



Evaluation of immunomodulatory activity of *Grewia asiatica* in laboratory animals

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ABSTRACT

Aim of the current study was to investigate the immunomodulatory potential of *Grewia asiatica* extract on cyclophosphamide induced myelosuppression, forced swimming test and carbon clearance test in Swiss albino mice. The assessment of immunomodulatory activity was studied by cyclophosphamide induced myelosuppression, forced swimming test and carbon clearance test. Cyclophosphamide (30 mg/kg, i.p.) was used to induced immunosuppression in mice, levamisole (50 mg/kg, p.o.) and ashwagandha (100 mg/kg, p.o.) used as standard immunostimulating agents. The ethanolic extract of *Grewia asiatica* (GAEE) fruits was administered orally at two dose levels of 200 and 400 mg/kg body weight in mice. Ethanolic extract of *Grewia asiatica* showed significant counteracting effect to cyclophosphamide-induced reduction in total WBC, % neutrophil and haemoglobin levels. Furthermore, after daily oral administration of *Grewia asiatica*, immobility time was decreased significantly in the *Grewia asiatica* administered group (200 and 400 mg/kg) on day 10. On the other hand, animals treated with GAEE, showed increase in the phagocytic index in carbon clearance assay. The present study clearly indicated immunostimulant property of GAEE and lends support for further development of new immunostimulant drug.

Key words: *Grewia asiatica*, cyclophosphamide, *Withania somnifera*, immunomodulatory.

INTRODUCTION

The immune system is a remarkably versatile defence system that has evolved to protect animals from invading pathogenic microorganisms and to eliminate disease. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders [1].

Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' is based on related principles. Rasayana, listed as a class in the texts of traditional Indian medicine literature, consists of a number of plants reputed to promote physical and mental health, improve defence mechanisms of the body and enhance longevity. Besides, a number of medicinal plants as Rasayanas have been claimed to possess immunomodulatory activities. Some of the Rasayana drugs as immunomodulatory agents are *Withania somnifera*, *Tinospora cordifolia*, *Asparagus racemosus* and *Mangifera indica* are well known for their traditional uses [2].

The concept of immunomodulation relates to a non-specific activation of the immune system. It implies primarily a non-antigen dependent stimulation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells, lymphocytes and also the production of various effector molecules by activated cells (Para-immunity). Being non-specific, it is expected to give protection against different pathogens including bacteria, fungi, viruses etc. and constitutes an alternative or adjunct to conventional chemotherapy [3].

Grewia asiatica L. belongs to the family Tiliaceae and is of commercial importance. It is a deciduous bushy plant and thrives best in the tropical climate [4]. In India this plant grows satisfactory and produces well up to an elevation

of 1,000 m and adequate sunlight and warm or hot temperature are required for fruit ripening, development of appropriate fruit colour, good eating and proper medicinal quality. Summer season is best for fruit quality and therefore *Grewia asiatica* fruits were collected in a very ripening state [5].

The fruit of *Grewia asiatica* is astringent and cooling. Infusion of bark is demulcent while leaves are used in postular eruptions. Its root bark is used as a remedy for rheumatism. 50% Ethanol extract of aerial parts of *Grewia asiatica* showed hypotensive activity while the aqueous extract of stem bark is reported to be antidiabetic. Its seed extract and seed oil exhibited antifertility activity. Fruit Extract of *Grewia asiatica* shows radioprotective effect. The fruit is astringent and stomachic. It is reported that unripe phalsa (*Grewia asiatica*) fruit alleviates inflammation and is administered in respiratory, cardiac and blood disorders, as well as in fever reduction. The antioxidant properties of vitamin C are well known and anthocyanin has recently emerged as a powerful antioxidant [6].

The fruit is like a berry and has a sweet and sour acidic taste. The fruits are claimed to be beneficial for heart, blood and liver disorders, anorexia, indigestion, thirst, toxemia, stomatitis, hiccough, asthma, spermatorrhoea, fevers and diarrhoea and are used for treating throat, tuberculosis and sexual debility troubles. *Grewia asiatica* fruits are a rich source of nutrients such as proteins, amino acids, vitamins and minerals and contain various bioactive compounds, like anthocyanins, tannins, phenolics and flavonoids [7].

Despite many therapeutic effects, so far no data is available on the immunomodulatory effects of *Grewia asiatica*. In this context, the objective of the present study was to scrutinize the immunomodulatory potential of fruits of *Grewia asiatica* in experimental animals.

EXPERIMENTAL SECTION

Plant material

The fruits of *Grewia asiatica* were collected from Lucknow, during the month of May. The plant material was authenticated by NISCAIR, Delhi.

Extraction of plant material

Fresh fruits of *Grewia asiatica* were collected locally in summer season, were washed, shade dried and powdered. Then *Grewia asiatica* powder was extracted in ethanol by soxhlet apparatus for 3 days. Then the extract was obtained [8].

Experimental animals

Swiss albino mice weighing 25-30g of either sex were used in the study after approval of the Institutional Animal Ethics Committee (BBDNIIT/IEAC/027/2014). They were maintained under standard laboratory condition at ambient temperature of $25\pm 2^{\circ}\text{C}$ and $50\pm 15\%$ relative humidity with a natural light dark cycle. Animals were fed with a standard pellet diet and water *ad libitum*.

Drugs

Cyclophosphamide 30 mg/kg.b.w. (Cadila Healthcare Limited, Daman, U.T.), Ashwagandha 100 mg/kg.b.w. (Divya Pharmacy, Uttarakhand), Levamisole 50 mg/kg.b.w. (Khandelwal Laboratories Pvt. Ltd., Mumbai).

Cyclophosphamide induced myelosuppression

Animals were divided in 4 groups on randomized basis and 6 animals were taken in each group. The drugs were administered as shown below:

Group I - control group (1% sodium CMC vehicle for 13 days).

Group II (Cyclophosphamide group) – 1% sodium CMC vehicle for a period of 13 days and cyclophosphamide (30 mg/kg, i.p.) injection on 11th, 12th and 13th days.

Group III and IV- Ethanol extract of fruits of the plant daily for 13 days (200 and 400 mg/kg, p.o. respectively).

The groups III and IV were injected with cyclophosphamide (30 mg/kg, i.p.) on the 11th, 12th, and 13th days, 1 hr after the last administration of the respective oral treatment. Blood samples were collected on 14th day of experiment by retro-orbital puncture and haematological parameters were studied for haemoglobin levels, total WBC and % neutrophil [9].

Forced swimming test

Briefly, a mouse was placed in a container 40 cm high and 18 cm in diameter containing 20 cm of water at 25°C and forced to swim for 6 min. Immobility duration was recorded. In the study, GAEE (200 and 400 mg/kg, p.o.) and

ashwagandha (100 mg/kg, p.o.) [10] were administered daily for 10 days and last treatments were administered 1 hr before behavioural tests. Control animals were treated with 1% sodium CMC vehicle [11].

Carbon Clearance Assay

Animals were divided in 4 groups on randomized basis and 6 animals were taken in each group. Group 1 was treated with vehicle, group 2 was treated with standard drug levamisole [12] and group 3rd and 4th was treated with test drug for 5 days. After 48 hr of the last dose of the drug, mice was injected with 0.1 ml of Indian ink via the tail vein. Blood samples were withdrawn at 0 min and 15 min after injection of Indian ink. A 50 μ l blood sample was mixed with 4 ml of 0.1% sodium carbonate solution. The absorbance of this solution was determined at 660 nm. The phagocytic index was calculated.

$$K = (\text{Log}_e \text{OD1} - \text{Log}_e \text{OD2})/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively [13].

Statistical analysis

Experimental results were expressed as means \pm SEM. The data were analyzed by an analysis of variance, one way ANOVA followed by Tukey post test.

RESULTS

Cyclophosphamide induced myelosuppression

Cyclophosphamide at the dose of 30 mg/kg. i.p. caused a significant reduction in total WBC count, Hb and % neutrophil as compared to control group. Ethanolic extracts of fruit showed highly significant ($P < 0.001$) increase in total WBC, Hb and % neutrophil when compared with cyclophosphamide group (Table 1).

Table 1. Effect of *Grewia asiatica* fruits on WBC, Hb and % neutrophil

S.N.	Treatment	WBC Count (X 1000/mm ³)	Hb (g/dl)	% Neutrophil
1.	1% Sodium CMC suspension	11.56 \pm 0.13	12.55 \pm 0.08	24.50 \pm 0.31
2.	1% Sodium CMC suspension +Cyclophosphamide	8.97 \pm 0.12 ^{###}	9.10 \pm 0.12 ^{###}	16.17 \pm 0.25 ^{###}
3.	GAE (Test 1)+Cyclophosphamide	11.95 \pm 0.07 ^{***}	12.85 \pm 0.09 ^{***}	22.50 \pm 0.31 ^{***}
4.	GAE (Test 2)+ Cyclophosphamide	12.78 \pm 0.10 ^{***}	13.27 \pm 0.09 ^{***}	24.80 \pm 0.19 ^{***}

Results expressed as mean \pm SEM, (n=6) ^{***} $P < 0.001$, test groups were compared to cyclophosphamide treated group, ^{###} $P < 0.001$, cyclophosphamide treated group was compared to control group by one way ANOVA followed by Tukey test.

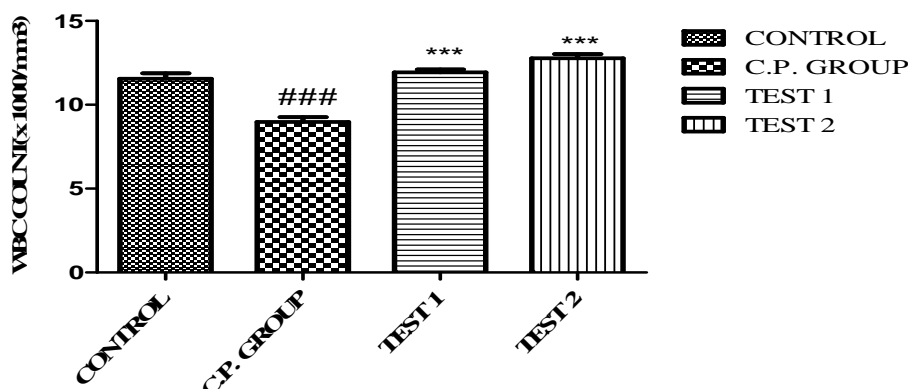
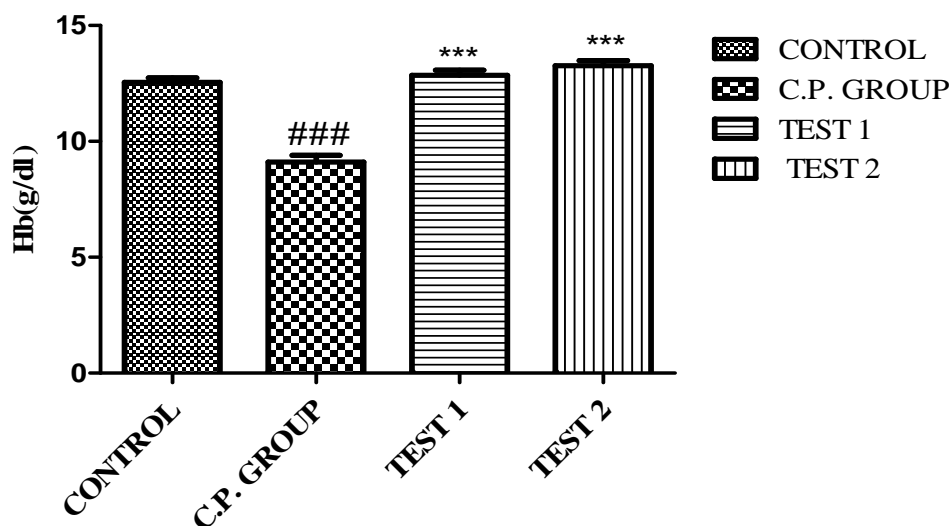
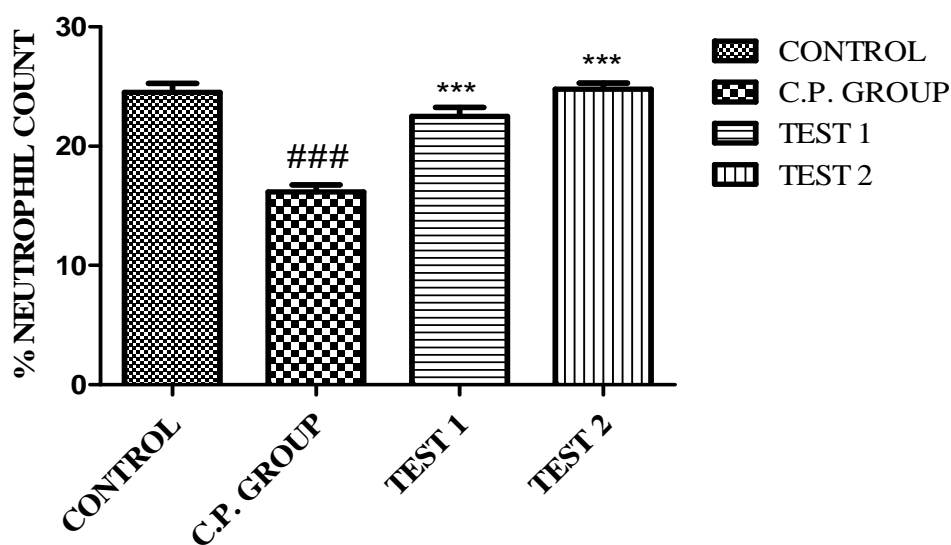


Figure1: Effect of *Grewia asiatica* fruits on WBC count

Forced swimming test

The measurement of immobility was performed 10 days after for all the treatment groups. Immobility time was decreased in the *Grewia asiatica* treated group in a dose dependent manner when compared with control group. Immobility time was also decreased in standard group when compared with control group.

Figure 2. Effect of *Grewia asiatica* fruits on HaemoglobinFigure 3. Effect of *Grewia asiatica* fruits on % NeutrophilTable 2. Effect of *Grewia asiatica* fruits on immobility time

S.N.	Treatment	Immobility time (sec)
1.	1% Sodium CMC suspension	171.50±1.29
2.	Standard (Ashwagandha)	118.17±1.09***
3.	GAE (Test 1)	135.67±0.74***
4.	GAE (Test 2)	133.50±1.32***

Results expressed as mean±SEM, (n=6) ***P<0.001, groups were compared to control group by one way ANOVA followed by Tukey test.

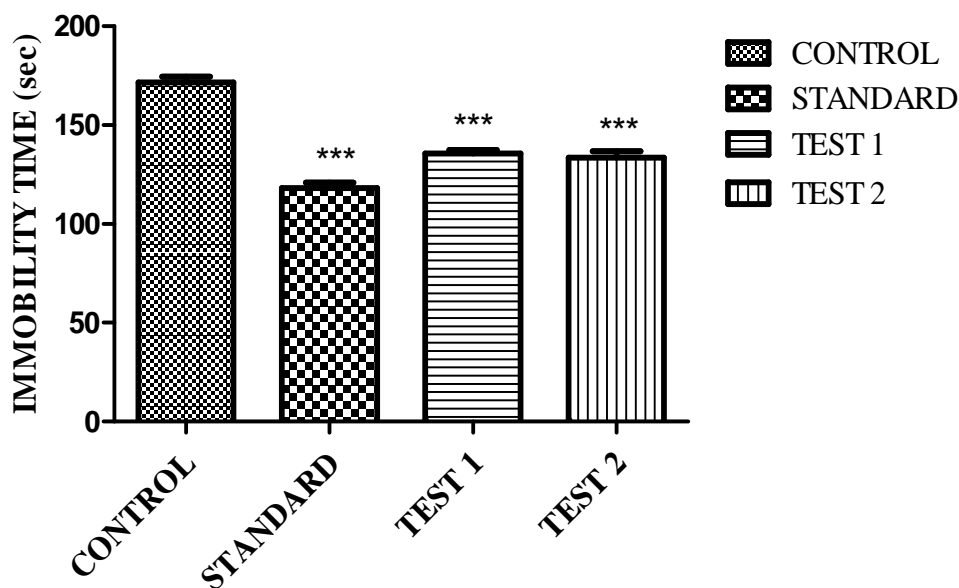


Figure 4. Effect of fruits of *Grewia asiatica* on immobility time

Carbon clearance test

The faster removal of carbon particles had been correlated with the enhanced phagocytic activity. The phagocytic activity of the reticulo-endothelium system was measured by the removal of carbon particles from the blood circulation. The oral administration of *Grewia asiatica* (200 and 400 mg/kg) for 5 days exhibited a dose-related increase in the clearance rate of carbon by the cells of the RES. *Grewia asiatica* showed phagocytic index as 0.0568 ± 0.0141 and 0.0739 ± 0.0087 with doses of 200 and 400 mg/kg., respectively. The phagocytic index of control (group I) was 0.0228 ± 0.0055 and standard was 0.1861 ± 0.0704 .

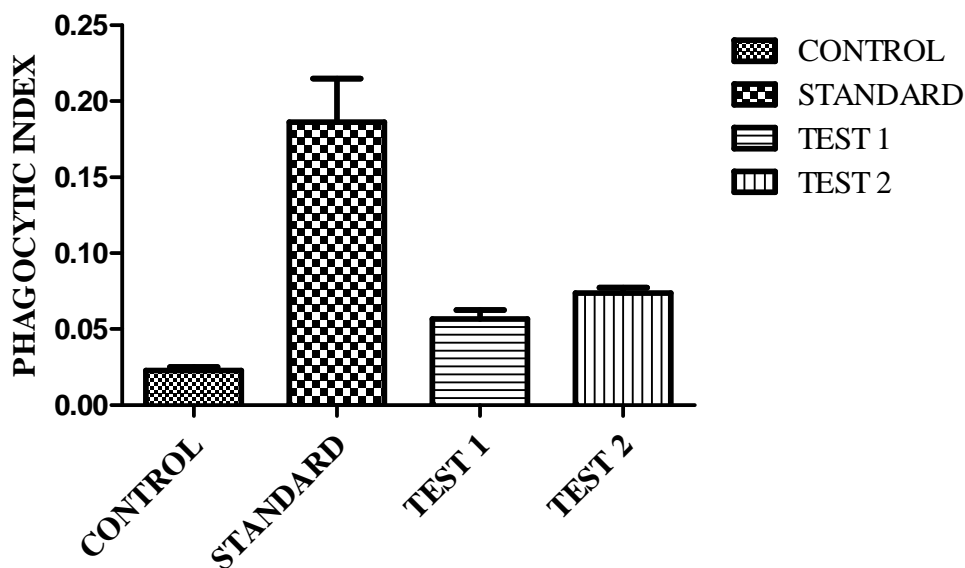


Figure 5. Effect of *Grewia asiatica* fruits on phagocytic index

Table 3. Effect of *Grewia asiatica* fruits on Phagocytic index

S.N.	Treatment	Phagocytic index
1.	1% Sodium CMC suspension	0.0228±0.0055
2.	Standard (Levamisole)	0.1861±0.0704
3.	GAEE (Test 1)	0.0568±0.0141
4.	GAEE (Test 2)	0.0739±0.0087

Results expressed as mean±SEM, (n=6) statistical analysis was done by one way ANOVA followed by Tukey test.

DISCUSSION

The results of scientific evidences obtained in this study support the traditional claim of *Grewia asiatica* for medicinal purposes. In the present study, the immunomodulatory activities of ethanolic fruit extract of *Grewia asiatica*, an important plant of indigenous system of Indian medicine were explored.

A high degree of cell proliferation renders the bone marrow was a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leucopenia. In the cyclophosphamide-induced myelosuppression assay, cyclophosphamide, a known antineoplastic agent of the alkylation class suppresses T-lymphocyte activity, leading to a suppressed immune function. It is used experimentally to mimic an immunodeficient condition. The *Grewia asiatica* on administration to immune compromised mice promoted leukocyte production when compared to the CP control group that received only the immune suppressant (cyclophosphamide) drug. GAEE significantly ameliorated the WBC count, neutrophils count and haemoglobin and also restored the myelosuppressive effects induced by cyclophosphamide [14].

In case of forced swimming test (FST), forced swimming involves physical exercise and psychological stress. Rodents, when forced to swim in a restricted space become immobile after an initial period of vigorous activity. The increased swimming time has been observed in mice, pre-treated with *Grewia asiatica* with enhanced physical performance significantly longer than untreated (control) group and thus confirming its immunostimulant nature. Increased swim duration in mice pretreated with *Grewia asiatica* are similar to the changes produced by reference drug *Withania somnifera* [15].

It has been reported that the FST exposure caused immune deregulation and a potent psychophysiological stressor that alters physiological endocrine and immune function. Furthermore, the relation between immune function and immobility time has also reported [16].

Based, on these data, forced swimming test has been used as an immune-enhancement test in many studies [17]. In the present study it was found that the immobility time during the forced swimming test was shortened by oral administration of the GAEE for 10 consecutive days.

The use of *Withania somnifera* (WS) as a general tonic to increase energy and prevent disease may be partially related to its effect on the immune system. Glycowithanolides and a mixture of sitoindosides IX and X isolated from WS were evaluated for their immunomodulatory and central nervous system effects (antistress, memory, and learning) in Swiss mice (15-25 g, 5-6 months old) and Wistar strain albino rats (120-150 g and 250-300 g). Both materials produced statistically significant mobilization and activation of peritoneal macrophages, phagocytosis, and increased activity of the lysosomal enzymes [18].

Phagocytosis is the process by which certain body cells, collectively known as phagocytes, ingest and remove microorganisms, malignant cells, inorganic particles and tissue debris [19]. The carbon clearance test was done to evaluate the effect of drugs on the reticuloendothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation. Since both doses of GAEE as well as levamisole showed remarkable augmentation in the phagocytic index, it is speculated that it might be due to increase in the activity of the reticuloendothelial system by prior treatment of animals with GAEE and levamisole [20].

CONCLUSION

On the whole, the results of this study on the *Grewia asiatica* fruits indicated that fruits hold immunostimulatory activity and may provide a scope for further detailed investigation on the fractions/individual constituents responsible for immunostimulant activity.

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