doi.org/10.36721/PJPS.2020.33.5.SUP.2269-2273.1

Hematopoietic effects of *Azadirachta indica* methanolic extract in cyclophosphamide mediated myelosuppressed albino rat

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Abstract: Myelosuppression or bone marrow suppression is one of the most common side effects caused by anti-cancer drugs. Certain nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics and viruses like B19 virus can also cause bone marrow suppression resulting in serious consequences like leukopenia, anemia and thrombocytopenia. Currently, it is mainly treated by Filgrastim, use of which is not without side effects. Certain natural drugs can be a safer alternative to treat myelosuppression. *Azadirachta indica*, commonly known as Neem, is an important medicinal plant of subcontinent. Keeping in view the traditional uses of Neem, present study aims to investigate its potential role in reversing myelosuppression. Albino rats were used to determine hematopoietic activity of Neem leaves after inducing myelosuppression by cyclophosphamide given subcutaneously. Filgrastim was used as reference standard to compare the antimyelosuppressant activity of the drug. The drug was evaluated in three doses i.e. 50mg/kg, 100mg/kg and 200mg/kg body weight, while blood samples were drawn on 0, 1st, 7th, 14th and 21st day. The drug was found to be effective in reversing bone marrow suppression in all three doses based on the hematological parameters (mean WBC, RBC, platelets, Hb, Hct etc.) which improved significantly. The results suggest that the drug can be used as antimyelosuppressant after establishing its safety and identifying its active constituents with their mechanism of action.

Keywords: *Azadirachta indica*, B19 virus, cyclophosphamide, filgrastim, myelosuppression, myelotoxicity.

INTRODUCTION

Myelosuppression or bone marrow suppression is a life threatening disorder resulting in decreased immunity (due to leukopenia), anemia (due to decreased RBCs) and poor blood clotting (due to thrombocytopenia). Myelo-suppression is frequently caused by cytotoxic chemotherapy. It is also associated with the use of other drugs like NSAIDs, antibiotics and viral infections e.g. by B19 virus (El-Radhi, 2018). Myelosuppression or myelotoxicity often requires dose adjustment of chemotherapeutic agents which, in turn, can cause a decrease in their response against cancer. Treatment of more serious cases of myelosuppression, however, may involve drug withdrawal, bone marrow transplantation or use of hematopoietic agent like Filgrastim (also known as granulocyte colony stimulating factor) which stimulate production of white blood cells (Moore *et al*., 1997). There are not many drugs that could treat myelosuppression so there is a need to develop safe and effective anti-myelosuppressants (Larson, 2002). This idea led us to investigate Neem tree for this purpose.

With advancements in the medical sciences, the significance of phytomedicines has been increased dramatically (Phillipson, 2001). Though various medicinal plants have gained importance in treating diseases such as anemia, diabetes and malaria, however, the medicinal properties of these plants are yet to be explored to their full potential (Gurib-Fakim, 2006; Padayachee & Baijnath, 2012).

Neem (*Melia azadiracchta* or *Azadirachta indica*) belongs to family Meliaceae (Pimple, Badole, & Menaa, 2013). Neem is an important medicinal plant of subcontinent used to treat various ailments (Rajasekaran, 2008). Almost all parts of the plant have some medicinal use. Leaves have antibacterial, antiviral, anti-allergic, hypoglycemic, hepatoprotective, hypolipodemic and antipyretic uses. Neem oil, obtained from fruit and seeds has insecticidal and insect repellent properties (Lokanadhan, Muthukrishnan, & Jeyaraman, 2012).

Different biologically active compounds have been isolated from different parts of the plant and their pharmacological properties have been determined e.g. azadirachtin, nimbin. Nimbidine, limonoids, azadirone and flavonoids (Sarah, Tabassum, Idrees, & Hussain, 2019).

Although lots of research work has been reported on Neem, yet not much work has been reported on its hematopoietic potential. Hence, the present study aims to evaluate the hematopoietic effects of the methanolic extract of neem leaves by inducing myelosuppression in white albino rats.

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MATERIALS AND METHODS

Collection of plant material

500g fresh leaves were collected from botanical garden of Government College University, Lahore, Pakistan. Plant material was authenticated by Prof. Dr. Zaheerud Din, Government College University, Lahore and were placed in herbarium vide voucher specimen number GC-Herb-Bot-3238 for *Azadirachta indica*. Afterwards, the collected material was washed and dried in shade for a period of 10-12 days.

Preparation of extract

The powdered material 150 g was soaked in methanol at room temperature for 48 hrs. The process was repeated thrice until the residual material was exhaustively extracted. The extract, so obtained was dried by using rotary evaporator (Heidolph, Germany) under reduced pressure at 50 ± 1°C to get a thick semi-solid residue. Required doses of 50 mg/kg, 100 mg/kg and 200 mg/kg were prepared by diluting the extract with distilled water. The doses were administered by an oral gavage (Lopes, Calvo, Vilegas, & Carlos, 2005).

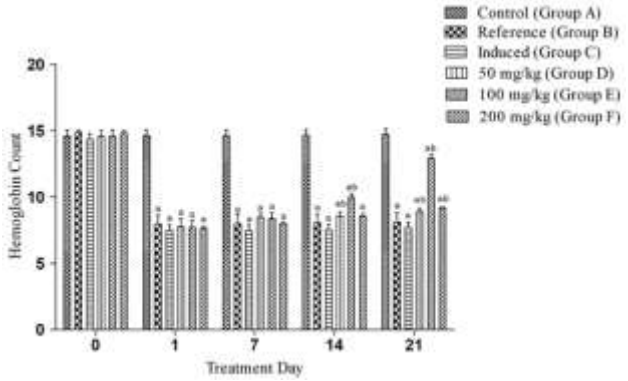

Fig. 1: Hemoglobin count (g /dL) in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

Animals

Normal, healthy and adult male albino rats weighing about 250-300 g, purchased from the University of Lahore, were included in this study. They were housed in plastic cages in the animal house at a temperature range of 25-30˚C and 50-55% relative humidity. The experimental animals were fed with laboratory grade food pellets and water *ad libitum*. The study was conducted with the ethical approval of the Research, Ethics and Higher Degrees Committee, of the UCP, Lahore. Animals were handled and cared in accordance with the WHO guidelines.

Experimental design

A total of 30 Albino rats were divided into 06 groups with 5 rats in each group. Baseline values of all hematological parameters were obtained before starting the experiment and myelosuppression was induced in 5 groups, B, C, D, E and F (n=25) by subcutaneous administration of cyclophosphamide in a dose of 50mg/kg body weight daily for 3 succeeding days (Patil, Shetty, Bhide, & Narayanan, 2013). Group A (control) was given rat food and water *ad libitum*, Group B (toxicant/negative control group) kept untreated, Group C (reference/positive control group) was treated with reference drug, namely filgrastim (5µg/kg/day subcutaneously) for 5 consecutive days (Sheng, Pero, & Wagner, 2000), Groups D, E and F received 50 mg/kg, 100 mg/kg and 200 mg/kg of dried methanolic extract for 21 days, respectively (Okpanyi & Ezeukwu, 1981). The doses were administered orally with an oral gavage.

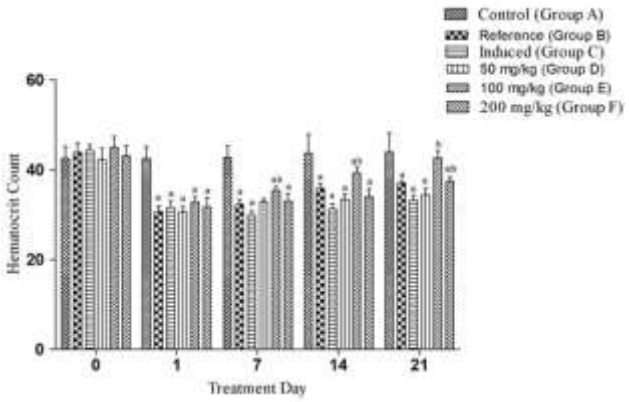

Fig. 2: Hematocrit percentage in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

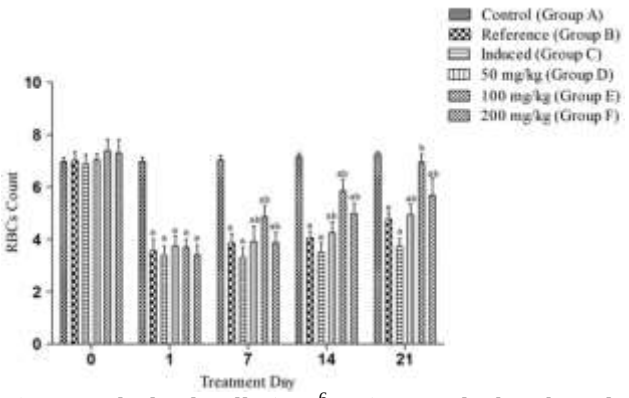

Fig. 3: Red Blood Cells ($\times 10^6$ /µL) in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B,

C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

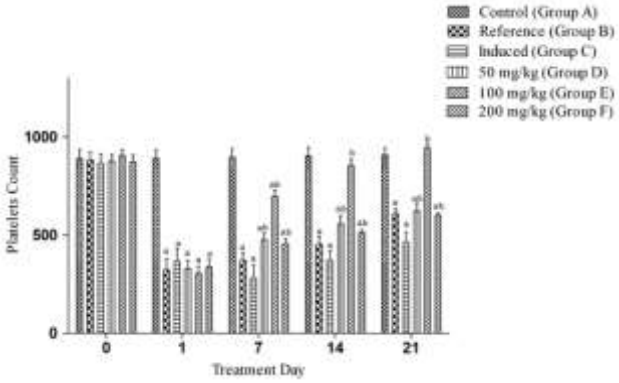

Fig. 4: Platelets levels ($\times 10^3$ /µL) in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

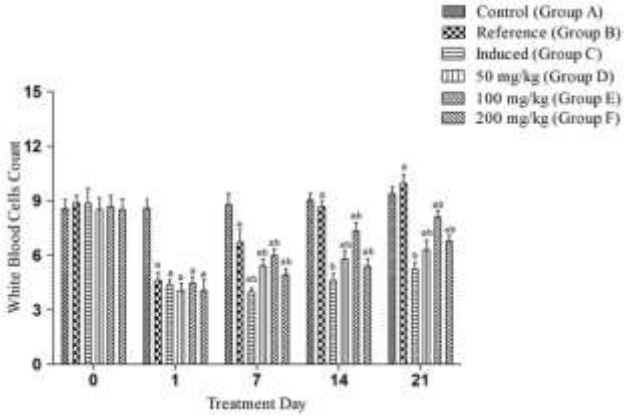

Fig. 5: White blood cells ($\times 10^3$/µL) count in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

Blood sample collection

Blood samples of 0.5-1 ml were drawn from each rat via the left ventricular cardiac puncture (Paulose & Dakshinamurti, 1987; Yoburn, Morales, & Inturrisi, 1984) under light ether anesthesia (Van Herck *et al*., 1991) on day 0 (before induction of myelosuppression with cyclophosphamide), 1st (after 3rd dose of cyclophosphamide), then on 7th, 14th, 21st day. The samples were collected using 3 ml syringe and EDTA tube and run in a hematology analyzer (Sysmex XP-300, America, Inc.) for cell counts of blood parameters.

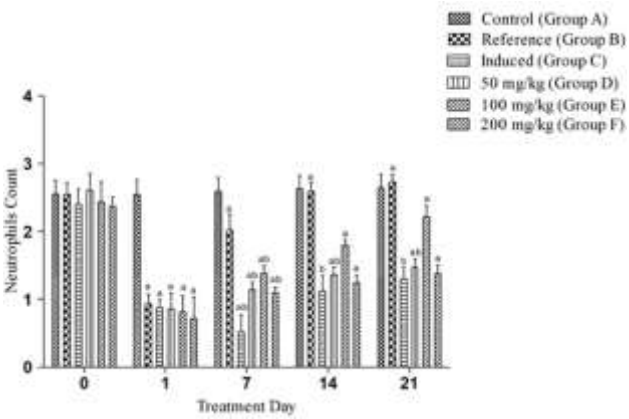

Fig. 6: Neutrophils count ($\times 10^3$/µL) count in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

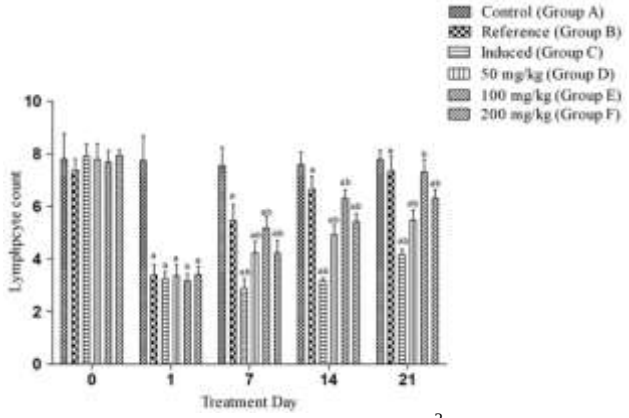

Fig. 7: Lymphocytes count ($\times 10^3$/µL) count in cyclophosphamide induced myelosuppressed rats (n=5). Data in bars are shown as Mean ± SEM. Bars with same superscripts do not differ significantly whereas bars with superscripts "a" & "b" indicates that a = significant difference of Group B, C, D, E & F with Group A (control) whereas b = significant difference of group C, D, E, & F with Group B (Induced) at $p < 0.05$

STATISTICAL ANALYSIS

Statistical evaluation of data was performed by using GraphPad Prism software version 5.00. The results were presented as mean ± standard error of mean (SEM) and were compared by using one-way analysis of variance (ANOVA) among groups. Tukey's test was used for post hoc analysis and p value ≤ 0.05 was taken as significant.

RESULTS

The efficacy of methanolic extract of the leaves of *Azadirachta indica*, at dose level of 50 mg/kg, 100 mg/kg

and 200 mg/kg was determined for the recovery of cyclophosphamide induced myelosuppression by evaluating different hematological parameters. Theses parameters include white blood cells count, differential WBCs count, red blood cells count, hemoglobin concentration, hematocrit (Hct) % and platelets count of animals (N=30. On day 0 (before induction of myelosuppression), the baseline values of peripheral blood parameters of all groups were monitored and there was no significant difference ($p > 0.05$) among all groups for each blood parameter. On day 01 (after the administration of cyclophosphamide 50 mg/kg s.c.) the levels of all hematological parameters decreased significantly as compared to observed levels in the control group. There was no marked variation among all induced groups for these parameters.

On day 07 and 14, Group C revealed no significant difference in mean hemoglobin (8.0±0.30), hematocrit (32.2±0.51 and 35.8±0.47 resp.), red blood cells (3.8±0.16 & 4.1±1.1 resp.) and platelets count (368.4±18.43 & 452.4±17.45), however, it showed significant improvement in WBCs (5.5±0.27 and 6.7±0.22), lymphocytes (2.8±0.16 and 3.1±0.05) and neutrophils counts (2.0±0.10 and 2.5±0.05) while other groups; D, E and F showed significant improvement in hematocrit, red blood cells, platelets, lymphocytes, WBCs and neutrophils counts as compared to the Group B as shown in fig. 1, 2, 3, 4, 5, 6 and 7. Mean values of all hematological parameters did not reach to their normal level and were significantly lowered than that of their values in Group A. On day 21 Group C showed no significant difference in mean hemoglobin and hematocrit count, however, showed significant improvement in RBCs, platelets, WBCs, lymphocytes and neutrophils counts while groups D, E and F showed significant improvement ($p < 0.05$) in Hct, RBCs, platelets, lymphocytes, WBCs and neutrophils counts as compared to the Group B. Hct and RBCs counts in Group D (50 mg/kg dose level) and Group E (100 mg/kg dose level) were normalized and revealed no significant variation with the control group.

DISCUSSIONS

The present study was designed to investigate effects of three different doses of methanolic extract of neem leaves in myelosuppressed rats. It demonstrated varying degree of changes in hematological parameters e.g., mean RBC, Hb concentrations, Hct, WBC, neutrophils, lymphocytes and platelets among myelosuppressed rats at the dose level of 50 mg/kg, 100 mg/kg and 200 mg/kg. Literature survey has revealed that ingestion of medicinal compounds or drugs through oral route can cause variations in the blood profile (Ajagbonna, Onifade, & Suleiman, 1999; Owoyele *et al*., 2011). These variations can cause positive or negative effects. In this study, significant increase in mean hematological parameters ($p<0.05$) were observed. The significant increase in red blood cells, hemoglobin and hematocrit after oral administration of methanolic extracts of neem leaves suggests that these extracts may contain biologically active chemical compounds that trigger the formation and/or release of erythropoietin in the bone marrow stem cells of myelosuppressed rats. Erythropoietin a "glycoprotein hormone" stimulates the stem cells and expedite the production of erythrocytes in the bone marrow (Ohlsson & Aher, 2010). At dose level (100mg/kg), methanolic extract of neem caused significant recovery to normal range in erythrocyte indices when compared with the control. This is in line with the work of Neboh, who reported that methanolic leaf extract of *Cassia occidentalis* revealed significant increase in erythrocytes, hemoglobin and hematocrit in comparison to the control group in cyclophosphamide induced myelosuppresed rats (Neboh & Ufelle, 2015). This infers that the erythropoietic property of neem is limited to 100mg/kg dose. Expectedly, rise in RBC counts on administration of neem leaves different doses resulted in an increase in hemoglobin and hematocrit profiles which is linked with the total population of RBCs in the blood, it might therefore be implied that the extract may increase the biosynthesis of erythrocytes and Hb, it might also stimulate the incorporation of Hb into RBCs and as a result of this oxygen exchange improves (Adebayo, Adesokan, Olatunji, Buoro, & Soladoye, 2005). Hematopoietic activity of the extract can be due to tannins, alkaloids, saponins, terpenoids, phenols and steroids. This outcome can be inferred from the results of Agbor and Odetola (Oyagbemi, Odetola, & Azeez, 2008). The significant increase in white blood cells and the differential leukocytes counts in the test animals show that the drug may possess the immune system stimulatory ability because oral administration of the extract at 100mg/kg significantly increased the WBC, neutrophils and lymphocyte count when compared with toxicant group. This correlates with the results of Iranloye (Iranloye, 2002). The improvement in leukocytes count may be due to improvement in the rate of entrance of WBCs into the circulation from the bone marrow and a reduced rate of removal from blood. The improvement in platelets after oral use of the drug (extract) indicates that it has the potential to be used as for thrombocytopenia like other plant drugs e.g. Subenthiran and co-workers (Subenthiran *et al*., 2013) stated that leaf juice of *Carica papaya* (Linn) taken during the period of dengue infection had the property to prompt the production of platelets. These outcomes propose that in particular during chemotherapy phytochemicals like alkaloids, flavonoids and tannins present in these extracts may have stimulated the process of thrombopoiesis in myelosuppressed rats by triggering megakaryocytes to form sufficient platelets in order to maintain suitable platelets level in mammals (Tahir *et al.,* 2014).

CONCLUSION

It is concluded from the result of the present study that the methanolic extract of Neem leaves have the potential to improve the hematological parameters and can be used as supportive treatment in hematological abnormalities. However, further studies are necessary to determine the safety profile and active constituents of the drug.

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