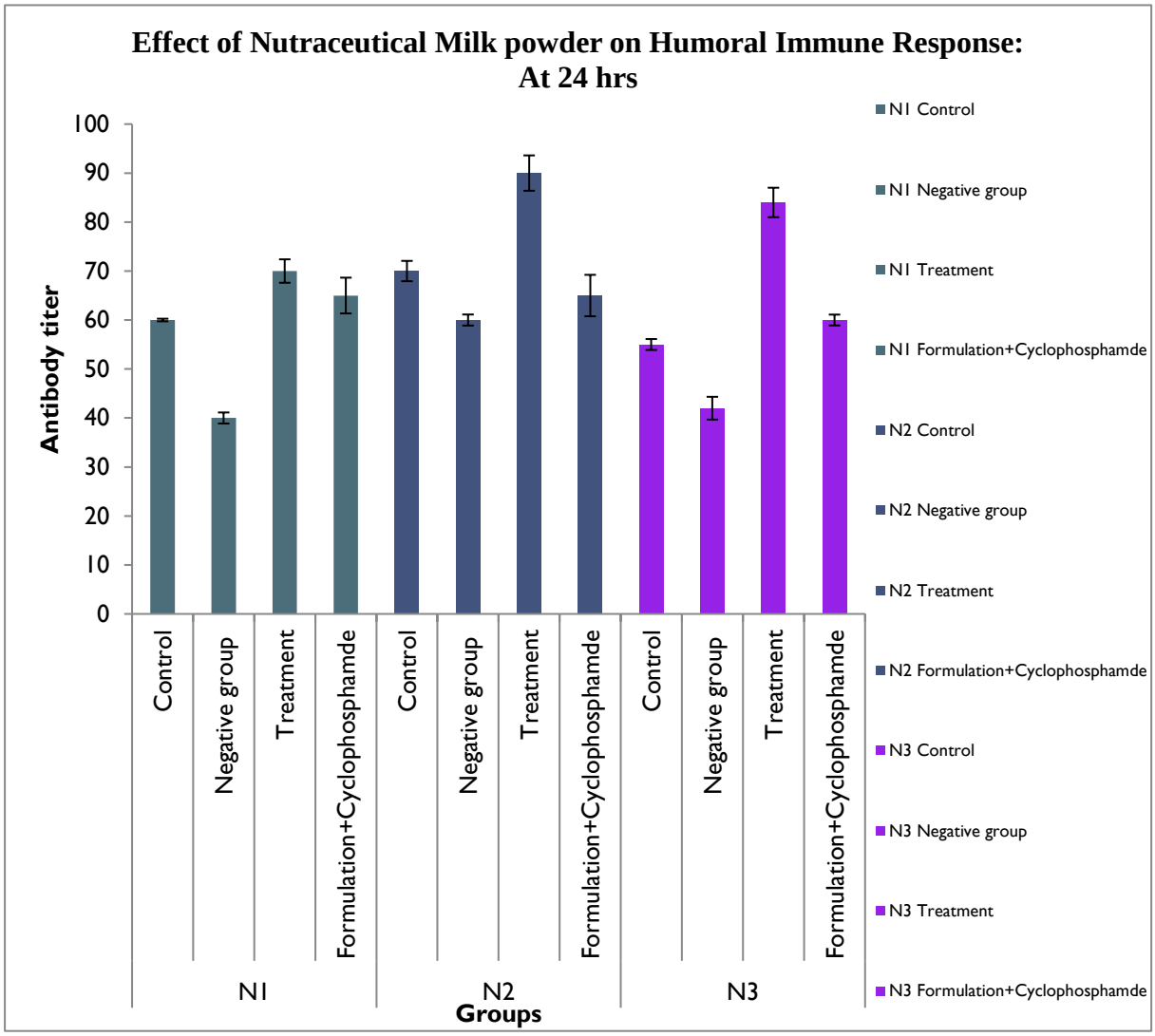

Figure 2- Effect of Oral Administration of Nutraceutical Milk powder blend formulations on Humoral Immune Response in cyclophosphamide induced immunosuppression in mice.

2. Assessment of Cell Mediated Immune Response by DTH

The results show a significant reduction in paw volume in the negative control group, which received Cyclophosphamide (30 mg/kg b.w.), suggesting suppressed cell-mediated immune responses. Cyclophosphamide is a potent immunosuppressive agent that works by depleting B-cells and T-cells, inhibiting DNA synthesis in rapidly dividing immune cells, and impairing both humoral and cellular immunity [35]. As a result, the negative control group displayed a reduced cell-mediated immune response, leading to lower paw volume and diminished footpad thickness.

In contrast, the treated groups showed a substantial increase in mean paw volume (**$p < 0.001$), with a significant rise in paw volume at 48 hours, indicating an enhanced cell-mediated immune response. The formulation group displayed a dose-dependent increase in footpad thickness, suggesting a strong DTH (Delayed-Type Hypersensitivity) response. These results align with previous studies indicating that formulation can enhance cellular immune responses by promoting lymphocyte activation and cytokine production [34, 36].

Notably, the Formulation + Cyclophosphamide group exhibited a partial counteraction of Cyclophosphamide-induced immunosuppression, as evidenced by the dose-dependent increase in footpad thickness. This suggests a synergistic effect

between the formulation and Cyclophosphamide, where the immunostimulatory properties of the formulation may help to enhance cellular immunity even in the presence of an immunosuppressive agent. Studies support the potential of combining immunostimulants with chemotherapeutic agents to enhance immune responses while mitigating adverse effects of immunosuppression [35, 37].

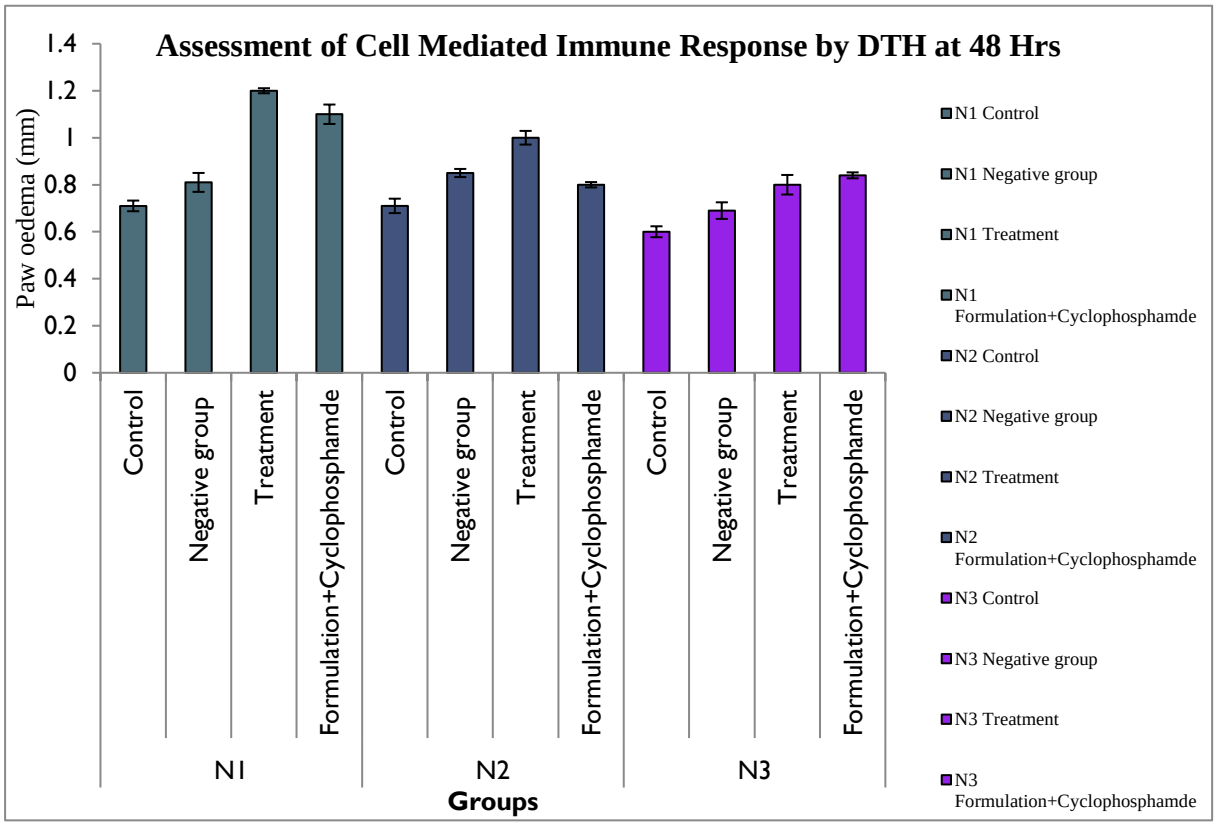

Figure 3- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Cell-Mediated Immune Response in Delayed Type Hypersensitivity Induced by Cyclophosphamide in Mice

1. Carbon Clearance Test:

The Carbon Clearance Test was conducted to assess the functional changes in macrophages of the reticuloendothelial system (RES) by evaluating their phagocytic ability. Cyclophosphamide, a known immunosuppressive agent, was used to induce macrophage dysfunction. Cyclophosphamide impairs the immune system by depleting immune cells, particularly macrophages, and inhibiting their ability to carry out vital immune functions such as phagocytosis. This is achieved through its cytotoxic effects on rapidly dividing cells, including those of the immune system, leading to reduced macrophage activity and compromised immune responses [35, 37].

The results showed that Cyclophosphamide administration (negative control group) caused a significant reduction in phagocytic activity ($p < 0.05$), indicating suppressed macrophage function. In contrast, the Treatment group and the Formulation + Cyclophosphamide group did not exhibit significant changes in their phagocytic abilities, suggesting that the Nutraceutical Milk Powder preserved macrophage function even in the presence of Cyclophosphamide. This finding suggests that the formulation may counteract the immunosuppressive effects of Cyclophosphamide and help maintain macrophage activity within the RES.

The oral administration of the formulation for seven days (F1, F2, and F3) demonstrated the potential of the Nutraceutical Milk Powder to maintain normal phagocytic activity in the RES, even when combined with Cyclophosphamide. This protective effect is consistent with previous studies that suggest certain nutraceuticals can enhance macrophage phagocytosis and overall immune function. These findings support the notion that the Nutraceutical Milk Powder may serve as an effective immunomodulatory agent capable of safeguarding macrophage function against the suppressive effects of Cyclophosphamide [34-36].

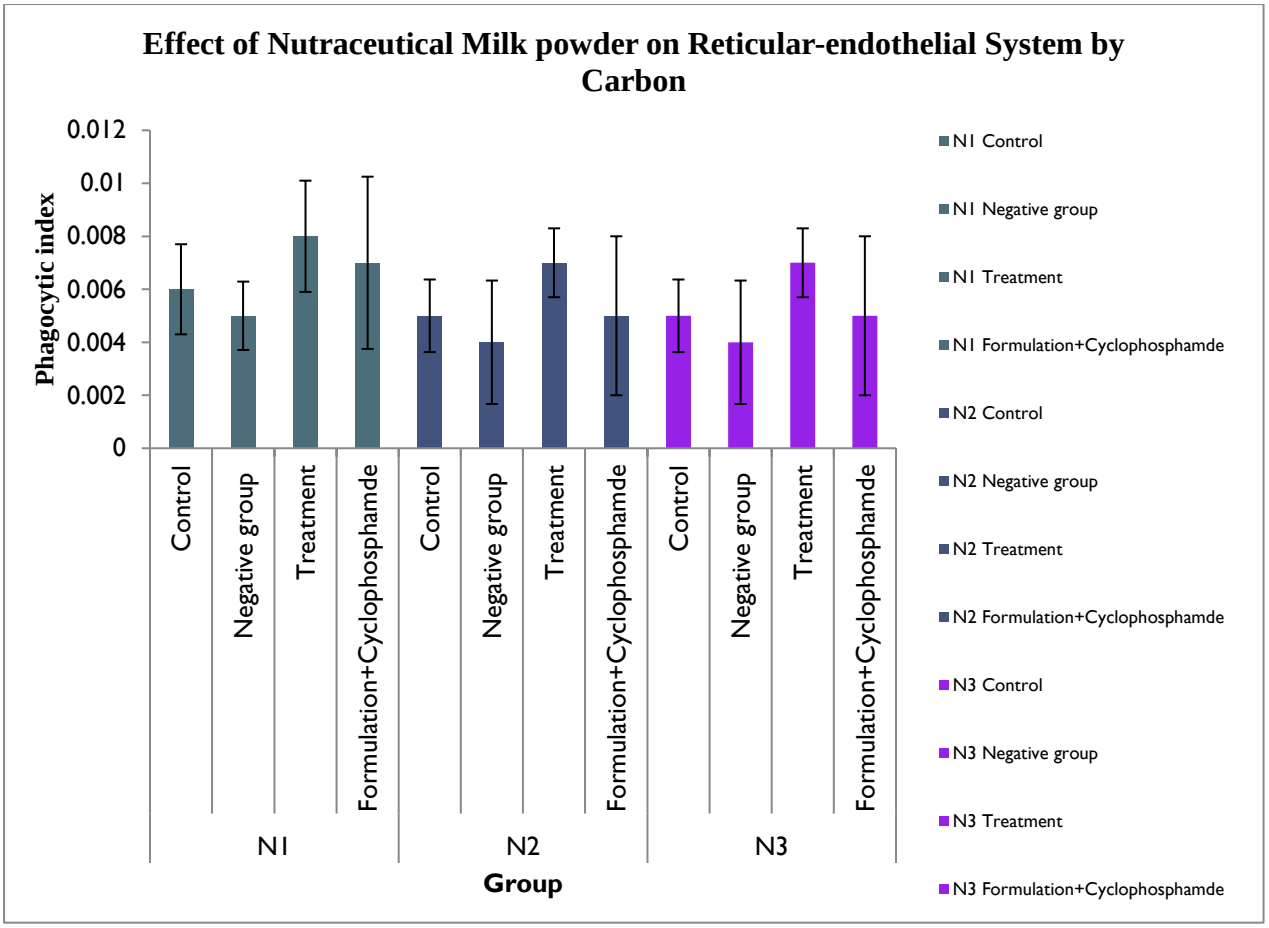

Figure 4- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on the Reticuloendothelial System Evaluated by Carbon Clearance Test in Mice

4. Cyclophosphamide induced myelosuppression

Cyclophosphamide induces myelosuppression by damaging bone marrow, leading to reduced production of white blood cells (WBCs) and hemoglobin, impairing immune response. This effect is due to the cytotoxic action of Cyclophosphamide on rapidly dividing hematopoietic cells. As expected, the Cyclophosphamide group showed a significant decrease in WBC count ($p < 0.05$) compared to the Control group.

In contrast, both the Treatment and Formulation + Cyclophosphamide groups exhibited a significant increase (***$p < 0.001$) in WBC and hemoglobin counts, suggesting that the Nutraceutical Milk Powder mitigated Cyclophosphamide-induced myelosuppression. These results align with previous studies showing that certain herbal formulations can enhance hematopoiesis and improve blood cell counts during chemotherapy, indicating the protective potential of the nutraceutical formulation against myelosuppression [38, 39].

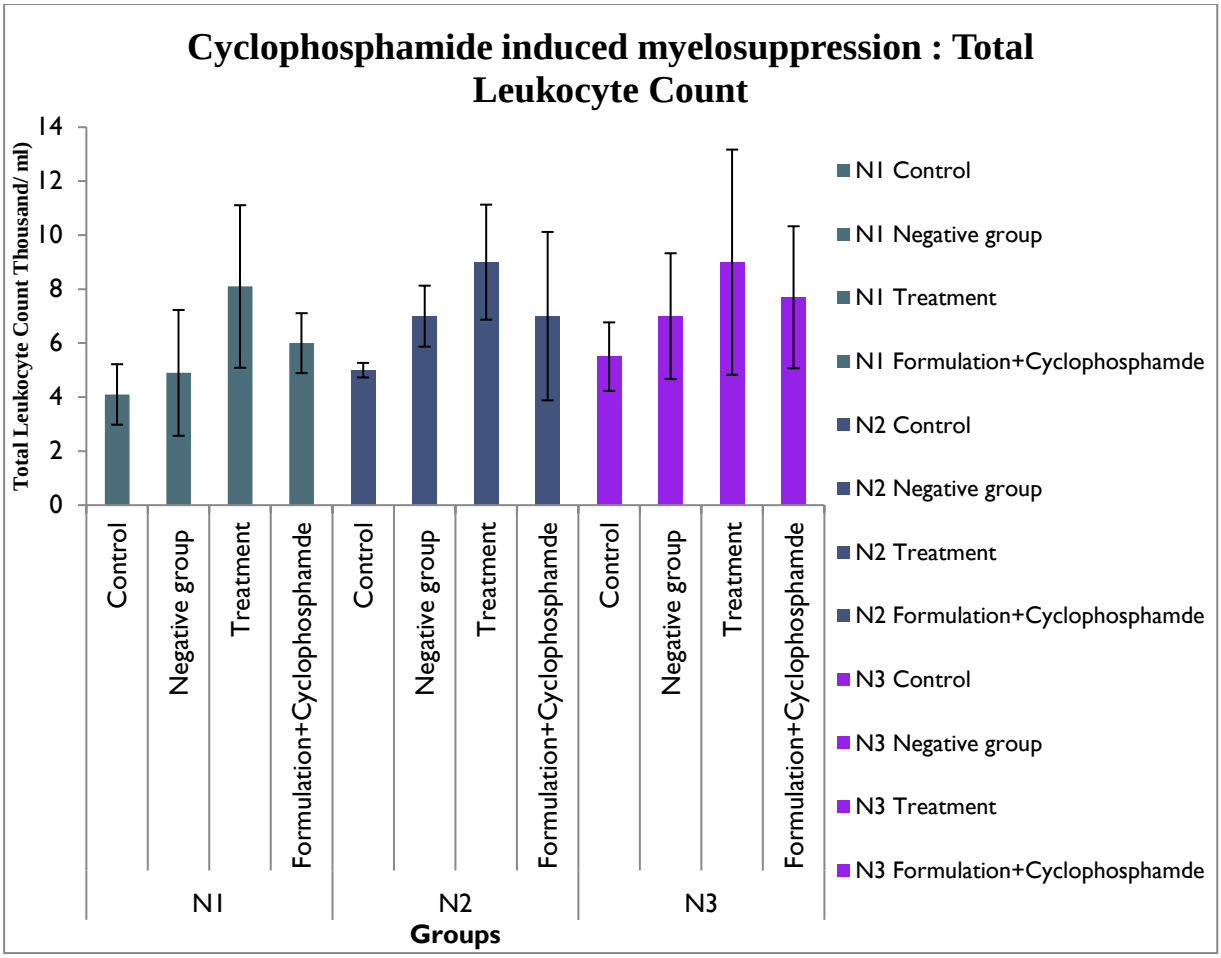

Figure 5- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Total Leukocyte Count in Cyclophosphamide-Induced Myelosuppression in Mice

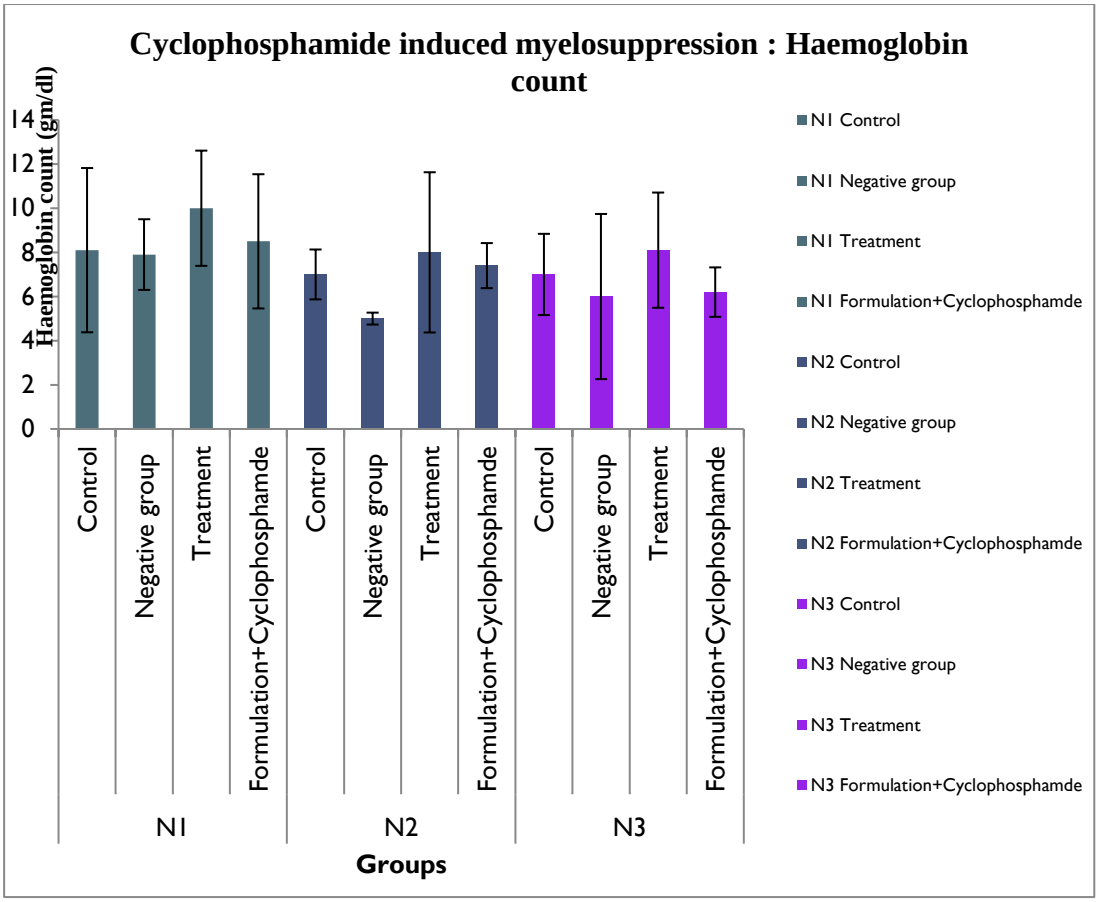

Figure 6- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Haemoglobin Count in Cyclophosphamide-Induced Myelosuppression in Mice

5. Effect on weight of lymphoid organ:

Cyclophosphamide, a potent chemotherapeutic agent, is known to act as an immunosuppressant by depleting lymphoid organs, particularly the spleen and thymus, and inhibiting the proliferation of immune cells. This results in a weakened immune response, reflected by reduced lymphoid organ weight and overall immune function. In the current study, the Cyclophosphamide group exhibited lower lymphoid organ weights compared to the Control group, indicating its suppressive effects on immune function.

In contrast, the Treatment group (F1, F2, F3) and the Formulation + Cyclophosphamide group demonstrated a significant increase in the weight of the thymus and liver ($p < 0.05$), suggesting that the Nutraceutical Milk formulation positively modulated immune function. The enhanced lymphoid organ weights in these groups indicate potential immune-stimulating effects, even when combined with Cyclophosphamide. These findings align with previous studies, which report that certain herbal formulations can promote lymphoid organ development and stimulate immune responses, thereby counteracting the immunosuppressive effects of chemotherapy agents like Cyclophosphamide [40, 41]

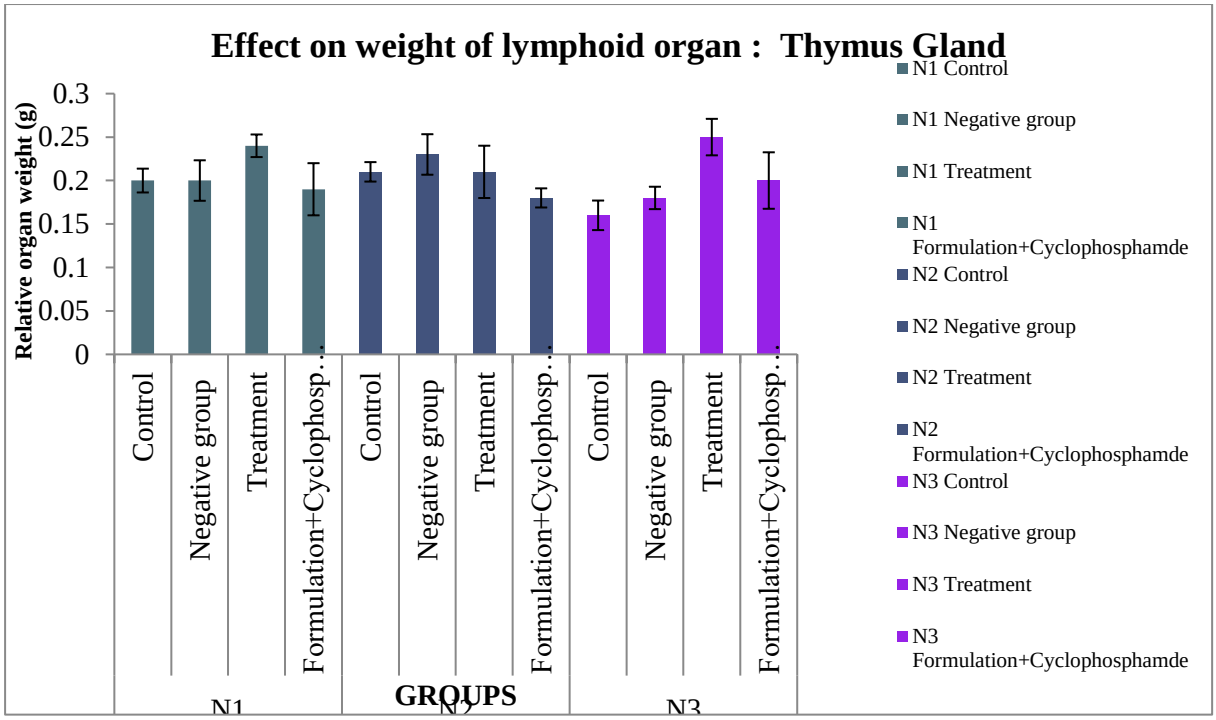

Figure 7- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Thymus Gland Weight in Cyclophosphamide-Induced Myelosuppression in Mice

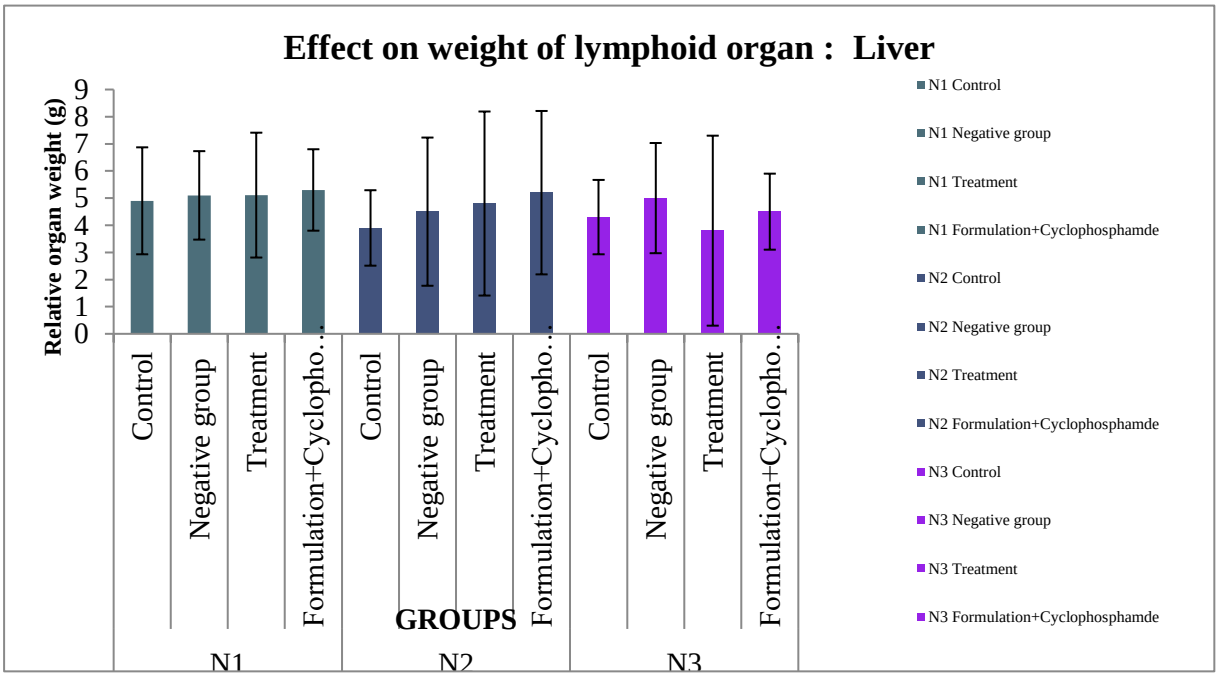

Figure 8- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Liver Weight in Cyclophosphamide-Induced Myelosuppression in Mic

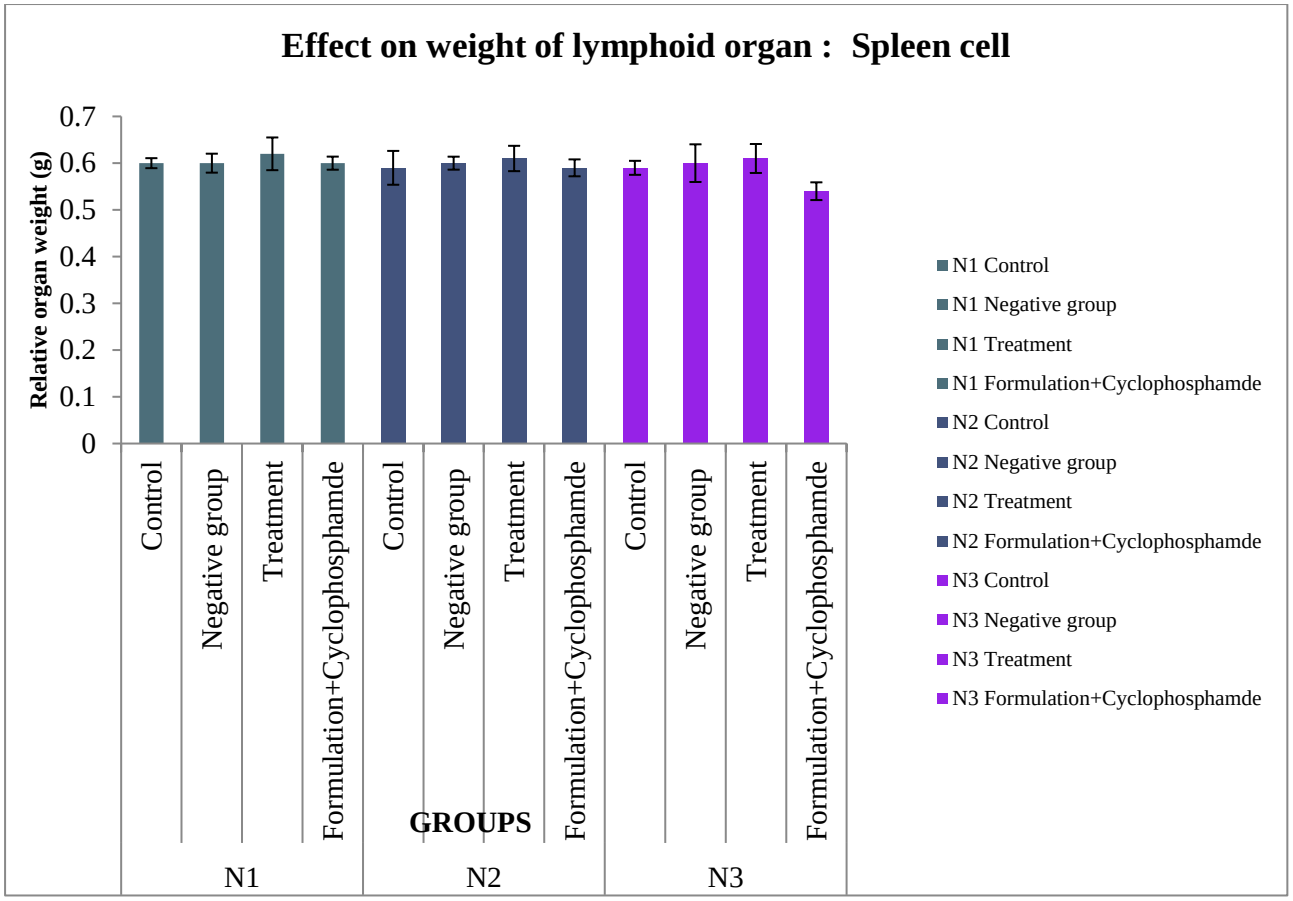

Figure 9- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Spleen Weight in Cyclophosphamide-Induced Myelosuppression in Mice

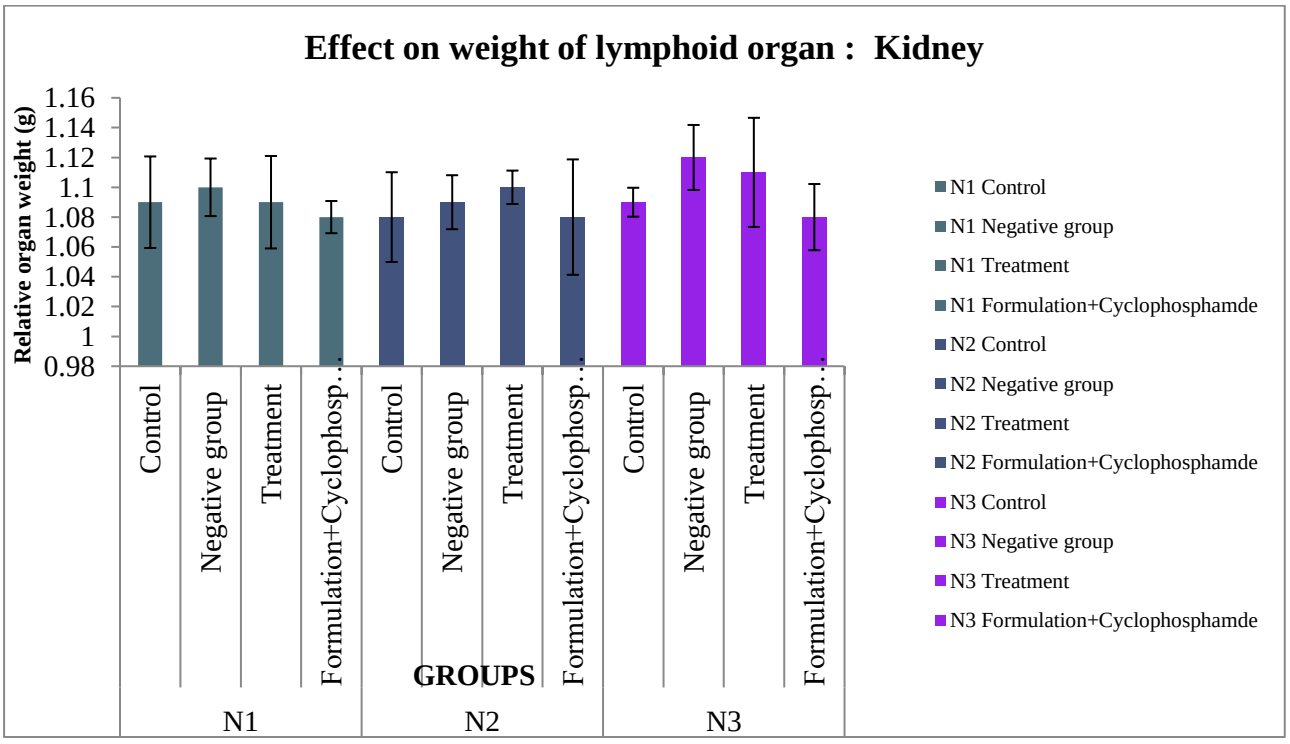

Figure 10- Effect of Oral Administration of Nutraceutical Milk Powder Blend Formulations on Kidney Weight in Cyclophosphamide-Induced Myelosuppression in Mice

This study comprehensively evaluated the immunostimulant effects of a nutraceutical formulation containing extracts of *N arbor-tristis*, *W somnifera*, *Z officinale*, *A racemosus*, and *B aristata* on various immunological parameters in mice. The findings indicate significant impacts on humoral and cell-mediated immune responses, as well as on the reticuloendothelial system, cyclophosphamide-induced myelosuppression, and lymphoid organ weights.

4. CONCLUSION

The findings from this study highlight the significant immunomodulatory potential of the Nutraceutical Milk formulation in enhancing both humoral and cell-mediated immune responses. The results demonstrated a marked increase in Haemagglutinating Antibody (H.A.) titers, indicating a robust humoral immune response attributed to the bioactive compounds present in the formulation, such as steroidal saponins from *Asparagus racemosus* and withanolides from *Withania somnifera*.

In addition to the humoral response, the study utilizing the Delayed Type Hypersensitivity (DTH) model revealed that the Nutraceutical Milk formulation significantly enhanced cell-mediated immunity, as evidenced by increased paw thickness measurements. The formulation exhibited a dose-dependent increase in footpad edema, suggesting a synergistic effect with Cyclophosphamide on cellular immunity.

Furthermore, the Carbon Clearance Test indicated that the Nutraceutical Milk positively influences the phagocytic activity of macrophages in the reticuloendothelial system, mitigating the immunosuppressive effects of Cyclophosphamide. This protective effect was further supported by the observed amelioration of Cyclophosphamide-induced myelosuppression, demonstrated by increased total white blood cell and haemoglobin counts in the treatment groups.

The impact on lymphoid organ weights further underscores the formulation's ability to modulate immune responses, with significant increases noted in the thymus and liver weights. These results collectively suggest that the Nutraceutical Milk formulation may serve as a promising therapeutic agent for enhancing immune function, particularly in conditions of immunosuppression.

Overall, this study provides a strong foundation for further research into the mechanisms underlying these effects and the potential clinical applications of the Nutraceutical Milk formulation in managing immune-related conditions.

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