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Screening of phytochemicals and bioactive compounds in *Punica granatum* peel and evaluation of haematological alterations in STZ induced diabetic male albino rats

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Abstract
Pomegranate is a curative plant from ancient times which belongs to punicaceae and Lythraceae family. It has been used for the treatment of various health issues such as cancer, diabetes, inflammation, dental plaque, dysentery and intestinal infections. This plant develops as small trees or shrubs in several countries of Iran, Iraq, India, Afghanistan, Pakistan, Russia and Mediterranean area which is an important source of many bioactive compounds. The present study was undertaken to screen phytochemicals and bioactive compounds in *Punica granatum* peel and to evaluate the bodyweight and haematological alterations in STZ induced diabetic male albino rats. The study included five treatment groups comprising of ten albino Wistar rats each. The various groups in this study included normal control (Group I), diabetic control (Group II), diabetic rats treated with metformin at the rate of 500 mg/kg bw, (Group III), diabetic rats treated with pomegranate fresh juice at 100% dosage (Group IV) and diabetic rats treated with pomegranate peel extract at 100% dosage (Group V). It was observed that the potential phytochemicals present in hydroalcoholic extract were saponins, tannins, flavonoids, terpenoids, cardiac glycosides and phenols. The results in addition showed that there was a significant decrease ($p \leq 0.05$) in the mean bodyweight, RBC, Hb values in diabetic rats when compared to normal control rats. The treatment groups treated with metformin, pomegranate juice and pomegranate peel extract (Group III to V) showed a significant improvement in all the parameters compared to diabetic control by the end of 45th day. It was inferred that metformin substantially alleviated the effects of STZ in diabetic rats compared to all the treatment groups. Pomegranate peel extract was observed to have marginally better antidiabetic effects compared to pomegranate juice.

Keywords: Diabetes mellitus (DM), pomegranate peel extract (PPE), pomegranate juice (PJ), metformin (MF), body weight (BW), red blood cell (RBC), haemoglobin (Hb), total leucocyte count (TLC)

Introduction
Diabetes mellitus is a common and most frequently diagnosed endocrine disorder of dogs and cats. Similar to humans, different types of diabetes occur in these animals also. Its incidence is increasing possibly due to sharing of common environment of humans with an increase in predisposing factors such as obesity, age, physical inactivity and other hormonal disorders in both the species (Hoenig, 2002) [10]. Ancient Indian medicinal systems like Ayurveda have described many medicinal plants possessing potent hypoglycaemic activity. The extracts of these herbs are used in the treatment of diabetes for lowering blood sugar level and many such herbs on pharmacological and clinical trials, have shown to possess hypoglycaemic effect and repair β-cells of islets of Langerhans (Gupta *et al*., 2008) [8]. Hence there, is a need to introduce traditional medicine in treatment and management of diabetes mellitus to reduce the side effects and cost of conventional treatment. Among the various plants, pomegranate also has shown to possess potent antidiabetic property.
Pomegranate (*Punica granatum* L.) is a granular apple delectable fruit consumed worldwide. The total production of pomegranate around the world is 1,500,000 tons and 60% of that is the weight of the peel itself. *Punica granatum* Linn. is a plant that belongs to Punicaceae family and grows in Iran, Egypt, India, Bangladesh, Sri Lanka, North Africa. Pomegranate peels are used as a popular remedy throughout the world and exploited in traditional medicine for its strong mordancy properties (Nitave and Patel, 2014) [17]. Several studies focused on prevention and treatment of cancer, cardiovascular disease, diabetes, dental

conditions, erectile dysfunction and skin allergy investigations have been carried out to determine antioxidant, anticarcinogenic and antiinflammatory properties of pomegranate constituents. Pomegranate peel consists of considerable amounts of phenolic compounds, including flavonoids such as anthocyanins, catechins and other complex flavonoids and hydrolysable tannins (punicalagin, punicalin, pedunculagin, ellagic and gallic acid) (Prakash and Prakash, 2011) [20]. The ethno pharmacological profile of pomegranate peel makes it a prized traditional asset for its antimicrobial, antimutagenic, antioxidant, anti-inflammnotry properties. The aim of the present study was to screen for phytochemicals and bioactive compounds in *Punica granatum* peel and to evaluate the haematological alterations in STZ induced diabetic Wistar male albino rats.

Materials and Methods

Experimental animals

Male albino Wistar rats weighing 180-200g obtained from Raghavendhra Enterprises, Bengaluru, were used for the present study. They were maintained under standard laboratory conditions and offered *ad libitum* quantity of standard commercial rat feed and clean drinking water. The animals were kept for acclimatization after procurement for 2 weeks. The study was carried out with prior approval from Institutional Animal Ethics Committee (IAEC) with IAEC No VCH/IAEC/2019/128. The experiment was carried out for a period of 45 days.

Experimental design

Group I	Normal control: normal rats administered orally with saline
Group II	Diabetic control: rats administered with STZ at 45mg/kg bw intraperitoneally
Group III	Diabetic rats treated with metformin at 500 mg/kg bw orally
Group IV	Diabetic rats treated with pomegranate fresh juice at 1mL/day
Group V	Diabetic rats treated with pomegranate peel extracts at 100mg/day

Drugs and chemicals

Streptozotocin (STZ)

Streptozotocin, was procured from Sigma Chemicals, St. Louis, USA (No. SO130). The working injectable STZ solution was made in freshly prepared 0.1 M citrate buffer with pH 4.5 and stored at 4-8° C.

Administration of STZ solution

The STZ solution was administered by intraperitoneal route at the rate of 45 mg/kg bw to all the rats of Groups II, III, IV, V.

Metformin

Metformin (500 mg) an oral hypoglycaemic drug, purchased from a local chemist shop was powdered and suspended in distilled water (20 ml) to make a concentration of 100 mg/ml. The solution was prepared every day and administered orally at a dose rate of 500 mg/kg bw for treatment (Group-III).

Plant extracts

Pomegranate juice (PJ)

The fruits of fresh pomegranate were washed and manually peeled, without separating the seeds. Pomegranate juice was obtained using a commercial blender and filtrated with a Buchner funnel to remove water insoluble materials.

Preparation of pomegranate peel extract solution (PPE)

Peel of fresh pomegranate fruit was removed, shade dried for 20 days, and then put in a freeze dryer for complete dryness. Dried peels were crushed and an amount of 500g of dried material was mixed with hydro-alcoholic solution prepared with water and methanol (40:60) containing two litres of distilled water and three litres of methanol for maceration in a closed conical flask at room temperature. The flasks were agitated by keeping in a rotary shaker and allowed to stand for one week. It was then filtered using Buchners funnel and Whatman No.1 filter paper. The resultant filtrate was taken and methanol was evaporated by using a rotary flask evaporator (Rotavapor, BUCHI, Switzerland) at room temperature not exceeding 45 °C. Crude PPE was obtained and kept at -80 °C and the extract was further subjected to lyophilization.

Phytochemical screening of hydroalcoholic extract of pomegranate peel

Hydroalcoholic extract of pomegranate peel was subjected to phytochemical screening by various qualitative tests for detection of bioactive molecules which included saponins, tannins, flavonoids, terpenoids, cardiac glycosides and phenols as per Harborne (1991), Khandelwal *et al*. (2009) and Karthikeyan and Vidya (2019) [9, 14, 12].

Test for saponins

Foam test: The extract was diluted with 20 mL of distilled water separately and shaken for 15 minutes in graduated cylinder observed for development of a layer of foam.

Test for tannins

Lead acetate test: The extract was treated with few drops of neutral lead acetate solution (10%) and observed for formation of yellow precipitate.

Test for flavonoids

Alkaline Reagent Test: To 2 mL of 2.0% NaOH mixture was mixed with aqueous extract. The solution was examined for development of intense yellow coloration and fading of the color with few drops of diluted acid.

Test for terpenoids

Salkowaski reaction: Chloroform was added to the dried extract followed by addition of a few drops of concentrated sulphuric acid, shook well and allowed to stand for some time and observed for color change in the lower layer.

Test for cardiac glycosides

Keller-kiliani test: To 2mL of hydoalcoholic extract, equal volume of water and 0.5 mL of lead acetate solution was added, and filtered. Filtrate was extracted with equal volume of chloroform. Chloroform extract was evaporated to dryness and residue was dissolved in 3 mL of glacial acetic acid followed by addition of few drops of $FeCl_3$ solution. The resultant solution was transferred to a test tube containing 2 mL of concentrated sulphuric acid.

Test for phenols

Ferric chloride test: To 2mL of the extract, 3mL of ethanol and a pinch of ferric chloride was added and was observed for development of red color.

Administration of the treatment solution
Throughout the period of experiment, the pomegranate juice, pomegranate peel extract and metformin were administered orally to their respective groups by using clean gavaging/rat feeding needle attached to an appropriate disposable syringe during morning hours daily for a period of 45 days.

Experimental induction of diabetes
The animals were fasted overnight and hyperglycaemia was induced in Group II to VII by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (45 mg/kg bw) in 0.1 M cold citrate buffer with a pH 4.5. Control **(**Group I) animals received citrate buffer alone.

Confirmation of diabetes
The blood glucose levels were estimated 72 hours post STZ injection and the rats with blood glucose level above 200 mg/dL were considered as diabetic. After confirmation of diabetic state, all the groups received their respective treatments daily for 45 days.

Clinical observations
Rats of all the groups were observed for feed and water intake, general behaviour, alertness, urine output, diarrhoea and any other clinical symptoms manifested.

Collection of serum sample
Blood was drawn from the retro-orbital plexus of the rats under light ether anaesthesia at different time intervals such as 3rd 15th, 30th and 45th day post STZ injection of the study. First few drops of blood were collected in ethylene diamine tetra acetic acid (EDTA) for haematological analysis, and about 2 mL of blood from each animal of all groups was collected in clot accelerator tubes, allowed to clot for 30 min and then centrifuged at 3000 rpm for 10 min. The separated serum was collected into 2 mL Eppendorff tube and subjected for glucose estimation immediately after collection and the remaining serum samples were stored at -20ºC for further analysis.

Sacrifice of animals
The effects of treatments given to different groups were studied by sacrificing two rats from each group under xylazine and ketamine anaesthesia on 3rd, 15th and 30th day and remaining rats on 45th day of the experiment. Sacrificed animals were subjected for detailed post mortem examination and gross changes if any, were recorded.

Haematological evaluation
On 3rd, 15th, 30th and 45th day, the blood collected in EDTA from all the treatment groups were subjected for estimation of haemoglobin, RBC and TLC and platelet count using the automated haematology analyzer (BC-2800 vet, Mindray).

Statistical analysis
Statistical analysis was performed using the statistical software Graph pad prism, version 8.4.3 for windows. Mean and standard error values were calculated and expressed as mean (±SE). The data were analysed by two way analysis of variance (ANOVA) for all parameters.

Results and Discussion
Phytochemical screening of hydroalcoholic peel extract of *Punica granatum*
In the present study, preliminary phytochemical constituents of hydroalcoholic pomegranate peel extract was evaluated. The extracts of pomegranate peel revealed the presence of saponins, tannins, flavonoids, terpenoids, cardiac glycosides and phenols (Table R-1). The yield of pomegranate peel extract was 25%.

Detection of saponins
Foam test: There was formation of 1 cm layer of foam which indicated the presence of saponins.

Detection of tannins
Lead acetate test: Formation of yellow precipitate was observed which indicated the presence of tannins.

Detection of flavonoids
Alkaline reagent test: Formation of yellow colour was noticed which became colourless on addition of few drops of dilute HCL, which indicated the presence of flavonoids.

Detection of terpenoids
Salkowaski test
Formation of blue-green ring at the junction of the two layers was observed which indicated the presence of terpenoids.

Detection of cardiac glycosides
Keller-Killani test
There was formation of reddish brown colour at the junction of two liquid layers which indicated the presence of cardiac glycosides.

Detection of phenols
Ferric chloride test
Formation of bluish black colour was observed which indicated the presence of phenols.

Table R- 1: Phytochemical constituents of *Punica granatum* (Pomegranate)

Sl. No	Phytochemical Constituents	Test performed	Observation	Result
1	Saponins	Foam test	Appearance of honeycomb froth	+
2	Tannins	Lead Acetate test	Appearance of Yellowish precipitate	+
3	Flavonoids	Alkaline reagent test	Appearance of yellow colour	+
4	Terpenoids	Salkowaski test	Formation of blue green ring	+
5	Cardiac glycosides	Keller-Killani test	Formation of brown ring	+
6	Phenols	Ferric Chloride test	Formation of deep blue colour	+

The phytochemicals and other chemical constituents of medicinal plants in general account for their medicinal value. The phytochemicals like saponins possess hypotensive and cardiodepressant properties (Olaleye, 2007) [18]. Glycosides are natural cardioactive substances which are employed in treatment of congestive heart failure and cardiac arrhythmia (Brian *et al.*, 1985) [5]. Flavonoids are antiproliferative and inhibit the promotion of growth and progression of tumours

(Stevens *et al.,* 1992) [22]. Phenols along with flavonoids exhibit multiple properties such as antioxidant, anticarcinogenic and anti-inflammatory properties (Asha *et al.,* 2011) [3]. Tanins are useful in healing wounds, varicose ulcers, haemorrhoids, frostbite, and burns (Igboko, 1983 and Maiduyi, 1983) [11, 15] and terpenoids have anti-inflammatory, antiviral, antimalarial, antibacterial activity and also cause inhibition of cholesterol synthesis (McGarvey and Croteau, 1995; Wang *et al.,* 2005) [16, 23]. With possession of phytochemical constituents such as saponins, tanins, flavonoids, terpenoids, cardiac glycosides and phenols, pomegranate peel extract is a natural product with antioxidant, anti-inflammatory, anticarcinogenic, cardioactive and other properties has promising place as a supplementation in various therapeutic regimes.

Evaluation of body weight and haematological parameters

To evaluate the efficacy of pomegranate juice and peel extract in improving bodyweight and hematological parameters, experimentally diabetes was induced in Wistar rats by streptozotocin a naturally occurring product produced by *Streptomycetes achromogenes,* which induces hyperglycaemia by massive reduction in the β cells of the islet of Langerhans.

The mean (± SE) body weight values of Group II diabetic rats progressively decreased from Day 3 to Day 45 with the values of 168.53±0.96, 162.59±0.96, 159.67±0.90, 151.88± 1.26 g on 3rd, 15th, 30th, and 45th day post STZ treatment respectively. In Group III diabetic rats treated with metformin, which is a synthetic analog of the natural product guanidine, the mean (± SE) body weight values improved, from 15th to 45th day of experiment in comparison with that of diabetic groups (Group II). Similarly the treatment groups (Group IV and V) treated with pomegranate juice and peel extract respectively, the mean body weight values improved from Day 15 to Day 45 in comparison with that of diabetic control group.

The improvement in the body weight in Group IV and V rats could be attributed to the bioactive phytochemical compounds in pomegranate juice and peel extract which have antihyperglycemic effects causing enhancement in weight gain, better utilization of nutrients as well as decrease the complications of hyperglycaemia (Aboonabi *et al.,* (2014); Faddladdeen and Ojaimi., 2019) [2, 7].

The haematological parameters evaluated in the present study included Hb percentage Red blood cell count and total leucocyte count. The mean value of Hb percentage declined drastically in diabetic control group (Group II) from 14.32±0.25 g/dL on 3rd day to 9.40±0.17 g/dL on 45th day of the experiment. Similarly, the mean values of total RBC declined from 7.70± 0.10 on 3rd day to 5.95±0.04 on 45th day. A decrease in the Hb percentage in diabetes is due to increased glycosylation of Hb, with hyperglycaemia. In diabetes, a minor Hb derivative HbA1c is produced by glycosylation which is helpful in monitoring diabetes (Pari *et al*., 2001 and Kaleem *et al.,* 2006) [19, 13].

The Hb and TRC mean values improved from 15th to 45th day in group III diabetic rats treated with metformin and Group IV and V with pomegranate juice and peel extract respectively. Metformin treatment reduces the imbalance between lipid peroxidation and antioxidant defence system in RBCs and further declines enzymatic glycosylation with improvement in Hb values (Aya *et al*. (2005) and Abdel- Moneim *et al.* (2019) [4, 1].

The improvement in Hb and RBC values in Group IV and V could be due to the phytoconstituents of pomegranate such as polyphenols and flavonoids which have antihyperglycemic and haemopoietic properties and prevent oxidation of the Hb and destruction of RBCs by reducing oxidative stress (Youdim *et al.,* 2000) [24].

In Group II diabetic rats, the total leucocyte count increased from 3rd day to 45th day and in Group III, Group IV, Group V diabetic rats with respective treatments the total leucocyte count improved by 45th day. Pomegranate phytoconstituents confer health benefits due to their antioxidant activity. They reduce inflammation by reducing MPO activity, TNF- α production and COX-2 and *i*NOS expression through the inhibition of MAPKs and NF- κB to scavenge ROS (Rosillo *et al.,* 2012 and Colombo *et al.,* 2013) [21, 6].

There was also gradual decrease in the severity of the clinical signs in the treatment groups IV and V. The improvement in the body condition could be attributed to the antihyperglycemic, antioxidant, hypolipidemic, hepatoprotective effects of the pomegranate.

It was observed that there was no significant ($p \geq 0.05$) variation between Group IV and V in any of the tested parameters throughout the study. The mean values of various parameters were also comparable with those of Group III treated with metformin. Nevertheless, It could be inferred that metformin substantially alleviated the effects of STZ in diabetic rats compared to all the treatment groups. Pomegranate peel extract was observed to have marginally better effects compared to pomegranate juice, in improving the bodyweight and haematological parameters.

Table 1: The mean (± SE) animal body weight (g) values of different treatment groups at different intervals of time

Groups	Mean (±SE) Body weights (g)			
	Days post-treatment			
	3rd day	15th day	30th day	45th day
Group -I	171.66 ± 0.77^{aW}	192.75 ± 3.72^{aX}	218.73 ± 1.29^{aY}	242.41 ± 1.78^{aZ}
Group -II	168.69 ± 0.96^{aW}	162.59 ± 0.96^{bX}	159.67 ± 0.90^{bY}	151.88 ± 1.26^{bZ}
Group -III	170.85 ± 0.96^{aW}	166.57 ± 0.93^{cX}	164.81 ± 1.44^{cY}	165.55 ± 1.90^{cZ}
Group -IV	169.53 ± 0.30^{aW}	175.32 ± 1.29^{dX}	184.96 ± 1.11^{dY}	192.41 ± 0.65^{dZ}
Group -V	170.93 ± 0.75^{aW}	178.24 ± 1.15^{dX}	186.38 ± 0.13^{dY}	195.07 ± 0.96^{dZ}

Note: Group I (NC), Group II (DC), Group III (MF), Group IV (PJ), Group V (PPE). All values are mean (± SE). Mean values with different superscript differ significantly. Values are statistically significant at $p \leq 0.05$

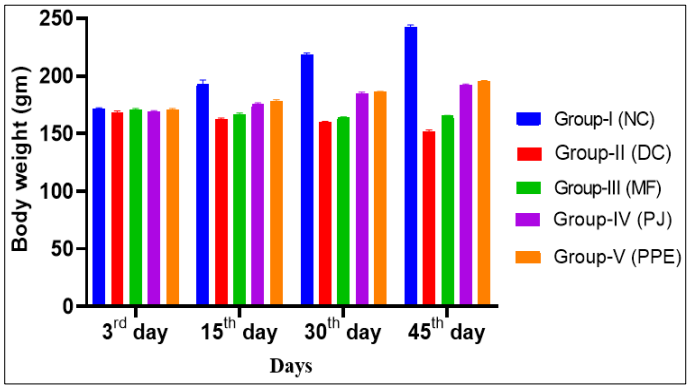

Fig 1: The mean (± SE) animal body weight (g) values of different treatment groups at different intervals of time

Table 2: The mean (± SE) haemoglobin (g/dL) values of different treatment groups at different intervals of time

Groups	Mean (±SE) Haemoglobin (g/dL)			
	Days post-treatment			
	3rd day	15th day	30th day	45th day
Group -I	14.91±0.11^{aW}	14.85±0.67^{aW}	14.79±0.19^{aW}	14.04±0.07^{aW}
Group -II	14.32±0.25^{aW}	12.36±0.27^{bX}	12.09±0.08^{bY}	9.40±0.17^{bZ}
Group -III	14.76±0.13^{aW}	13.05±0.04^{cX}	14.20±0.12^{cY}	14.12±0.07^{aZ}
Group -IV	14.84±0.13^{aW}	13.01±0.04^{cX}	14.83±0.13^{cY}	14.20±0.10^{aZ}
Group -V	14.22±0.04^{aW}	13.79±0.38^{cX}	14.65±0.32^{cY}	14.36±0.14^{aZ}

Note: Group I (NC), Group II (DC), Group III (MF), Group IV (PJ), Group V (PPE). Mean values with different superscript differ significantly. Values are statistically significant at $p \leq 0.05$

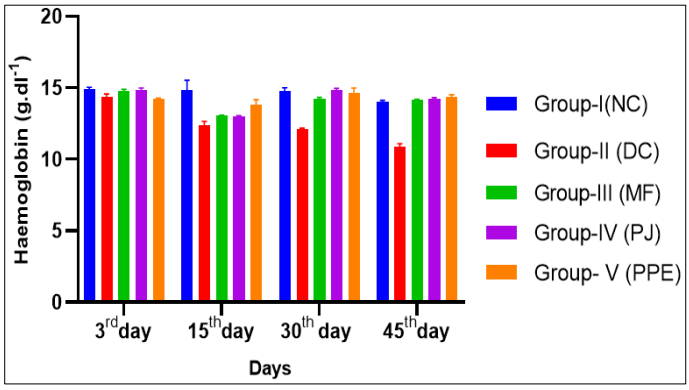

Fig 2: The mean (± SE) haemoglobin (g/dL) values of different treatment groups at different intervals of time

Table 3: The mean (± SE) RBC count (x10^{12}/L) values of different treatment groups at different intervals of time

Groups	Mean (±SE) RBC count (x10^{12}/L)			
	Days post-treatment			
	3rd day	15th day	30th day	45th day
Group -I	8.30±0.20^{aW}	8.54±0.19^{aW}	8.13±0.03^{aW}	9.95±0.02^{aW}
Group -II	7.70±0.10^{aW}	6.83±0.05^{bX}	6.40±0.15^{bY}	5.95±0.04^{bZ}
Group -III	7.09±0.03^{aW}	7.41±0.07^{cX}	7.64±0.16^{cY}	8.03±0.01^{cZ}
Group -IV	7.04±0.01^{aW}	7.34±0.17^{cX}	7.29±0.03^{cY}	7.44±0.07^{cZ}
Group -V	7.94±0.02^{aW}	7.49±0.11^{cX}	7.75±0.12^{cY}	7.80±0.08^{cZ}

Note: Group I (NC), Group II (DC), Group III (MF), Group IV (PJ), Group V (PPE), All values are mean (±SE). Mean values with different superscript differ significantly. Values are statistically significant at $p \leq 0.05$

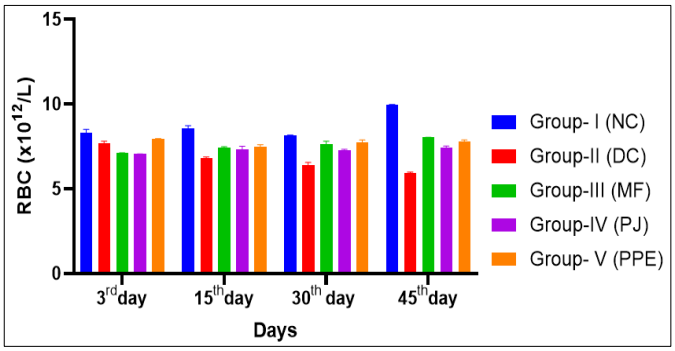

Fig 3: The mean (± SE) RBC count (x10^{12}/L) values of different treatment groups at different intervals of time

Table 4: The mean (± SE) WBC count (x10^3 µL) values of different treatment groups at different intervals of time

Groups	Mean (±SE) WBC count (x10^3 µL)			
	Days post-treatment			
	3rd day	15th day	30th day	45th day
Group -I	9.46±0.25^{aW}	9.73±0.09^{aW}	9.79±0.16^{aW}	9.97±0.01^{aW}
Group -II	13.17±0.01^{bW}	15.45±0.13^{bX}	16.06±0.01^{bY}	17.93±0.01^{bZ}
Group -III	12.33±0.03^{bW}	11.23±0.10^{cX}	10.66±0.33^{cY}	10.01±0.04^{cZ}
Group -IV	12.41±0.06^{bW}	12.54±0.02^{dX}	11.44±0.02^{dY}	11.30±0.01^{dZ}
Group -V	12.79±0.01^{bW}	12.41±0.04^{dX}	11.25±0.03^{dY}	11.19±0.10^{dZ}

Note: Group I (NC), Group II (DC), Group III (MF), Group IV (PJ), Group V (PPE). All values are mean (±SE). Mean values with different superscript differ significantly. Values are statistically significant at $p \leq 0.05$

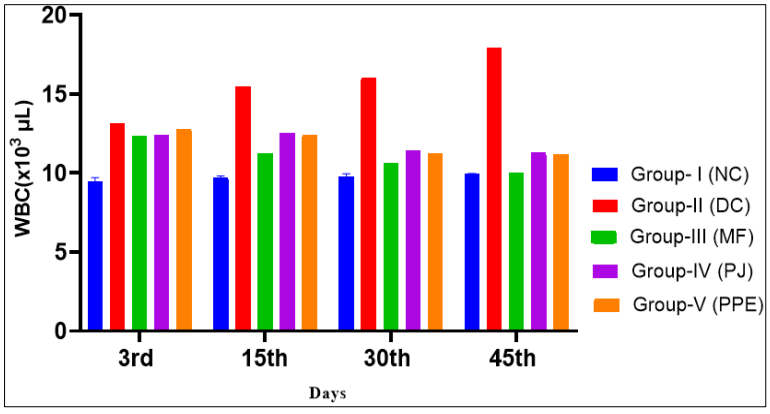

Fig 4: The mean (± SE) WBC count (x10^3 µL) values of different treatment groups at different intervals of time

Conclusion

- The hydroalcoholic extracts of pomegranate peel consists of saponins, tannins, flavonoids, terpenoids, cardiac glycosides and phenols and phytochemicals.
- Metformin substantially alleviated the effects of STZ in diabetic rats
- Treatment with pomegranate juice and pomegranate peel extract could moderately alleviate the STZ induced diabetic effects.
- Pomegranate peel extract was observed to have marginally better antidiabetic effects compared to pomegranate juice.

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