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In vitro Antioxidant, *In vivo* Bioavailability, and Immunomodulatory Effects of a Polyherbal Formulation (Storg B) Induced by Cyclophosphamide in an Experimental Animal Model

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The immunomodulatory properties of plants are being extensively explored these days as they contain a wide range of bioactive compounds that can treat immunomodulatory disorders. The aim of the present investigation was to determine the efficacy of the standardized polyherbal formulation (Storg-B). The Storg-B combines *Psidium guajava, Mangifera indica*, and *Citrus limon*.
Methods: The aqueous extract of the Storg B was investigated for DPPH radical scavenging activity to determine antioxidant activity. SD rats were selected for the evaluation of the

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immunomodulatory activity of Storg-B. The haemagglutination and hematology methods were used to evaluate the immunomodulatory studies. For the pharmacokinetic studies, the LC-MS/MS technique is employed to quantify Storg B.
Results: The percentage inhibition of DPPH radial IC_{50} values of standard BHT and Storg-B were 60.52 and 68.35µg/ml, respectively. In the *in vivo* animal studies, Storg-B showed significant immunomodulatory activity by increasing the primary and secondary antibody titer values by 6.58 and 8.25 mg/mL, respectively, for the SRBC antigen at a dose of 100 mg/kg. There's also an increase in the neutrophil adhesion percentage at the testing doses. Results of hematological parameters revealed that Storg-B restored all the levels of blood components at a 100 mg/kg dose. A Storg-B showed greater potency in the bioavailability studies.
Conclusion: Thus, the Storg-B used as an efficient and secure formulation in the treatment of immune deficiency by improving the immune response.

Keywords: Polyherbal formulation (Storg-B); antioxidant; immunomodulatory; bioavailability; B vitamins; In vitro and In vivo.

1. INTRODUCTION

The majority of the world's population has recently witnessed a shift from synthetic to natural medicine. It incorporates the use of natural herbal elements to replenish the body's vital nutrients and fundamentals for leading a healthy lifestyle [1]. Currently available chemical-based immunomodulators, such as levamisole, glycans, tellerones, and L-fucose, continue to cause immunosuppression, leading to adverse health effects in humans, particularly flu-like symptoms, fatigue, nausea, neutropenia, thrombocytopenia, and depression. They can also cause other diseases. Due to the emergence of side effects associated with chemical drugs, natural immunomodulators are considered potential tools that can replace chemical agents in treatment programs [2].

The prevention and treatment of diseases with herbal medicines can be traced throughout human history, in all cultures, and throughout the ages for all kinds of ailments. According to the World Health Organization (WHO), approximately 80% of the world's population mainly uses herbal medicines in their primary health care [3,4]. Immunomodulation with herbal medicines can stimulate both specific and non-specific immunity and thus has immunomodulatory properties.

In comparison to a single drug, herbal combinations frequently show strong potential in the treatment of diseases. A combination of herbals may act on multiple targets at the same time to provide thorough relief [5]. The polyherbal formulation removes the need for patients to take many distinct medicines at once, improves patient convenience, and indirectly improves therapeutic impact and compliance. When compared to a single herbal formulation, polyherbal formulation 's popularity in the market has been promoted by all these benefits [6].

Oxidative stress has been determined to be a major cause of many chronic and degenerative diseases, including atherosclerosis, ischemic heart disease, aging, diabetes, cancer, immunosuppression, neurodegenerative diseases, etc [7]. The most effective way to eliminate and reduce the activity of free radicals that cause oxidative stress is through the antioxidant defense system. Antioxidants are substances that have the ability to break the chain reactions of free radicals. Recently, there has been increased interest in medicinal plants with curative potential as reactive antioxidants to reduce tissue damage caused by oxidative stress [8].

The immune system's primary function is to differentiate between self and non-self. This non-self might be a body's own cell that can be misinterpreted as a foreign object, an infectious organism, or an organ that has been transplanted [9]. Immunomodulators are biological or synthetic compounds that have the ability to activate, inhibit, or modify any part of the immune system, including the innate and adaptive systems. Extensive study and innovation are needed for natural products, plants, and their extracts to understand their immunomodulatory efficacy. It might be an essential commodity to develop a novel herbal formulation to supplement existing chemotherapies.

Pharmacokinetics research is an important part of innovation in herbal medicine.

Pharmacokinetics deals with the absorption, distribution, metabolism, and absorption of compounds in the body. Bioavailability is the part of a drug dose that is absorbed from the place of administration and reaches the systemic circulation unchanged. The drug, its route of administration, and its composition determine the amount of the administered dose that is absorbed into the bloodstream. Knowing the absorption and metabolism properties of the drug makes it easy to prepare and administer medicines for maximum benefit and minimum risk.

Our product, Storg B, is an enriched B vitamin supplement from the three plants that have been used for decades for traditional medicinal and neutraceutical uses, namely *Psidium guajava, Mangifera indica*, and *Citrus limon.* We used an animal model of cyclophosphamide-induced immunosuppression in SD rats for the immunomodulatory studies.

Psidium gujava, commonly referred to as guava belongs to the Myrtaceae family. It is regarded as a native of Mexico and can be found in Asia, Africa, Europe, and South America. Guava leaves have been used in traditional medicine to treat dysentery, diarrhea, and digestive problems. A pharmacological analysis revealed that *Psidium guajava* has anti-inflammatory, anti-tussive, anti-allergic, anti-hypertensive, hypoglycemic, anti-cancer, and wound-healing properties. It also has analgesic and antioxidant properties [10].

Mangifera indica is a member of the Anacardiaceae family, which is widely employed in Indian traditional medicine and has been shown to have antiviral, antibacterial, and anti-inflammatory properties [11]. The alcoholic extract of the stem bark of Mangifera indica has also been reported to possess immunomodulatory activity with immunostimulant properties [12].

Citrus limon, commonly called lemon, belongs to the Rutaceae family and has evergreen leaves and edible yellow fruits. Lemon fruit consumption helps in treating various types of disorders. These fruits are extremely valuable for many industrial and medical purposes due to their polyphenol and vitamin C content. *Citrus limon* (lemon) peel contains bioflavonoids such as hesperidin, eriocitrin, narigenin, and rutin, among others. These substances exhibit a wide range of biological activities, such as antibacterial, antifungal, diabetic, anticancer, and antiviral properties [13]. These flavonoids have the ability to perform as direct antioxidants and free radical scavengers, have the potential to alter enzymatic activity, and safeguard membranes [14].

Storg B consists of eight water-soluble components: thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and cobalamin (B12). They are functionally related and cooperate in the synthesis of proteins, lipids, and nucleic acids, energy production, and immune defense; they also have many effects on brain activity. These essential nutrients are isolated from food and act as coenzymes in various processes. In general, B vitamins are synthesized in plants, with the exception of cobalamin [15,16].

2. MATERIALS AND METHODS

2.1 Preparation of Sample

Storg B is manufactured and registered by Star Hi Herbs PVT Ltd Jigani, Bangalore, Karnataka, India. Storg B Indian patent application number 202043054117.

2.2 Chemicals

All standards were purchased from Sigma-Aldrich. The Standards were stored in the original packages at -18°C prior to use. Methanol (JT baker for LC-MS), formic acid (Optima from Fisher Chemicals, LC-MS grade), Milli-Q-water from Sartorius ARIUM MINI.

2.3 Antioxidant Activity of Storg-B

Storg-B's ability to scavenge free radicals was evaluated using the DPPH radical scavenging assay, as described by Blois [17]. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of Storg-B in methanol at different concentrations (20, 40, 60, 80, and 100µg/mL). The sample was vortexed thoroughly and left in the dark at RT for 30 min. The absorbance of the sample was measured spectrophotometrically at 517 nm. BHT was used as a reference drug at different concentrations (20, 40, 60, 80, and 100). The percentage of DPPH radical scavenging activity was calculated by the following equation:

$$\%\ \text{of DPPH radical scavenging activity} = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the Storg-B. Then the percent of inhibition was plotted against concentration, and the IC_{50} was calculated from the graph. All analyses were carried out in 3 replicates, and absorbance was recorded at 517 nm.

2.4 *In vivo* Immunomodulatory activity Storg B

2.4.1 Animals and experimental protocol

In this investigation, Sprague-Dawley (SD) rats weighing between 150g and 250g were employed. They were supplied by the animal house, Bharathi College of Pharmacy, Mandya, Karnataka, India. The rats were kept in standard laboratory settings (temperature 24 ± 1 ºC with a 12/12 h dark/light cycle) in polypropylene cages (38 x 23 x 10 cm) with a maximum of six animals per cage. Before the trial, all of the animals were acclimatized to laboratory conditions for a week.

2.4.2 Grouping of animals

The SD rats were divided into 5 groups each group consisting of 8 animals:

Group I: Control received only normal saline
Group II: 20 mg/kg of Cyclophosphamide alone treated animals
Group III: Cyclophosphamide and 50 mg/kg of Levamisole treated animals
Group IV: Cyclophosphamide and 50 mg/kg of Storg B treated animals
Group V: Cyclophosphamide and 100 mg/kg of Storg B treated animals

2.4.3 Sheep red blood cell (SRBC) preparation and standardization

Blood from the sheep was drawn out via the external jugular vein using a newly made 1:1 ratio of Alsever's solution. Centrifuging the blood at 2500 rpm for 10 minutes allowed the SRBCs to be separated, and the collected blood was then rinsed with pyrogen-free normal saline (0.9% w/v). After that, the concentration was adjusted to 1×10^8 SRBC in 1 mL of sheep blood [18].

2.4.4 Hemagglutination assay

On days 7 and 14, all the groups except control were administered with 0.1 ml of 20% SRBC. On the 9th and 16th days, all the groups except control were administered cyclophosphamide to suppress immunity. This is also called the immunization of animals. Blood was withdrawn on the 14th and 21st days from the retro orbital plexus under mild ether anesthesia from all the antigenically sensitized and challenged rats, and serum was separated for antibody titer checks. 20µL of serum was serially diluted with 20µL of saline and filled into a microtiter plate. 20µL SRBC was added to each of these dilutions, and the plates were incubated at 37°C for one hour and then observed for hemagglutination and antibody reactions.

2.4.5 Neutrophil adhesion test

On the 21st day of drug treatment, blood samples were collected by puncturing the retro orbital plexus into heparinized vial and analyzed for total leucocyte counts (TLC) and differential leucocyte counts (DLC) by fixing blood smears and staining with Field stain I and II- Leishman's stain. After initial counts, blood samples were incubated with 80mg/ ml of nylon fibers for 15 min. at 37º C. The incubated blood samples were once again analyzed for TLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. The percent neutrophil adhesion was calculated as follows:

$$\%\ \text{Neutrophil Adherence} = \frac{\text{NI}_\text{u} - \text{NI}_\text{t}}{NI_u} \times 100 \quad (2)$$

Where, NI_u is neutrophil index of untreated blood, NI_t is neutrophil index of fibre treated blood.

$$\text{Neutrophil Index(NI)} = \text{TLC} \times \%\text{neutrophil} \quad (3)$$

2.4.6 Hematological analysis

The blood was collected from each group by retro orbital plexus into heparinized collecting tubes. The hematological analysis was carried out to determine the red blood cells (RBC), hemoglobin, total platelet count by using auto-analyzer.

2.5 *In Vivo* Bioavailability Study of Storg B

2.5.1 Animals used

Healthy SD rats (n = 6) weighing 150-200g and 8 to 12 weeks aged were selected for pharmacokinetic and bioavailability study. All rats were kept for acclimatization for seven days prior to start pharmacokinetic study. Body weight

variation among the animals did not exceed 20% of the mean body weight. The room was environmentally-controlled at a temperature of 22^{o} C with relative humidity of 60%. The photoperiod was 12 h light and 12 h darkness. Prescribed feed and ad libitum drinking water were provided during experimental period. The experiment was carried out in accordance with the guidelines of Institutional Animal Ethics Committee.

2.5.2 Experimental design

Study design six SD rats weighing about 150-200 g were selected as the animal model. The age of the rat was 8−12 weeks. The study involved rats had no medication for two weeks food was withdrawn 12 hours before drug administration and water was available throughout the study. The Storg B dose of 200 mg/kg was administered to rats using a gavage needle. The study was approved by Institutional Ethical committee (BCP/IAEC/06/2022). The blood samples were taken at the time intervals of 0.5, 1, 2, 4, 8, and 24 hours from the retro orbital plexus of the rats and centrifuged at 6000 rpm for 10 minutes. EDTA disodium salt was used as an anticoagulant. Blood samples were collected into labeled heparin tubes, and stored in -20^{0}C freezer until use. The concentration of plasma, after Storg-B consumption were determined by LC-MS/MS method. The value of C_{max}, T_{max} and $t_{½}$ of Storg-B containing vitamin B_1, B_2, B_3, B_5, B_6 and B_9 were obtained by plotting the graph between plasma concentration and time.

2.5.3 LC-MS/MS analysis

Liquid chromatography and Mass Spectrometry was performed by a Shimadzu LCMS-8050, high-end model of its UFMS (Ultra-Fast Mass Spectrometry) with Nexera X3 UHPLC series. Binary Analytical system was used which consisted of a Solvent Delivery Unit LC-40 Series with Mobile Phase Monitor MPM-40, Autosampler SIL-40, Column Oven CTO-40 Series. Heated ESI source which improves desolvation and escalate ionization efficiency with the addition of a heated gas used in combination with the nebulizer gas. UF sweeper® III collision cell increases CID efficiency by optimizing the collision cell pressure and a PC with Lab solution and Insight software.

The LC-MS/MS system was enabled with a chromatographic column Shim pack GIS C18-120 (100 x 4.6 mm, 3µm) maintained at 40°C. Mobile phase A was prepared by adding 1mL of formic acid to 1000mL of water (0.1% formic acid in water). Mobile phase B used was 100% methanol. All mobile phases were prepared daily, filtered through a 0.2µm nylon filter and degassed before use.

Chromatographic partition was achieved at a flow rate 0.5mL/min using isocratic condition of mobile phase A and B as (60:40). Injection volume was 10µl.

Mass spectrometry analysis was achieved by multiple reactions monitoring (MRM) in ESI positive mode using the following optimized parameters:

Nebulizing gas flow (Nitrogen) = 3 mL/min, Heating gas flow (Zero Air) = 5 mL/min, Interface Temperature = 300°C, DL Temperature = 180°C, Heat Block Temperature = 350°C, Drying Gas Flow (Nitrogen) = 10°C, Interface voltage = 4kV, CID gas (Argon) = 270kPa.

The determination of system suitability, specificity, method precision/repeatability, linearity, range, limit of quantitation, detection limit, accuracy (recovery), and intermediate precision are presented in this validation report. All results met their respective acceptance criteria, as outlined in the validation protocol. All method system suitability requirements were satisfied for each set of analyses performed during the validation exercise.

2.5.4 Standard preparation

Standard stock solutions were prepared individually by dissolving an appropriate amount of each Standard in Water (Vitamin B1, B3, B5 and B6). While preparing Vitamin B2, and B9, add approximately 1mL of 0.1N NaOH in water for better solubility. Prepare a mixture of all vitamins where the concentration was 20000 ng/mL. All standard stock solutions were stored at -18°C. The standard mixture solution 20000 ng/mL was prepared daily in an amber colored glass volumetric flask (5 mL) by diluting the calculated amount of each standard stock solution with water.

The calibration solutions were prepared daily in brown glass HPLC vials (1.8 mL) diluting the standard mixture solution 20000 ng/mL. Six different concentrations of stock standard solution were prepared 3 ng/mL to 300 ng/mL. Total volume of each calibration solution was 1mL for stock Standard solution prepared.

2.5.5 Sample preparation

For the sample evaluation, a 2 mL eppendorf tube containing 40 mL of serum sample followed with 160 µL of methanol. After being vortexed for 3 min, the mixture was centrifuged at 6000 rpm for 10 min at 4°C. After centrifugation, 100 µL of the supernatant and 200 µL of water were transferred to the brown glass HPLC vial (1.8 mL) for additional analysis. Vortex for 2 min and then inject.

For the spiked sample evaluation, 40 µL of serum was taken in a 2 mL eppendorf tube, to which 160 µL of methanol and 10 µL of the appropriate standard solution were added. The mixture was vortexed for 3 minutes at 4°C, followed by a 10-minute centrifugation run at 6000 rpm. After centrifugation, 100 µL of the cleared supernatant and 200 µL of water were transferred to the brown glass HPLC crimp vial (1.8 mL) for further evaluation. Vortex for 2 min, then inject.

2.6 Statistical Analysis

The statistical analysis of the experimental data was carried out using one-way ANOVA, followed by Tukey's multiple comparison tests, and the results were expressed as Mean ± SEM.

3. RESULTS AND DISCUSSION

3.1 Effect of Storg B on Antioxidant Activity

The analysis specification of Storg B is shown in Table No.1. Storg-B antioxidant activity was compared with that of standard butylated hydroxyl toluene (BHT). The ability of the Storg-B to donate hydrogen atoms was determined by decolorizing a methanol solution with 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produced a violet color in methanol solutions and faded to shades of yellow in the presence of antioxidants. The free radical antioxidant scavenging activity of standard BHT and Storg-B was found to have IC_{50} values of 60.52 and 68.35 µg/ml, respectively, as shown in Fig. 1. The results obtained in the present study indicate that Storg-B exhibits significant free radical scavenging activity as compared with the standard drug BHT.

3.2 Effect of Storg B on Humoral Immune Response

The hemagglutination test was used to assess the humoral immune response. Cyclophosphamide, an immunosuppressant agent, significantly decreases the Humoral antibody titer (HA titer) value compared to the control group. After administration of test doses, Storg B 100 mg/kg produced a significant increase in the primary and secondary antibody HA titer values, as shown in Fig. 2, which were significant compared to the positive control group, as evidenced by hemagglutination after incubation of serum with SRBCs. Results demonstrate that the addition of Storg-B significantly increased (P = .05) antibody titers, causing the rat serum to produce more IgG and IgM antibodies against sheep's red blood cells.

3.3 Effect of Storg B on Neutrophil Adhesion Test

Cyclophosphamide treatment reduced the percentage of neutrophil adhesion, but with the use of Storg-B, it substantially increased. Following therapy with Storg-B 50 and 100 mg/kg, the mean neutrophil percentage was found to be 28.03% and 29.36%, respectively, as shown in Table 2. In a dose-dependent way, the Storg B 100 mg/kg treated groups successfully increased the percentage of neutrophils.

3.4 Effect of Storg B on Hematological Parameters

The harmful effects of cyclophosphamide on the animals disrupted their normal blood component levels. In comparison to the positive control group, the treatment group Storg-B 100 mg/kg was effective in improving the normal level of blood parameters, as shown in Table No.3. The outcomes of the study suggest that Storg B has beneficial hematological effects.

3.5 Storg B on Bioavailability

LC-MS/MS is the technique that is employed to quantify the Bioavailability study of Storg B. A single dose of Storg B, 200 mg/kg, was orally administered to the SD rat. The main pharmacokinetic parameters C_{max}, tmax, Area under curve (AUC), and $t_{1/2}$ are calculated, and the values are shown in Table No.4 and Fig.3. The peak concentration (C_{max}) and time to reach the peak concentration (T_{max}) of B vitamin in the plasma were obtained from the concentration-time curve. In the graph, the plasma concentration versus time curve represents the total amount of the drug reaching the circulatory system. Storg B showed greater potency.

Table 1. Analysis specification of Storg-B

TEST	SPECIFICATION	PROTOCOL
PHYSICAL		
Description	Light brown hygroscopic powder	Organoleptic
Identification	To comply by TLC	TLC
Solubility	Soluble in water Not less than 90% w/w	USP
Loss on drying	Not more than 6.00% w/w (dried at 105^0 C)	USP<731>
Sieve test (passes through)-40#	Not less than 98% w/w	USP<786>
CHEMICAL ANALYSIS		
Each 1 gram contains (Assay of actives)		
Natural Vitamin B1	Not less than 3.0 mg (0.30%)	HPLC
Natural Vitamin B2	Not less than 3.33 mg (0.33%)	HPLC
Natural Vitamin B3	Not less than 35mg (3.4%)	HPLC
Natural Vitamin B5	Not less than 17mg (1.7%)	HPLC
Natural Vitamin B6	Not less than 4.2mg (0.42%)	HPLC
Natural Vitamin B9	Not less than 1300 mcg (0.13%)	HPLC
HEAVY METALS		
Lead	Not more than 3.0 ppm	ICP-MS
Arsenic	Not more than 1.0 ppm	ICP-MS
Cadmium	Not more than 1.0 ppm	ICP-MS
Mercury	Not more than 0.1 ppm	ICP-MS
PESTICIDES RESIDUE	Should be Absent	USP
MICROBIOLOGICAL PROFILE		
Total Plate count	Not more than 10000 cfu/g	USP<2021>
Total Yeast and Mould count	Not more than 1000 cfu/g	USP<2021>
Salmonella	Negative/10g	USP<2022>
E. Coli	Negative/10g	USP<2022>
Staph. Aureus	Negative/10g	USP<2022>
P. aeuriginosa	Negative/10g	USP<62>
Coli forms	Negative/10g	USP<62>
ADDITIONAL INFORMATION		
Sanitizing treatment	Non-irradiated and Not-treated with ETO	
Certification Status	Kosher and Halal certified	
Genetic Modification status	GMO free	
BSE/TSE status	BSE/TSE free	
Country of Origin	India	
Cultivated or wild crafted	Cultivated	
Manufactured by	Star Hi Herbs Pvt Ltd, Plot No 50,3rd Road,1St phase ,KIADB Ind Area, Jigani ,Bangalore 560105	
Shelf Life	3 years	

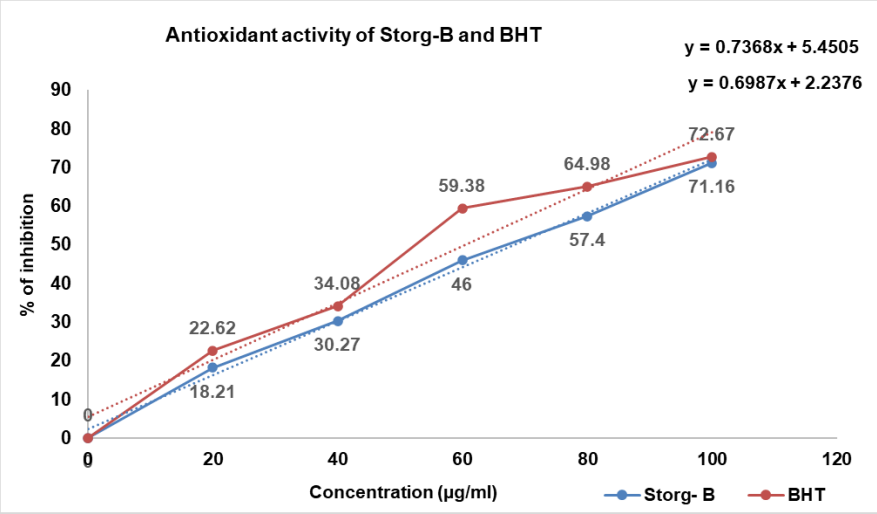

Fig. 1. DPPH free radical scavenging activity and IC_{50} of Storg-B and Standard BHT

Table 2. Effect of Storg-B on Neutrophil adhesion test

Groups	TLC (10^3 /mm^3) (A)		Neutrophil (%) (B)		Neutrophil Index (A X B)		Neutrophil Adhesion (%)
	UTB	NFTB	UTB	FTB	UTB	FTB	
Normal	6.55	5.32	23.72	18.47	155.3	98.26	36.72
Cyclophosphamide 20mg/kg	3.35	2.09	17.47	22.12	58.52	46.23	21.00
Cyclo + 50mg/kg Levamisole	5.20	4.47	21.6	17.53	112.32	78.35	30.24
Cyclo + Storg-B 50mg/kg	4.97	2.97	19.53	23.53	97.06	69.88	28.03
Cyclo + Storg-B 100mg/kg	5.18	4.51	22.31	18.10	115.56	81.63	29.36

***UTB= Untreated Blood, NFTB = Nylon Fibre Treated blood*

Table 3. Effect of Storg-B on Hematological parameters

Groups	WBC (10^9 /L)	RBC (10^{12}/L)	Hb (g/dL)	PLT (10^9/L)	DLC		
					Lymphocytes %	Monocytes %	Granulocytes %
Normal	6.55±0.1	5.3±0.13	12.6±0.32	15.9±1.2	62.72	16.48	25.25
Cyclophosphamide 20 mg/kg	3.35±0.3	4.1±0.43	12.42±0.65	9.8±1.3	43.98	10.28	30.93
Cyclo+Levamisole 50 mg/kg	5.20±1.7	4.8±0.14	12.54±0.17	12±1.02	56.33	15.47	24.8
Cyclo + Storg-B 50 mg/kg	4.97±0.88	4.74±0.12	12.56±0.32	11.8±1.36	54.88	14.88	26.71
Cyclo+ Storg-B 100 mg/kg	5.18±1.27	4.82±0.18	12.62±0.50	12.4±1.02	57.03	16.13	28.49

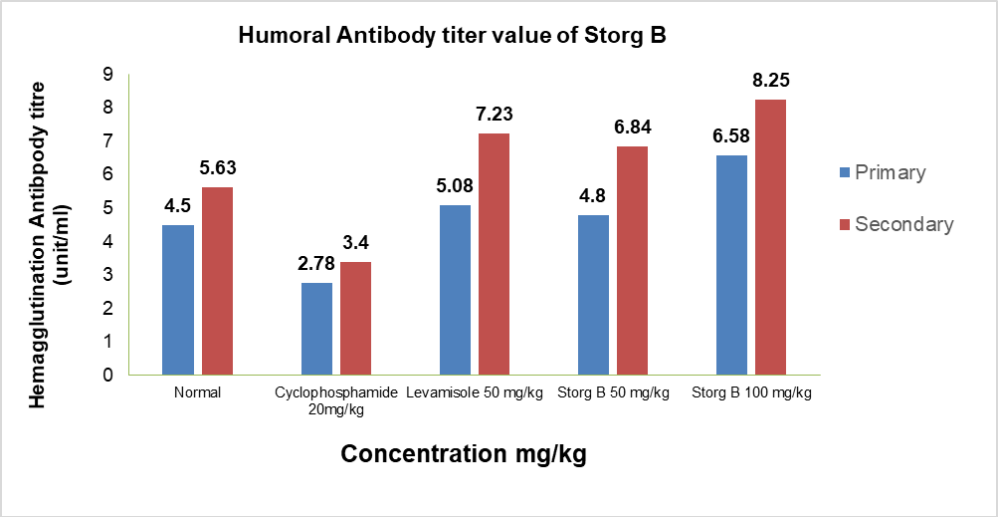

Fig. 2. Effect of Storg B on primary and secondary antibody response on HA titer

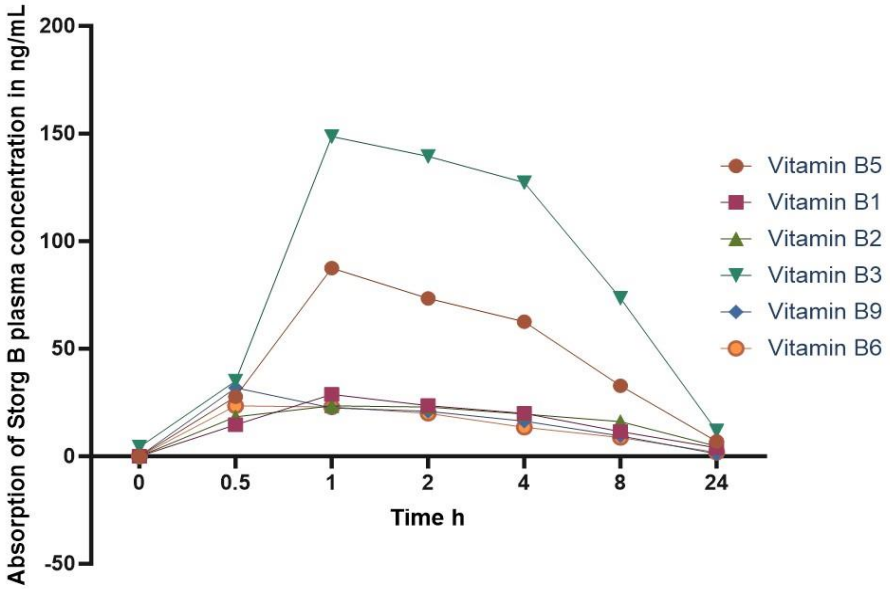

Fig. 3. Plasma concentration of Storg B time profile curve

Table 4. Bioavailability results of Storg-B

Parameters	C_{max} (ng/mL)	T_{max}	$t_{1/2}$	AUC (ng h/mL)
Vitamin B1	28.74	1 hour	5 hour 15 minutes	269.3
Vitamin B2	23.44	1 hour	13 hour	318.3
Vitamin B3	148.59	1 hour	7 hour	1430
Vitamin B5	87.5	1 hour	5 hour 30 min	759.4
Vitamin B6	23.4	30 min	4 hour 30 min	200.4
Vitamin B9	31.8	30 min	3 hour 45 min	216.8

3.6 Discussion

Polyherbal formulations are becoming widely popular due to their effectiveness in treating a variety of diseases and infectious disorders, particularly via altering the host defense mechanism [19]. Herbal formulation has been used all around the earth due to its medicinal and

therapeutic applications. In comparison to a single herbal formulation, polyherbal formulation offers more benefits [20]. Storg B is a standardized polyherbal supplement made from the extracts of the medicinal herbs Psidium guajava, Mangifera indica, and Citrus limon that contains enriched natural B vitamins and co-nutrients such as carotenoids and polyphenols. B-vitamin-mediated immunological regulation is specific to different immune cells and immune responses; that is, different B vitamins are required for different immune responses [21]. The B vitamins that are present in Storg-B are identified as follows: thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), and folate (B9). B vitamins play an essential role in catabolic and anabolic metabolism and act as coenzymes in numerous enzymatic processes that support each aspect of biological functioning, including major functions within the brain and nervous system. Any B vitamin deficiency may adversely impact the mitochondrial metabolism of amino acids, glucose, and fatty acids through the citric acid cycle and electron transport chain [15]. By playing a variety of roles in the immune system, B vitamins serve as essentials in the normal body's defense mechanism. In recent investigations, it has been recognized that vitamin B enhances a significant function in boosting immune responses [22].

In this study, a herbal immunomodulatory blend was prepared by synergized formulation of herbal extracts, scientifically quantified for their bioactive metabolites, tested *in vitro* and *in vivo, and* evaluated in a cyclophosphamide(CP) induced immunosuppression model in SD rats.

Antioxidants are considered to have an impact on DPPH due to their ability to donate hydrogen [23]. Radical scavenging activity is very essential in order to prevent free radicals from playing a harmful role in a variety of disorders, including cancer. The polyphenols primarily act as free radical scavengers; the majority of plant polyphenols have strong antioxidant qualities [24]. Since these plant polyphenols are co-nutrients in our Storg B, they may be contributing to antioxidant activity, which is demonstrated by its 68.35µg/ml free radical antioxidant scavenging activity in comparison to the standard BHT.

Immunomodulatory agents of plant origin improve the immune responsiveness of an organism against a pathogen by nonspecifically activating the immune system [25]. The humoral immune system includes the interaction of B cells with the antigens, which replicate and differentiate into plasma cells that secrete antibodies [26,27]. In the study, the Storg B-treated group produced increased primary and secondary antibody titres against SRBCs. The antibodies produced were intended to bind to the antigen and neutralize or facilitate recognition by phagocytes, which leads to the ultimate elimination of the antigen [28,29]. Storg B enhanced the humoral immune response to SRBCs in rats, as evidenced by an increase in antibody titer. This shows that the T and B lymphocyte subsets became more responsive, which are vital for antibody formation [30].

An animal's hematological characteristics can be employed to observe and quantify one of the immune responses. The total leukocyte count for both the control group and the groups that received Storg-B medication was determined based on these characteristics. When non-self-components invade the body, blood cells react first. Any immunological material with immunomodulatory properties would first cause a shift in the leukocyte count [31]. The study group V, which was administered the testing medication, exhibited the highest leukocyte count of 5.18 $10^3/mm^3$, indicating the early activation of blood cells in order to develop a potent immune response. The findings indicate that higher concentrations of the medication Storg-B can stimulate a significant immunological response. In the neutrophil adhesion test, adherence to nylon fibers was increased. This may be due to the upregulation of β-2 integrins and also to a decreased corticosterone level. The results are further supported by the highest percentage of neutrophils circulated among the group. Finally, the results revealed that Storg B has potential humoral immunity and can be a potential therapeutic candidate for several immune-suppressed conditions.

Any molecule's bioavailability (BA) is determined by the interaction of its pharmacodynamics (PDs) and pharmacokinetics (PKs) factors [32]. PKs (ADME) and PDs are two aspects that influence the therapeutic efficacy of drugs. The water-soluble vitamins biotin, folate, niacin, pantothenic acid, pyridoxine, riboflavin, and thiamin are absorbed through the intestinal tract by particular carrier-mediated pathways [33]. The estimations of C_{max} and area under the curve (AUC) parameters were included in the pharmacokinetic dataset. The time of peak concentration was not

significantly different, but it was significantly different in terms of AUC. The area under the curve is the most important parameter to evaluate the bioavailability of the drug in its dosage form because it indicates the extent of absorption [34]. From the results, the AUC value of the B vitamins is followed by B3 > B5 > B2 > B1 > B9 > B6. Where B3 (1430 ng h/mL) entered the systemic circulation for distribution to target cells and tissues, maybe with a mild alteration compared to other B vitamins present in the Storg B. Vitamin B1 (269.3 ng h/mL), B9 (216.8 ng h/mL), and B6 (200.4 ng h/mL) are acidity and temperature-sensitive. Since the pH of the small intestine is greater than that of the stomach phase, reductions in pH-sensitive vitamins may generally take place [35]. Hence, they have a slightly lower absorption rate. While Vitamin B5 (759.4 ng h/mL) and B2 (318.3 ng h/mL) are showing moderate absorption rates, since there aren't many bioavailability studies currently available for B vitamins. As a result, Storg B supplementation appears to be a viable strategy for antioxidant therapy and treating immunological deficiencies by increasing the immune response.

4. CONCLUSION

The findings clearly reveal that Storg B stimulates the humoral immune response to SRBC antigen in rats. Storg B has immune-boosting properties and could be useful in immunological illnesses where the antioxidant system is damaged.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments have been examined and approved by the Bharathi College of Pharmacy ethics committee (BCP/IAEC/06/2022).

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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