In Vivo Immunostimulatory Evaluation of Flavonoid-Rich Fraction from *Nerium oleander* Leaves Using the Carbon Clearance Assay

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Abstract

The present study investigates the immunomodulatory activity of a flavonoid-rich fraction obtained from the alcoholic extract of Nerium oleander leaves (l-NO-FF-ALC) through an in vivo carbon clearance assay in Swiss albino mice. The assay aimed to assess the functional activity of macrophages, key components of innate immunity, by evaluating their ability to clear colloidal carbon particles from the bloodstream. Five experimental groups (n = 5 per group) were established: a normal control group receiving saline, an immunosuppressed group treated with cyclophosphamide (25 mg/kg), a positive control group treated with Immunosin (50 mg/kg), and two test groups treated with l-NO-FF-ALC at 100 mg/kg and 250 mg/kg, respectively. Carbon ink was administered intravenously via the tail vein, and blood samples were collected at 5 and 15 minutes post-injection. The phagocytic index (K) was calculated based on the rate of carbon clearance. Cyclophosphamide significantly reduced the phagocytic index, confirming immune suppression. In contrast, treatment with l-NO-FF-ALC at both doses significantly restored macrophage activity ($p < 0.0001$), with the 250 mg/kg dose closely approaching the immunostimulatory efficacy of Immunosin. These results indicate that the flavonoid-rich fraction enhances innate immune function by stimulating macrophage-mediated phagocytosis. The findings support the potential of l-NO-FF-ALC as a natural immunostimulant and encourage further bioassay-guided studies for its use in managing immunosuppressive disorders or viral infections such as COVID-19.

Keywords: *Nerium oleander, Immunomodulatory activity, Carbon clearance assay, Macrophage function, Flavonoid-rich fraction, Cyclophosphamide, Immunosin.*

1. Introduction

The immune system serves as a critical defense mechanism that protects the host from infectious agents, malignancies, and various external threats. Its proper functioning is essential for maintaining physiological homeostasis. However, under certain conditions such as chronic stress, infections, malignancies, autoimmune disorders, and during chemotherapy or radiotherapy, the immune response becomes compromised, making the body more susceptible to opportunistic infections and disease progression [1]. In such scenarios, immunomodulatory agents are vital for either stimulating or suppressing specific components of the immune system to restore immunological balance [2].

Immunomodulators are broadly classified into immunostimulants and immunosuppressants based on their mechanism of action. Synthetic immunomodulatory drugs such as corticosteroids, cyclophosphamide, and methotrexate have shown efficacy but often come with a host of adverse effects including cytotoxicity, gastrointestinal disturbances, hepatotoxicity, and general immunosuppression [3]. As a result, there has been growing interest in identifying safer, plant-based alternatives that can offer immunomodulatory benefits with fewer side effects. Traditional systems of medicine, including Ayurveda and Traditional Chinese Medicine, have long recognized the value of certain herbs and plant-derived bioactives in modulating immune function [4].

One such plant with ethnomedicinal significance is *Nerium oleander* L., belonging to the Apocynaceae family. Commonly known as Indian oleander or Kaner, it is widely distributed in tropical and subtropical regions. Traditionally, various parts of the plant have been employed in the treatment of skin ailments, inflammation, asthma, cardiac disorders, and even as a general tonic [5]. Scientific investigations have revealed that *Nerium oleander* contains a diverse range of bioactive constituents including cardiac glycosides (oleandrin), triterpenoids, steroids, and notably, flavonoids [6]. Among these, flavonoids have garnered significant interest due to their potent antioxidant, anti-inflammatory, and immunomodulatory activities [7; 8].

Flavonoids, a class of polyphenolic compounds ubiquitously present in plants, are well-documented for their health-promoting properties. Their immunomodulatory potential arises from their ability to modulate the production of cytokines, enhance antigen-presenting cell function, influence the activity of T and B lymphocytes, and stimulate macrophage phagocytic function [9]. Specifically, flavonoids can increase nitric oxide production, stimulate the release of pro-inflammatory cytokines like TNF-α and IL-1β, and activate transcription factors such as NF-κB, all of which are critical in orchestrating an effective immune response [10].

Macrophages, key players in the innate immune system, act as the first line of defense by engulfing pathogens and presenting antigens to adaptive immune cells. Their functional activity is considered a reliable indicator of innate immune responsiveness. The carbon clearance assay is a well-established method to evaluate macrophage phagocytic activity in vivo. It is based on the principle that carbon particles injected intravenously are removed from circulation by the reticuloendothelial system, primarily the liver and spleen macrophages [11]. The rate of carbon clearance is directly proportional to the activity of these phagocytic cells and thus serves as a quantitative measure of immunomodulation.

The present study focuses on the in vivo evaluation of the immunomodulatory potential of a flavonoid-rich fraction isolated from the alcoholic extract of *Nerium oleander* leaves

(l-NO-FF-ALC) using the carbon clearance method in Swiss albino mice. Cyclophosphamide, a well-known immunosuppressive chemotherapeutic agent, was used to induce immunosuppression in mice, while Immunosin served as the positive immunostimulant control. The primary objective was to assess whether the flavonoid-rich fraction could restore or enhance macrophage phagocytic activity in immunocompromised animals. Given the increasing interest in phytoconstituents as complementary therapies in immune-related disorders, the findings from this study may contribute to the development of novel herbal immunotherapeutics, especially in the context of viral infections and immunodeficiency syndromes such as COVID-19.

This research is also aligned with the broader goal of integrating traditional knowledge with modern pharmacological validation to identify safer, effective, and affordable alternatives to conventional immunomodulators. The promising immunopharmacological attributes of *N. oleander*, particularly its flavonoid-rich components, make it a compelling candidate for further exploration. Future research directions may include bioassay-guided isolation of specific flavonoids, mechanistic studies on cytokine regulation, and in vitro evaluations using immune cell lines to elucidate the underlying pathways of action.

2. Materials and Methods

2.1. Preparation of Plant Extracts

Fresh, mature leaves of *Nerium oleander* L. were collected from the botanical garden of [Institution/Region], authenticated by a botanist, and voucher specimens were deposited in the herbarium. The leaves were shade-dried for 7–10 days to prevent degradation of thermolabile phytoconstituents, then ground into a coarse powder using a mechanical grinder. Successive solvent extraction was performed using Soxhlet apparatus with solvents of increasing polarity—petroleum ether, chloroform, and ethanol [12]. The ethanol extract was selected for its high flavonoid content and subjected to liquid-liquid partitioning using ethyl acetate and water, followed by precipitation using chilled ethanol to isolate the flavonoid-rich fraction (l-NO-FF-ALC) [13]. The obtained fraction was concentrated under reduced pressure and stored at −20°C in amber-colored bottles until further use to preserve phytochemical integrity.

2.2. Animals

Healthy adult Swiss albino mice (20–25 g) of either sex were procured from the animal facility of School of Pharmacy, Chouksey Engineering College, Bilaspur, Chhattisgarh, India. Animals were housed in polypropylene cages under standard laboratory conditions (temperature 22 ± 2°C, humidity 55 ± 10%, 12 h light/dark cycle), with ad libitum access to a standard pellet diet and water. The study was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and was approved by the Institutional Animal Ethics Committee (IAEC) under protocol no. SOP/IAEC/2024/11/06 [14].

2.3. Experimental Design

Mice were randomly assigned into five groups with five animals each (n = 5):

Group I: Normal control (received normal saline, 10 mL/kg, orally)

Group II: Cyclophosphamide control (25 mg/kg, intraperitoneal) to induce immunosuppression (Wagner & Proksch, 1999)

Group III: Standard drug group (Immunosin, 50 mg/kg, orally), a known immunostimulant

Group IV: l-NO-FF-ALC at 100 mg/kg, orally

Group V: l-NO-FF-ALC at 250 mg/kg, orally

Cyclophosphamide was administered once daily for three consecutive days to induce immunosuppression, while the test and standard groups received treatment for five days prior to the carbon clearance assay.

2.4. Carbon Clearance Assay:

The carbon clearance test was conducted to evaluate the phagocytic activity of the mononuclear phagocyte system, particularly hepatic and splenic macrophages [11]. Mice received an intravenous injection of India ink (10 µL/g body weight) via the tail vein. Blood samples (20 µL) were collected retro-orbitally at 5 and 15 minutes post-injection and immediately lysed with 2 mL of 0.1% sodium carbonate solution. The absorbance of each lysed sample was measured spectrophotometrically at 675 nm using a UV-Vis spectrophotometer [9]. The phagocytic index (K), representing the rate of carbon particle clearance from the bloodstream, was calculated using the following formula:

$$K = [\log_e(OD1) - \log_e(OD2)] / (t2 - t1)$$

Where OD1 and OD2 are the optical densities of blood samples at 5 and 15 minutes, and t2 – t1 is the time interval between the samples. A higher K value indicates increased phagocytic activity.

3. Results and Discussions

3.1. Macrophage Activity via Carbon Clearance Method

The results of the carbon clearance assay are presented in Table 3.1 and Figure 3.1. The normal control group (Group I) exhibited a baseline phagocytic index (K) of 0.0575 ± 0.0005, reflecting normal macrophage activity. In contrast, the cyclophosphamide-treated group (Group II) showed a significant suppression of phagocytic activity, with a markedly reduced K value of 0.0332 ± 0.0002 ($p < 0.0001$ vs. Group I), confirming the immunosuppressive effect of cyclophosphamide, a known alkylating agent that impairs immune cell proliferation and function.

Administration of the standard immunostimulant Immunosin (Group III) significantly reversed the suppression, increasing the K value to 0.0616 ± 0.0004 ($p < 0.0001$ vs. Group II). This result supports its efficacy as a positive control in modulating immune responses.

Notably, the groups treated with the flavonoid-rich fraction of *Nerium oleander* leaf ethanolic extract (l-NO-FF-ALC) exhibited a dose-dependent restoration of macrophage activity. At 100 mg/kg (Group IV), the phagocytic index was significantly increased to 0.0568 ± 0.0003, while the 250 mg/kg dose (Group V) further elevated the index to 0.0604 ± 0.0003 (both $p < 0.0001$ vs. Group II). These results indicate that l-NO-FF-ALC effectively restored and enhanced phagocytic function in immunocompromised animals.

Table No. 3.1 Macrophage Activity via Carbon Clearance Method

Group	Treatment	Phagocytic Index (K)
I	Normal Saline	0.0575 ± 0.0005
II	Cyclophosphamide (25 mg/kg)	0.0332 ± 0.0002 a****
III	Immunosin (50 mg/kg)	0.0616 ± 0.0004 b****
IV	l-NO-FF-ALC (100 mg/kg)	0.0568 ± 0.0003 b****
V	l-NO-FF-ALC (250 mg/kg)	0.0604 ± 0.0003 b****

Note: a = comparison with Group I, b = comparison with Group II, **** = p < 0.0001; Values are mean ± SD, n = 5.

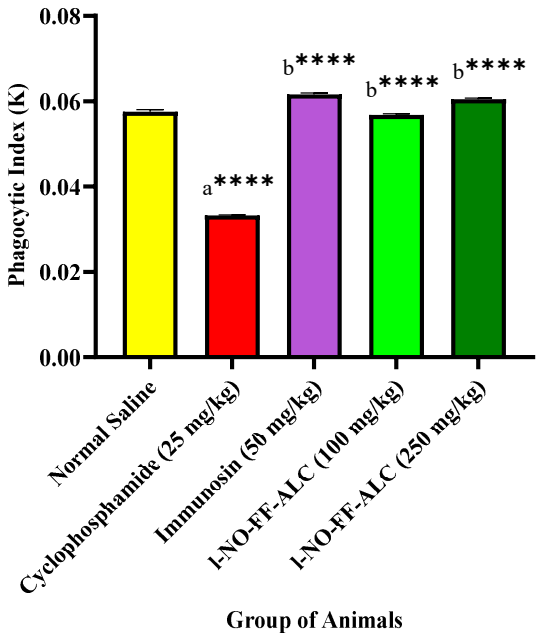

Fig. No. 3.1 Macrophage Activity via Carbon Clearance Method

The carbon clearance test is a well-established method for evaluating the activity of the mononuclear phagocyte system, specifically the ability of hepatic and splenic macrophages to clear colloidal carbon from circulation. In this study, the assay confirmed the immunosuppressive effect of cyclophosphamide and the restorative potential of Immunosin and l-NO-FF-ALC.

The immunostimulatory action of l-NO-FF-ALC may be attributed to its rich content of flavonoids. Flavonoids have been widely recognized for their ability to modulate innate and adaptive immunity by promoting macrophage activation, enhancing antigen presentation, and stimulating cytokine release such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Additionally, their antioxidant properties may contribute to cellular protection and enhanced phagocytic function by mitigating oxidative stress.

The observed dose-dependent increase in phagocytic activity suggests that higher concentrations of flavonoids provide more effective stimulation of the reticuloendothelial system. The 250 mg/kg dose of l-NO-FF-ALC demonstrated comparable efficacy to the standard drug Immunosin, highlighting its therapeutic potential as a natural immunomodulatory agent.

These findings are consistent with earlier reports on the immunopharmacological activities of flavonoid-containing plant extracts and align with the traditional uses of *Nerium oleander* in ethnomedicine for immune-related conditions.

4. Conclusions

The present study demonstrates that the flavonoid-rich fraction derived from the alcoholic extract of *Nerium oleander* leaves (l-NO-FF-ALC) possesses potent immunostimulatory properties, as evidenced by its ability to significantly enhance macrophage-mediated phagocytosis in cyclophosphamide-induced immunosuppressed mice using the carbon clearance assay. The observed dose-dependent increase in the phagocytic index suggests a robust activation of the mononuclear phagocyte system, indicating the extract's efficacy in restoring innate immune function.

These findings provide scientific support for the traditional use of *Nerium oleander* in immunomodulation and highlight the therapeutic potential of plant-derived flavonoids in developing adjunct treatments for immune deficiency and immunosuppression. However, while the results are promising, further research is warranted to isolate and characterize the specific bioactive flavonoid constituents responsible for the observed effects. Advanced molecular and cellular studies, including cytokine profiling, receptor interaction assays, and gene expression analyses, will be essential to elucidate the underlying immunomodulatory mechanisms and ensure safety and efficacy in future clinical applications.

In conclusion, the flavonoid-rich extract of *Nerium oleander* leaves holds potential as a natural, plant-based immunomodulatory agent, meriting further investigation and development for pharmaceutical or nutraceutical use.

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