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IN VITRO ANTIOXIDANT AND *IN VIVO* IMMUNOMODULATORY STUDY OF LEMON PEEL EXTRACT (STORG-FA) AND ITS BIOAVAILABILITY ASSESSMENT

H. M. Firoz, C. T. Sadashiva * and A. K. Sreedrisya

R & D Department, Star Hi Herbs Pvt. Ltd, Jigani, Bangalore - 560105, Karnataka, India.

Keywords:

In vitro, *In vivo*, Antioxidant, Immunomodulatory, Bioavailability, Lemon Peel

Correspondence to Author:
C. T. Sadashiva

General Manager,
R&D Department, Star Hi Herbs Pvt. Ltd, Jigani, Bangalore - 560105, Karnataka, India.

E-mail: research@starhiherbs.com

ABSTRACT: Background: Natural bioactive compounds of plant origin have attracted comprehensive attention due to their multiple biological effects, including antioxidant and immunomodulatory activities. However, scientific underpinnings of these health benefits are inadequate. **Objective:** Present investigation was undertaken to evaluate the bioavailability, antioxidant, and Immunomodulatory activity of the herbal formulation Lemon Peel extract (Storg-FA), which contains Lemon Peel extract standardized for 5% Folic Acid, along with co-nutrients including various water-soluble natural folates. **Methods:** The invitro antioxidant activity of Storg-FA was evaluated using the DPPH radical scavenging assay whereas cyclophosphamide induced immunosuppressed rat model was established to investigate the immunomodulatory activity of Storg-FA in Sprague-Dawley (SD) rats. Haemagglutination test was used to assess their effects on humoral response. After 14 and 21st days of drug administration, the extent of protection against immunosuppression caused by Cyclophosphamide was assessed by estimating hematological parameters. The invivo bioavailability study of Storg FA was conducted using LC-MS/MS analysis. **Results:** Storg-FA showed pronounced invitro antioxidant activity against DPPH radical. IC_{50} of Storg FA was found to be 109.35µg/ml, as compared to BHT used as a standard (IC_{50} of 60.52µg/mL). Storg-FA stimulated the humoral immune response. Increased haemagglutination inhibition was observed with Storg FA in both low and high dose. It was identified that these herbal formulations significantly improved the haematogram parameters. **Conclusion:** The results suggest that Storg-FA can be considered as promising antioxidant and immunomodulatory agents.

INTRODUCTION: The investigation of the antioxidant and immune-enhancing properties of natural products represents an active area of current research [1-3]. Immunomodulatory and antioxidant activities of products derived from nature are widely used to treat many ailments, including autoimmune disease, inflammatory disorders, and cancer [4, 5].

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Natural products have the advantage of having a biological origin, being biodegradable, and having a favorable effect on human health and the environment [6]. The use of natural products to heal diseases has been documented since ancient times.

Recently, there has been growing interest in inexpensive and less toxic natural products over the use of synthetic chemotherapeutic agents. The present work aimed to explore the bioavailability, antioxidant and immunomodulatory activities of lemon peel extract (Storg FA). Lemon is well-known for its diverse phytochemicals and ample supply of vitamin C, folic acid, potassium, and pectin, which serve as essential supplements for

diseases [7, 8]. A disturbance between the process of generation of reactive oxygen species (H_2O_2, oxy radicals $O_2^{\bullet-}$ and the hydroxyl radical HO•) and subsequent removal by antioxidant defense mechanisms is known as oxidative stress. This disturbance is the root cause of several health issues, including cancer, heart disease, aging, and neurodegenerative diseases [9, 10]. Oxidative stress has an effect on the immune response of our body as well. The endogenous antioxidant system safeguards the immune cells against deleterious influence of the free radicals and preserves their proper function. An impaired immune system leads to overutilization of endogenous antioxidants [11]. Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are primarily added to food products particularly to lipid containing foods to prolong shelf life [12], but they are suspicious of causing toxicity, and may result in cancer, and liver damage, prompting a growing interest in natural antioxidants, particularly those derived from plants.

The immune system is a sophisticated association of different immune cells and molecules that work together to protect the body against infections and foreign invaders. Many of the studies have shown that antioxidant micronutrients protect immune responses from the immunosuppressive effects of environmental factors, such as exposure to UV and cigarette smoke that also increase the risk of cancer [13]. Recent clinical studies have revealed that antioxidant supplementation, specifically vitamins C, E, A and beta-carotene, can significantly enhance certain immune responses in the elderly, particularly enhanced the activation of cells involved in tumor immunity [14].

The human body needs certain micronutrients, such as vitamins and minerals, and people must meet their daily requirements through dietary sources. Micronutrients prevent pathogenic organisms from evading the immune system by acting as immunomodulators and strengthening the host immune response [15]. Their antioxidant properties show benefits in building body resistance and boosting energy. Numerous experimental investigations have been conducted in order to evaluate the antioxidant properties and immunomodulatory effects of vitamins and minerals, especially plant-based immunity-enhancing compounds are in the limelight due to their potential health benefits [16]. Our Storg-FA is a certified organic and 100% natural lemon peel extract standardized for 5% Folic Acid content, as well as co-nutrients including various water-soluble natural folates from lemon peel. Water-soluble Vitamin B9 is necessary for several body functions. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in biological reactions involving folate [17]. It is crucial for rapid cell division and growth, such as infancy and pregnancy. Folic acid is essential for both adults and children to produce healthy red blood cells and prevent anemia [18].

Citrus fruits are one of the most significant natural product crops in the world owing to their nutritional and therapeutic values. Every year, the citrus-juice processing industry generates a large amount of citrus peel waste. The citrus by-products are considered an inexpensive source of bioactive compounds with versatile biological activities beneficial for the food and pharmaceutical industries that are less exploited. Lemon is one of the most popular Citrus species in the world after orange and mandarin [19]. While fresh fruit and juice are consumed in large quantities all over the world, the lemon peel is typically thrown away as waste [8]. However, customers are unaware, that lemon peels have a wide range of beneficial bioactive compounds with strong antioxidant activity that may be similar to those found in lemon pulps [20]. The measurement of antioxidant and immunomodulatory activities may serve as a means for functionally relevant quality assessment of these natural products. Therefore, the present work was designed to explore the following endpoints. (1) Invitro antioxidant activity by DPPH (2) Invivo immunomodulatory activity in cyclophosphamide induced rat model (3) Invivo bioavailability study by LC-MS/MS method.

MATERIALS AND METHODS:

Plant Materials: Storg-FA is an extract made entirely of natural lemon peel standardized for 5% Folic Acid that is certified organic and contains co-nutrients such as several water-soluble natural folates from lemon peel. Storg-FA is manufactured and registered by Star Hi Herbs Pvt. Ltd. Jigani, Bangalore, Karnataka, India. This study was performed at Invivo Biosciences, Bangalore,

Karnataka, India from September 2022 to October 2023.

Chemicals: All chemicals and reagents used for the conduct of this study were of analytical grade. All standards were purchased from Sigma-Aldrich, USA. The Standards were stored in the original packages at -18°C prior to use. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and analytical grade methanol were purchased from Sigma-Aldrich. Cyclophosphamide was purchased from Sigma-Aldrich, USA. Methanol (JT baker for LC-MS), formic acid (Optima from Fisher Chemicals, LC-MS grade), and Milli-Q-water (Sartorius ARIUM MINI).

***In vitro* Antioxidant Activity of Storg-FA:** The Storg-FA ability to scavenge free radicals was evaluated using the DPPH radical scavenging assay, as described by Blois [21]. The ability of the Storg-FA (Lemon peel) to donate hydrogen atoms was assessed by decolorizing a methanol solution with 2, 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produces violet or purple color in methanol solutions and in the presence of antioxidants fades to shades of yellow. A 0.1Mm DPPH solution in methanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of Storg-FA in methanol at different concentrations (50, 100,150,200, and 250µg/mL). The sample was vortexed thoroughly and left in the dark at room temperature for 30 minutes and its absorbance was measured spectrophotometrically at 517 nm.BHT was used as reference drug at different concentrations (20, 40, 60, 80 and 100). The percentage of DPPH radical scavenging activity was calculated by the following equation:

$$\%\boldsymbol{of DPPH\ radical\ scavenging\ activity} = (\boldsymbol{A_0} - \boldsymbol{A_1})/\boldsymbol{A_0} \times \mathbf{100}$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the Storg-FA. Then the percentage of inhibition was plotted against concentration, and the IC_{50} was calculated from the graph. The experiment was repeated three times at each concentration. The antioxidant activities were expressed as the half maximal inhibitory concentration (IC_{50}), which is the antioxidant concentration, required decreasing the absorbance of the control by 50%, calculated by plotting the obtained inhibition percentages against the concentrations of the solutions used in the assay.

***In vivo* Immunomodulatory Activity of Storg-FA:**

Animals and Experimental Protocol: The studies were conducted in accordance with ethical clearance obtained from the Institutional Animal Ethics Committee (IAEC) before the experiment (Invivo/017/2023). It carried it out in compliance with the guidelines laid by the Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India. SD rats weighing 150-250 grams were used in this study. They were supplied by the animal house, Invivo Biosciences, Bangalore, Karnataka, India. The rats were housed in polypropylene cages (43 x27 x15 cm) with a maximum of six animals per cage and kept under standard air-conditioned laboratory conditions (temperature maximum of 24°C and minimum of 23°C and relative humidity maximum of 63% and minimum of 48%) with a 12h light and 12 h dark cycle. The mice were acclimatized to the laboratory condition for a week before the experiment and handled throughout the experiment following the guideline for the care and use of laboratory animals [22].

Antigen Preparation:

Sheep Red Blood Cell (SRBC) Preparation: To prepare sheep red blood cells (SRBC), blood was collected from the external jugular vein of sheep and mixed in 1:1 proportion with freshly prepared Alsever's solution. SRBCs were isolated from the collected blood by centrifuging at 2500 rpm for 10 min. Plasma and buffy coat are removed by washing cells with pyrogen-free normal saline (0.9% w/v). The concentration was adjusted to $1x10^8$ SRBC in 1 mL of sheep blood for Immunization and challenge.

Haemagglutinating Antibody (HA) Titre: The rats were divided into 4 groups, each containing 8 animals. Group I served as the control group. Group II received 100 mg/kg of cyclophosphamide (Sigma-Aldrich, USA), while Groups III and IV received 50 mg/kg and 100 mg/kg of Storg-FA, respectively. On days 7 and 14, all the groups except control were administered with 0.1 mL of 20% SRBC. On the 9^{th} and 16^{th} days, all the groups except control were administered CP 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 at37°C for one hour and then observed for hemagglutination and antibody reactions. The rank of minimum dilution that exhibited hemagglutination was considered as the antibody titer. The level of antibody titer on day 14 of the experiment was considered as the primary humoral immune response whereas the one estimated on day 21 of the experiment was the secondary humoral immune response.

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 Hemoglobin (Hb), WBC, RBC, total Platelet count, and Differential leukocyte count using automated hematology analyzer (Beckman Coulter, Inc., Fullerton, CA, USA).

***In vivo* Bioavailability:**

Animals: Healthy male Sprague-Dawley (SD) rats (n = 6) weighing 150-200g were selected for pharmacokinetic and bioavailability study. All the rats were acclimatized for seven days before commencing the pharmacokinetic study. Body weight variation among the animals did not exceed 20% of the mean body weight. The room was maintained at a temperature of 22°C and a relative humidity of 60%. The experiment was conducted under a 12 hour light and 12 hour dark photoperiod, with prescribed feed and *ad libitum* drinking water. The experiment was carried out in accordance with the guidelines set by the Institutional Animal Ethics Committee.

Experimental design: Study design SD rats (weighing about 150-200g) were selected as the animal model. The rats were 8–12 weeks old. The rats selected for the study were not given any medication for two weeks before the trial began. Twelve hours before drug administration, food was denied to rats until 24 hours post-dosing, although they were allowed access to water throughout the research. The Storg FA dose of 200 mg/kg based on the animal body weight was administered to rats using a gavage needle. The study was approved by Institutional Ethical committee (Invivo/017/2023). Blood samples (0.6 ml) were withdrawn from the retro-orbital plexus before dosing (zero time) and at time intervals of 0.5, 1, 2, 4, 8, 24 hours after administration. The anticoagulant used was EDTA disodium salt. Plasma was separated by centrifugation at 6000 rpm for 10 min and the extracted plasma sample from each blood sample was divided into two aliquots and kept in appropriately labeled heparine tubes at –20°C until used. Estimation of C max, T max and $t_{1/2}$ of Storg FA was done by using LC-MS/MS method.

LC-MS/MS Analysis: Liquid chromatography and Mass Spectrometry were 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, auto sampler SIL-40, Column Oven CTO-40 Series. Heated ESI source which improves desolvation and enhances ionization efficiency with the addition of a heated gas used in combination with the nebulizer gas. UF sweeper®III collision cell enhances CID efficiency by optimizing the collision cell pressure and a PC with Lab solution and Insight software. The LC-MS/MS system was equipped with a chromatographic column Shimpack GISC18-120 (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 separation 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 completed by multiple reactions monitoring (MRM) in ESI positive mode using the following optimized parameters:

Nebulizing gas flow (Nitrogen) =3mL/min, Heating gas flow (Zero Air) = 5mL/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.

Standard Preparation: Standard stock solutions were prepared by dissolving an appropriate amount of Standard (Vitamin B9) in Water containing 8% Acetic acid. Prepare a mixture of Storg-FA where the concentration was 20000ng/mL). The standard stock solutions were stored at -18°C. The standard mixture solution of 20000ng/mL was prepared freshly before analysis in an amber- colored glass volumetric flask (5mL) by diluting the calculated amount of each standard stock solution with water. The calibration solutions were prepared freshly before in brown glass HPLC vials (1.8mL) diluting the standard mixture solution 20000ng/mL as per **Table 1**. Concentrations of Stock standard solution were prepared using from 3ng/mL to 300ng/mL. The total volume of each calibration solution was 1mL for stock Standard solution prepared.

Sample Preparation: For the Sample analysis, 40µ L of serum sample was taken in a 2mL Eppendorf tube followed by 160µL of Methanol into the tube. The mixture was vortexed for 3 minutes and then centrifuged at 6000rpm for 10 minutes at 4°C. After Centrifuge, the transfer of 100µL of Clarified supernatant and 200µL of water were transferred to the brown glass HPLC vial (1.8mL) for further analysis. Vortex for 2 min and inject. For the Spiked Sample analysis, 40µL of serum sample was taken in a 2mL Eppendorf tube followed with 10µL of an appropriate standard solution and 160µL of Methanol into the tube. The mixture was vortexed for 3mins and then centrifuged at 6000rpm for 10 min at 4°C., after Centrifuge, transfer 100µL of Clarified supernatant and 200µL of water was transferred to the brown glass HPLC crimp vial(1.8mL) for further analysis. Vortex for 2 min and inject.

Statistical Analysis: Average of all data was compiled, and SEM was calculated. All the data were compiled using one-way ANOVA followed by Dunnett's multiple comparison tests. *P* values<0.05 were considered as statistically significant (Graphpad prism version. 10.0.3(273).

RESULTS:

Antioxidant Activity: Table 1 and **Fig. 1** illustrate the antioxidant activity of the Storg-FA. The free radical scavenging activity of Storg-FA was determined and compared to that of standard butylated hydroxyl toluene (BHT). The antioxidant activity of standard BHT and Storg-FA was found to have IC_{50} values of 60.52 and 109.35µg/ml, respectively. The results obtained in the present study indicate that Storg-FA exhibits significant free radical scavenging activity. The results of the present investigation imply that Storg-FA may be a promising natural antioxidant source that may play a significant role as therapeutic agents in averting or delaying the onset of aging and age-associated oxidative stress-related degenerative diseases.

TABLE 1: DPPH FREE RADICAL SCAVENGING ACTIVITY AND IC50 OF STORG- FA AND STANDARD BHT

	Storg-FA		Standard BHT	
Sl. no.	**Concentration (µg/ml)**	**Inhibition of Storg-FA**	**Concentration (µg/ml)**	**%Inhibition of BHT**
1	Control	0	Control	0
2	50	30.66	20	22.62
3	100	58.19	40	34.08
4	150	71.03	60	59.38
5	200	82.91	80	64.98
6	250	90.56	100	72.67

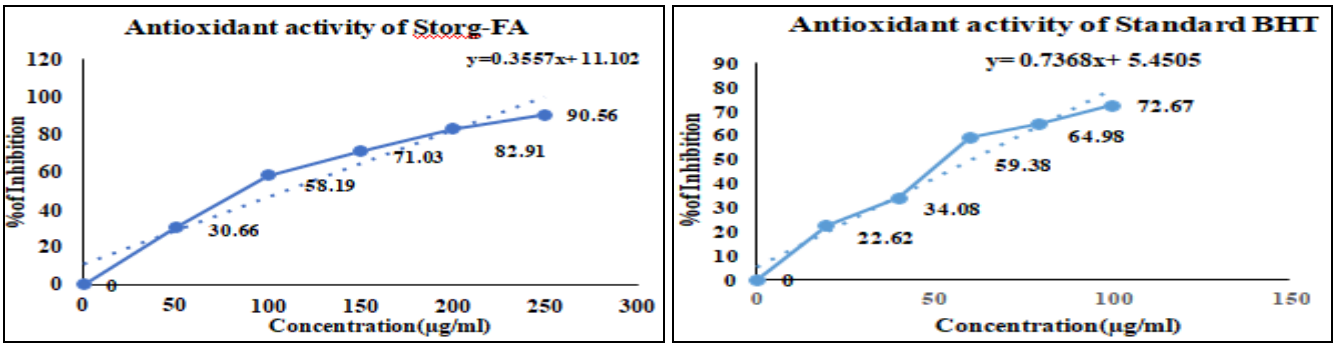

FIG. 1: DPPH FREE RADICAL SCAVENGING ACTIVITY AND IC50 OF STORG-FA AND STANDARD BHT

***In vivo* Immunomodulatory Activities of Storg-FA:**

Humoral Immune Response Model: The effect of Storg FA on primary and secondary antibody response on HA titer is shown in **Table 2** and **Fig. 2.** Primary antibody response on day 14th in Storg FA (50mg, 100mg/kg/p.o) treated group with normal immune status showed a significant increase ($p<0.05$) in HA titer when compared with the control group. A significant decrease in the antibody titer was observed in the Cyclophosphamide–treated group when compared with the control group. In immunosuppressed groups, where the immunity was suppressed by administration of Cyclophosphamide on day nine, Storg-FA (100mg/kg/p.o) administration produced significant ($p<0.05$) rise in the antibody titer when compared with the Cyclophosphamide – treated group. Secondary antibody titer on the twenty-first day in Storg FA both low-dose and high-dose treated groups with a normal immune status group showed a significant rise ($p<0.05$) in the antibody titer when compared with the control group. In the immunosuppressed groups where the immunity was suppressed by administration of Cyclophosphamide on day sixteenth Storg FA both low dose and high dose showed a significant rise ($p<0.05$) in HA titer when compared with the Cyclophosphamide group.

TABLE 2: EFFECT OF STORG FA ON HUMORAL IMMUNE RESPONSE IN CYCLOPHOSPHAMIDE-INDUCED IMMUNOSUPPRESSED RATS

Group	Dose (mg/kg)	Hem agglutination Antibody titer (unit/ml)	
		Primary	Secondary
Normal control		4.5±0.33	5.63±0.18
Negative control (SRBC+Cyclophosphamide)	0.1ml+100	3.0±0.27	4.13±0.30
SRBC+Cyclophosphamide Storg-FA	50	5.9±0.37	6.75±0.33
SRBC+Cyclophosphamide+Storg-FA	100	7.63±0.18	9.63±0.18

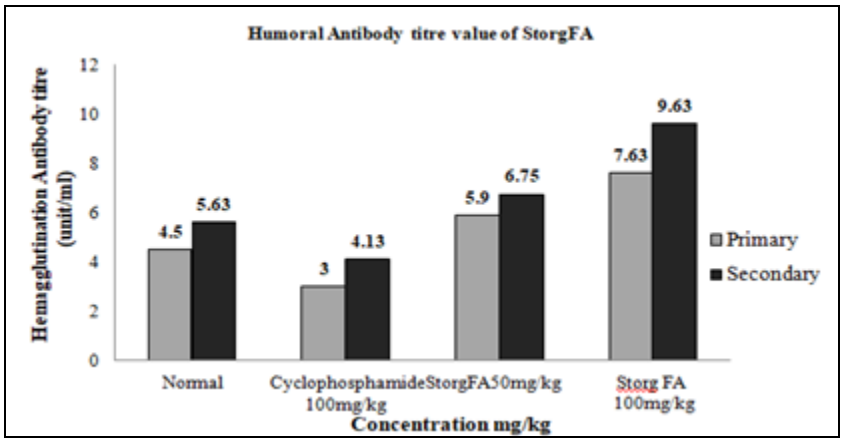

FIG. 2: EFFECT OF STORG-FA ON PRIMARY AND SECONDARY ANTIBODY RESPONSE ON HA TITER

Hematological Analysis: The effect of Storg-FA on the hematological indices of the rats demonstrated a substantial increase in the white blood cell (WBC) count of the extract-treated animals. The rise in WBC count is suggestive of the ability of Storg-FA to activate the immune system, which in turn produces more white blood cells. White blood cells have a major role in enhancing defense mechanism against infection and in phagocytosis. Neutrophils are also equipped with bacterial uptake (phagocytosis). This also serves as a strong pointer to the immunostimulatory activity of Storg-FA. However, when comparing the treated animals to the control, there was no discernible difference in the levels of hemoglobin or red blood cells (RBCs). Lymphocytes are crucial for cellular and humoral immunity, while monocytes, acting as monocyte-macrophages, phagocytose bacteria and are involved in inflammatory reactions through which they act on antigenic substances, attracting T-lymphocytes in the immune system. The extract-treated groups showed significantly higher platelet levels compared to the controls, indicating their role in protecting against microbial invasion, mobilizing innate immune cells, and enhancing their activity, thus promoting adaptive immune responses **Table 3** and **Fig. 3.**

TABLE 3: EFFECT OF STORG-FA ON HEMATOLOGICAL PARAMETERS

Parameters	Hb(g/dl)	WBC (10^9/L)	RBC (10^{12}/L)	Plate let count (10^9/L)	Neurophiles (%)	Lymphocyte s (%)
Normal	12.64±0.32	9.5±0.10	5.3±0.13	15.9±1.2	26.65±0.73	72.00±1.16
Cyclophosphamide 100mg/kg	12.42±0.65	8.6±0.3	4.1±0.43	9.8±1.3	35.16±2.21	66.79±1.93
Cyclophosphamide+ Storg FA 50mg/kg	12.23±0.45	9.7±0.22	5.26±0.19	16.88±0.83	34.67±0.52	66.65±0.89
Cyclophosphamide+ Storg FA100mg/kg	12.75±0.22	10.1±0.74	5.93±0.23	17.97±1.18	31.50±0.96	68.84±2.01

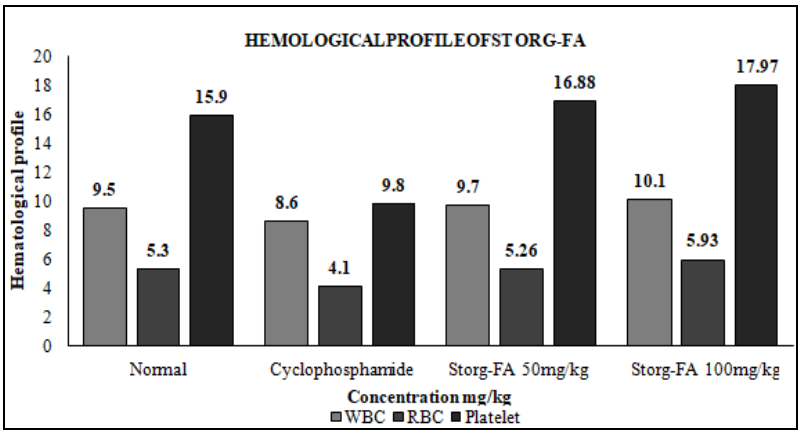

FIG. 3: HEMATOLOGICAL PROFILE OF STORG-FA

***In vivo* Bioavailability Study of Storg- FA:** In the present study, the LC-MS/MS method has been successfully applied to pharmacokinetic studies of oral administration of Storg FA (at a dose containing 200 mg/kg) in rats. The main pharmacokinetic parameters C_{max} (maximum concentration), t_{max} (time taken to reach maximum concentration), and $t_{1/2}$ (Half-life period) are shown in **Table 4** and **Fig. 4**. The t_{max} and $t_{1/2}$ observed for Storg FA were 1hour and 5 hour respectively. The area under the curve (AUC) determines the extent of exposure of a drug with respect to the time. AUC was found to be 580.6ngh/L for Storg FA. Storg FA exhibited a modest rate of absorption and metabolism in the blood circulatory system following oral dosing to rats after a single oral administration. These findings indicate more efficient increase in plasma concentrations with folic acid at the dose of 200mg/kg. The chemical analysis of Storg-FA is shown in **Table 5.**

TABLE 4: RESULTS OF BIOAVAILABILITY STUDY OF STORG FA

Parameters	Storg FA (200mg/kgb.w.)
Cmax	67.24ng/mL
Tmax	1hour
$t_{1/2}$	5hour
AUC	580.6ngh/mL

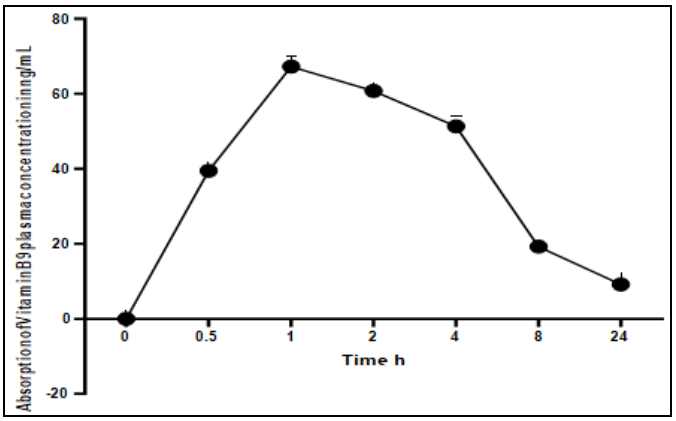

FIG. 4: PLASMA CONCENTRATION-TIME PROFILE OF STORG-FA

TABLE 5: ANALYSIS SPECIFICATION OF STORG FA

Test	Specification	Protocol
	Physical	
Description	Brown 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 105^0C)	USP<731>
Sieve test (passes through)-40#	Not less than 98% w/w	USP<786>
	Chemical analysis	

Natural Folic acid	Not less than 5.0 % (50,000mcg/g)	HPLC
Folate as Dietary Folate equivalent	Not less than 83, 333mcg/g	As per NLEA regulation
	Heavy metals	
Lead	Not more than 3.0ppm	ICP-MS
Arsenic	Not more than 1.0ppm	ICP-MS
Cadmium	Not more than 1.0ppm	ICP-MS
Mercury	Not more than 0.1ppm	ICP-MS
Pesticides residue	Should be Absent	USP
	Microbiological profile	
Total Plate count	Not more than 3000 cfu/g	USP<2021>
Total Yeast and Mould count	Not more than 100 cfu/g	USP<2021>
Salmonella	Negative/10g	USP<2022>
E. coli	Negative/10g	USP<2022>
Staph. Aureus	Negative/10g	USP<2022>
P. aeurginosa	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	3years	

DISCUSSION: In recent years, there has been extensive investigation into the recycling of agro-industrial waste for the production of bioactive compounds and their value-added applications. The food processing industry produces huge amounts of citrus peel waste which are fed to animals or disposed of, increasing the burden on the environment. These citrus peels constitute an excellent repository of phytochemicals and antioxidants, potentially serving as an affordable resource. However, there has been very restricted data into immunomodulatory activity and bioavailability of bioactive contents from the citrus peels, in particular lemon peels. Previous studies have reported that the administration of Lemon peel extract has been found to enhance immune function and antioxidant activity [23-25].

The concentration of the bioactive compounds in the extract determines its antioxidant activity. Consequently, greater extract concentrations exhibit greater antioxidant activity [26]. The present study showed that Storg-FA (Lemon peel extract) exhibited DPPH radical scavenging activity in a concentration-dependent manner. The effect of antioxidants on DPPH is believed to be because of their ability to donate hydrogen. DPPH free radical scavenging is a commonly used mechanism for evaluating the antioxidant activity of plant extracts. Due to the relatively shorter time required for analysis, this method has been widely used to predict antioxidant activities [27]. The results obtained in this study suggest that Storg-FA (lemon peel extract) exhibited radical scavenging activity through their electron transfer or hydrogen donating ability.

Plant derived immunomodulatory agents strengthen the immune responsiveness of the organism against a pathogen by stimulating the immune system [28]. The study conducted a hemagglutination test to assess the impact of lemon peel extract on the humoral immune response, highlighting the need for systemic studies to validate their therapeutic claims made with regard to their clinical utility. Humoral immunity is the result of B cells interacting with the antigen, which leads to their proliferation and differentiation into plasma cells that release antibodies. Hence, antibodies serve as the effectors of the humoral response by binding to the antigens, neutralizing them, or facilitating their removal by cross-linking to create clusters that are then ingested by phagocytic cells [29]. The study results demonstrated that Storg-FA (Lemon peel extract) had a stimulatory effect on the humoral immune response. This was evidenced by the mean

hemagglutination antibody titer to SRBC, which exhibited a dose-dependent increment for the rats dosed with Storg-FA (Lemon peel extract) in comparison to the rats that were given cyclophosphamide alone. Results from this study therefore demonstrated that the lemon peel extract contains compounds that can stimulate the production of antibodies in immunocompromised animals, thus justifying its use as an immune stimulant.

In this study, we first showed that our Storg-FA could reverse the immunosuppression caused by cyclophosphamide. Cyclophosphamide is a crucial chemotherapeutic drug in tumor therapy, but it is not beneficial for healthy cells, and it can cause side effects like bone marrow suppression, immunosuppression, oxidative stress, and occasionally life-threatening effects [30]. In previous studies, cyclophosphamide induced rats have been treated as immunosuppression models, revealing that Cyclophosphamide induced immunosuppression significantly lowers red blood cell, WBC, and platelet counts [31]. Hematopoietic stem cells produce mature blood cells such as WBCs, red blood cells, and platelets which are multi potent and self-renewing [32]. Consistent with prior reports [33], we observed that cyclophosphamide treatment decreased these cells. However, Storg-FA administration significantly restored these cell counts in a dose-dependent manner. The present study suggests that the aqueous extract of lemon peels (Storg-FA) enhances the Production of RBC, WBC and platelets. These results suggest that Storg-FA (lemon peel extract) enhances immunity by protecting against cyclophosphamide-induced immunosuppression.

Natural products with antioxidant properties and immunomodulatory activity have been extensively utilized in the pharmaceutical and food industry and have also been very popular as health-promoting herbal products [34]. Establishing the pharmacokinetics and bioavailability of herbal products is a constant challenge due to their complex composition and the ever-increasing list of their active phytochemical constituents. In pharmacology, the area under the plasma level–time curve (AUC) is a measure of drug bioavailability. The AUC gives insights into the total amount of active drugs that reach systemic circulation and its clearance rate from the body. $t_{½}$ represents the time required to reduce the concentration of the drug by 50% due to the elimination process [35]. The present study was a single-dose study that included the calculation of the area under the curve, C_{max}, T_{max} and $t_{1/2}$. Storg FA exhibited a modest rate of absorption and metabolism in the blood circulatory system following oral dosing to rats after a single oral administration (200 mg/kg). The C_{max} and T_{max} results indicated that Storg FA was rapidly absorbed. Further studies can be carried out at various dose levels and measures can be taken for improving the bioavailability.

CONCLUSION: The results evidenced the potential of Lemon peel extract (Storg-FA) as an effective drug with antioxidant and immunomodulatory activity and could be used for the preparation of nutraceuticals for pharmaceutical use in the treatment of various human ailments and its complications. The DPPH assay demonstrated high antioxidant content in the aqueous extract of lemon peels. The present investigation communicates the immunomodulatory effects of Storg- FA on cyclophosphamide-induced immunosupression in rats. The findings revealed that Storg- FA produced significant increase in RBC, WBC and platelet count and stimulated humoral response as evidenced by an increase in antibody titre in rats. The ensemble of results indicates that Storg-FA possess good bioavailability. It is evident that drugs of natural origin are potential sources of drug with antioxidant activity as well as immune response mediators. Further research is needed to understand the effects of these products on health and their mechanisms of action. This will allow us to find the best form of administration of the drug and the conditions in which they could deliver better benefits.

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