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RESEARCH ARTICLE

Anti-thrombocytopenic and Haemostatic Effect of an *Unani* Formulation on Busulfan-induced Thrombocytopenia in Rats

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Abstract

Background: The *Unani* medical system uses single medications or formulations, which are combinations of multiple drugs, to manage a variety of ailments. The *Unani* physician used these formulations in their clinics. A Polyherbal *Unani* Formulation (PUF) was selected to explore its Anti-thrombocytopenic activity. This formulation is used in fever, and based on its ingredients, the Anti-thrombocytopenic activity was explored. **Aim:** The research aimed to examine the anti-thrombocytopenic and hemostatic effect of a compound *Unani* herbal formulation in the busulfan-induced male Sprague Dawley (SD) rats. **Methods:** Busulfan-induced thrombocytopenia method was used in this study. Preventive and curative regimens were tested, and platelet counts, bleeding time, clotting time, Haematological and biochemical blood tests were carried out. The histopathology was also done at the end of the experiments. The decoction of the PUF was used in the male SD rats for this study. **Results:** The PUF did not show anti-thrombocytopenic activity in the preventive regimen, but in the curative regimen, it exhibited increased platelet count, much better in animals than busulfan control animals and showed mild anti-thrombocytopenic activity. It has also shown hepatoprotective and nephroprotective potential in the preventive and curative regimen. **Conclusion:** PUF exhibited mild anti-thrombocytopenic activity in the curative study and hepatoprotective and nephroprotective potential in this study.

Major Findings: Present study demonstrated the mild anti-thrombocytopenic activity in curative regimen, and hepatoprotective as well as nephroprotective potential of a traditional *Unani* formulation in preventive and curative regimen.

Keywords: Compound Formulation, Decoction, *Joshanda*, Thrombocytopenic Activity, *Unani*

1. Introduction

In clinical practice, thrombocytopenia, or a decrease in platelets in the blood, is a common occurrence and the most common cause of bleeding. It happens as a result of decreased platelet production, increased platelet breakdown, and sequestration[1,2]. Thrombocytopenia is a low platelet cell count (150,000/L), which can be acquired or inherited, and is brought on by a variety of reasons. Platelets are blood cells that aid in blood clotting and wound healing; associated hazards with thrombocytopenia range from no risk to risks of bleeding and thrombosis. Uncertainty exists regarding the relationship between bleeding risk and the degree

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of thrombocytopenia[3]. Even though the risk of bleeding varies slightly depending on the cause of the thrombocytopenia, haemorrhage is uncommon in isolated thrombocytopenia at counts below 50,000/µl [1]. One or more of the following three processes contribute to thrombocytopenia. The most prevalent and significant causes of thrombocytopenia include (i) Agranulocytosis, a bone marrow condition, which is usually linked to insufficient platelet production. These include Idiopathic aplastic anaemia and related disorders (congenital megakaryocytic hypoplasia, Fanconi syndrome, others), and some viral infections. Chemicals (chlorothiazides, ethanol, tolbutamide, *etc*.) and physical agents, chemotherapy drugs, prolonged alcohol use, folic acid and vitamin B12 deficiencies, paroxysmal nocturnal hemoglobinuria, some hereditary forms, *etc*., can result in low platelet production. (ii) Accelerated platelet destruction, despite adequate platelet production by the bone marrow, a low platelet count may nevertheless be present. Autoimmune disorders, certain drugs, infections, surgical procedures, pregnancy, and other conditions that cause abnormal blood clotting might result in a decrease in platelet count in the body. (iii) Abnormal platelet distribution or pooling, and a splenic enlargement may be associated with liver disease (cirrhosis with portal hypertension), the diseases sarcoidosis and Gaucher's illness, Hodgkin's disease and additional lymphomas, *etc*[1-5].

The methods available for treating thrombocytopenia depend on the underlying cause[6]. Corticosteroids and lithium carbonate are used to increase the platelet count, but have their side effects[7,8]. Platelet transfusion is also used to improve the platelet count, but in autoimmune thrombocytopenia, it has a minimal effect[9]. All these treatment modes are expensive and increase hospital stays, which also contributes to increasing the risk of mortality and morbidity[10].

No specific treatment is available for dengue, chikungunya, and influenza. Dengue, on the other hand, has posed a serious health risk in recent times. It causes the immune system to be destroyed and leads to symptoms such as headaches, inflammation, and thrombocytopenia[11]. Neither potent vaccines nor drugs are available for these viral diseases, and symptomatic and supportive therapies manage them. Paracetamol and NSAIDs have been generally used for symptomatic relief in these diseases.

Preclinical investigations have demonstrated that certain herbal medicines have the ability to enhance platelet count in animals, *e.g.*, *Gandum* (*Triticum aestivum*) increases platelet counts in thrombocytopenia and pancytopenia conditions, and in another study, the platelet count was significantly increased with *Doodhi Kalan* (*Euphorbia hirta*). *Asparagus racemosus* showed anti-thrombocytopenic and anti-anaemic effects, and the water extract of *Tinospora cordifolia* probably acts by improving the production of platelets from bone marrow. *Carica papaya* (leaf extract) boosted the platelet count, as proved by animal studies and clinical trials. The Apple Fruit Extract-enriched diet (AFE) increased platelet counts in both normal and thrombocytopenic rats[6,10,12-16]. Numerous researchers have investigated the immune-modulating action of plant phytochemicals. Phytochemicals, including alkaloids, glycosides, flavonoids, polysaccharides, tannins, lectins, phenolic compounds, saponins, anthocyanins, terpenoids, and sterols, have an impact on immunomodulatory capabilities[17]. This study aims to evaluate the efficacy and safety of a PUF in treating low platelet count in laboratory animals.

2. Materials and Methods

2.1 Plant Material Collection and Authentication

The crude drugs of PUF (Table 1)[18] were obtained from the GMP-certified pharmacy at NRIUMSD. Dr. Mohd Kashif Husain, SMP Unit, NRIUMSD, Hyderabad, authenticated the botanical identity of herbal drugs. The voucher specimens were deposited at the SMP unit for records and future reference for *Chiraita Shireen, Gilo-e-Sabz, Gul-e-Ghafis, Gul-e-Nilofar, Beikh-e-Kasni,* and *Khaksi* as voucher No. SMPU/CRI-Hyd-14115, 14116, 14117, 14118, 14119, and 14120, respectively.

2.2 Chemicals and Reagents

The chemicals utilised for the study were obtained from Sigma Aldrich Chemicals Pvt. Ltd. and SR Life Science Solutions.

2.3 Composition of the Test Drug

Joshanda (decoction) was prepared in the drug standardisation research unit of NRIUMSD, Hyderabad,

according to the composition given in *Unani* literature (Table 1)[18].

2.4 Mode of Administration of Test Drug

Joshanda was given *via* oral administration using a feeding cannula. The test drug, *Joshanda*, was prepared fresh daily before administration to the animals.

2.5 Method of Preparation of *Joshanda*

After cleaning, the 18 g of coarse powder of drugs was soaked in 180 ml of water overnight and boiled in the morning till the quantity was reduced to half. After straining, the decoction volume was adjusted to 90 ml, which was considered per day human dose[18-20].

For administering the test drug as a decoction, the clinical dose of the decoction prepared as mentioned above was 90 ml per day in humans. The clinical dose was translated to rats as per body surface area; accordingly, equivalent doses were 9 ml/kg in rats. In addition, a second dose of 18 ml/kg (double dose) was administered to rats to examine the dose-dependent effect of the experimental medication. The percentage yield of *Joshanda* was evaluated by converting it into lyophilised powder and represented the drug PUF in mg/kg in the study.

After the lyophilisation process, the dried extract of decoction for humans was ≈ 1.40 g/day (1400 mg/day) and considered an adult dose. Rats were given a dose of 144 mg/kg (≈150 mg/kg) after the adult human dose was converted based on body surface area. A second dose, *i.e.*, a double dose of 300 mg/kg for rats, was also calculated and used.

2.6 Experimental animals

The animals were obtained from the NIN, Hyderabad. The study employed SD strain male rats, aged 8-10 weeks. The study obtained approval from the IAEC of the NRIUMSD, Hyderabad. The approval number, dated 18/12/2018, was CRIUM/IAEC/2018/02/P04. The male SD rats were kept in controlled conditions, including a 12-h light-dark cycle, 23±2°C temperature, and 45-55% humidity. They had unhindered access to water and a standard dry pellet diet. The animals were acclimated to the laboratory setting before starting the experiment.

3. Anti-thrombocytopenic Activity

3.1 Busulfan-induced Thrombocytopenia

There were six randomly allocated groups of adult male SD rats in total, consisting of six rats. These groups included a standard control group, a busulfan control group (serving as a negative control), and four test groups used to evaluate the effectiveness of various doses of PUF. Subcutaneous injection of busulfan on day 1, day 5, day 10, and day 15 was given to induce Thrombocytopenia. In the preventive regimen, PUF was administered in treatment Group III and Group IV from the same day of busulfan dosing for 20 days. In the curative experiment, treatment was commenced on the 20th day for a duration of twenty days in treatment Group V and treatment Group VI. *Joshanda* of PUF was orally administered. Platelet counts were analysed on day one before the dosing and after treatment using an automated haematology analyser. Biomarkers of liver and kidney function were also evaluated using biochemical kits. Animals were euthanised following the final administration of treatment using CO_2 asphyxiation. The percentage increase in body weight and the relative weights of the liver and spleen were measured, and the organs were subjected to histopathology[10,12,21,22].

Table 1. Ingredients of PUF[18]

S. No.	Name	Botanical Name	Part used	Quantity
1	Gul-e-Ghafis	*Gentiana olivieri* Griseb.	Flower	3 gm
2	Gul-e-Neelofar	*Nymphaea nouchali* Burm.f.	Flower	3 gm
3	Chiraita Shireen	*Swertia chirayita* (Roxb.) H. Karst.	Whole herb	3 gm
4	Beikh-e-Kasni	*Cichorium intybus* L.	Root	3 gm
5	Khaksi	*Sisymbrium irio* L.	Seeds	3 gm
6	Gilo-e-Sabz	*Tinospora cordifolia* (Willd.) Hook. f. and Thomson	Stem	3 gm

3.2 Experimental Design

3.2.1 Group I (Normal Control)

This group was treated with 0.9% w/v normal saline orally for 20 days and served as a standard control to assess the spontaneous change in platelet count.

3.2.2 Group II (Busulfan Control)

This group received a 5mg/kg subcutaneous injection (sc. inj.) of busulfan on days 1, 5, 10, and 15 to induce thrombocytopenia.

3.2.3 Group III PUF-150 (Preventive)

This group received a 5mg/kg sc. inj. of busulfan on days 1, 5, 10, and 15 and 150 mg/kg PUF orally daily for 20 days starting on the same day.

3.2.4 Group IV PUF-300 (Preventive)

This group received a 5mg/kg sc. inj. of busulfan on days 1, 5, 10, and 15 and 300 mg/kg PUF orally daily for 20 days starting on the same day.

3.2.5 Group V PUF-150 (Curative)

This group received 5mg/kg busulfan sc. inj. on days 1, 5, 10, and 15 and 150 mg/kg PUF orally daily from day 20 to 40 after thrombocytopenia induction.

3.2.6 Group VI PUF-300 (Curative)

This group received 5mg/kg busulfan sc. inj. on days 1, 5, 10, and 15 and 300 mg/kg PUF orally daily from day 20 to 40 days after thrombocytopenia induction.

The animals were weighed after every ten days to estimate the change in body weight.

3.2.7 Clotting Time

Clotting time was determined through puncture/pricking retro-orbital plexus under mild isoflurane anaesthesia conditions. Immediately, the stopwatch was started. At regular intervals of 30 seconds, a timer was used to break a small section of the capillary tube. This process was continued until a fibrin thread became visible at the fractured end of the tube. The measured duration between the puncture of the retro-orbital plexus and the initial observation of fibrin thread at the fractured ends of the capillary tube was measured[12,22-24].

3.2.8 Bleeding Time

Duke's method, with some modifications, was used to determine the bleeding time. The exposed tail (between middle to lower) for the blood extraction was dipped into lukewarm water to clear the visibility of veins and cleaned with distilled water and sterile gauze. A needle was horizontally placed into the tail vein to a depth of 1 cm and promptly removed. The bleeding tail was dabbed onto a Whatman filter paper until all traces of blood were no longer visible on the paper. The duration between the initial application and the cessation of blood was documented[13,25,26].

3.2.9 Blood Collection for Haematological and Biochemical Investigation

Blood was withdrawn from the retro-orbital plexus on the 0 day (before the initiation of the study), as well as on the 20th and 40th days, after subjecting the animals to anaesthesia using isoflurane inhalation.

Serum vacutainers were used for biochemical analysis, and EDTA vacutainers were used for haematological examinations while collecting blood samples. A completely automated haematology analyser was used to measure haematological parameters such as haemoglobin, RBC, WBC, differential WBC, and platelet count. An automated analyser (Erba EM200) was used to analyse serum biochemical parameters, including ALT, AST, ALP, total bilirubin, creatinine, and blood urea nitrogen.

3.2.10 Histopathological Examination

Following the completion of the anti-thrombocytopenic study, all animals were sacrificed by euthanasia. The internal organs and tissues underwent visual examination, isolation, trimming, and quantification by weight. The spleen and liver were weighed to measure relative organ weight. The liver and spleen tissues were fixed in a formalin solution, neutral buffered 10% and then analysed using histological examination.

3.2.11 Statistical Analysis

Tukey's test was conducted following the Analysis Of Variance (ANOVA) for statistical computation. The statistical test was conducted using GraphPad Prism Version 5.0, with P-values less than 0.05 considered statistically significant. All findings were presented as Mean ± SEM.

4. Results

The body weight of the animals receiving the test drugs (PUF) did not differ significantly as compared of the control groups (Figures 1 and 2).

4.1 Relative Organ Weight

4.1.1 Liver

The liver weight of the animals in the control group (normal or busulfan) did not differ significantly from that of the test group (PUF) in both the preventive (Figure 3) and curative studies (Figure 4).

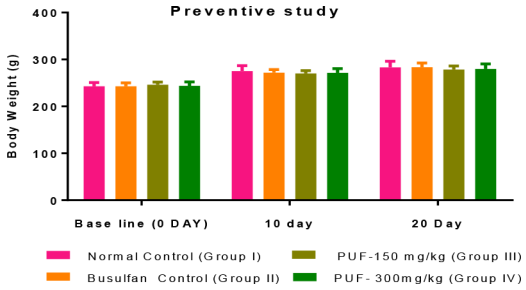


Figure 1. Mean body weight of rats in the control and treatment groups (preventive).

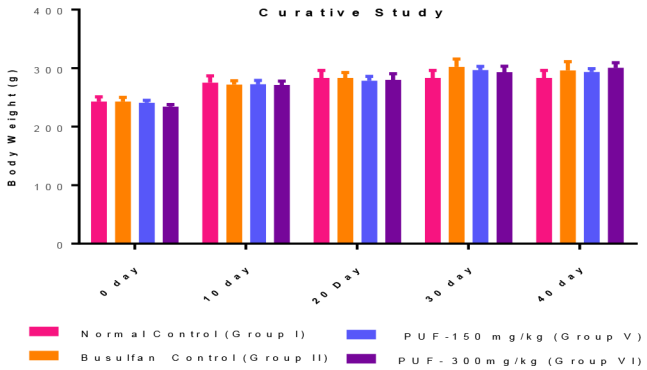


Figure 2. Mean body weight of rats in the control and treatment groups (curative).

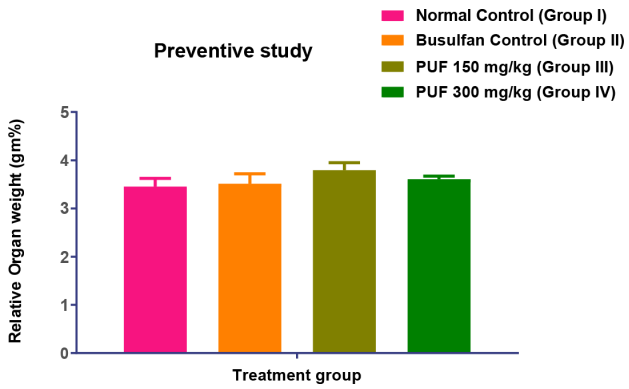


Figure 3. Relative organ weight of liver for controls and PUF-treated rats.

4.1.2 Spleen

There were no notable variations in the relative weight of the spleen in animals treated with the PUF vs. control groups (normal or busulfan), in the preventive (Figure 5) and the curative study (Figure 6).

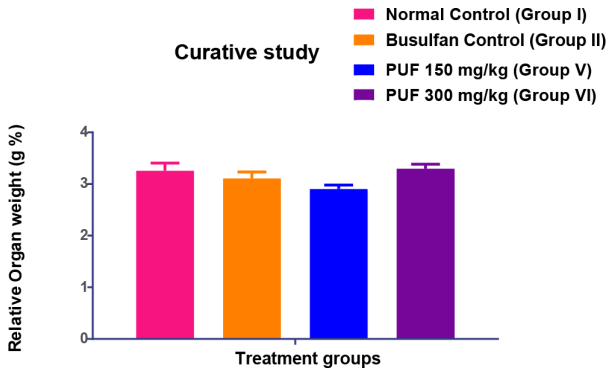


Figure 4. Relative organ weight of liver for controls and PUF-treated rats.

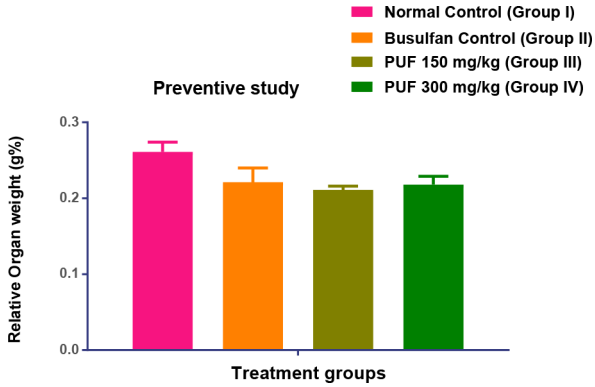


Figure 5. Relative organ weight of the spleen for controls and PUF-treated rats.

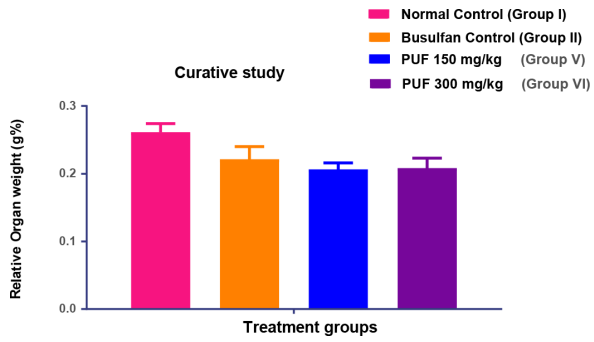


Figure 6. Relative organ weight of the spleen for controls and PUF-treated rats

4.1.3 Platelet Count

Prior to the onset of thrombocytopenia, there was no notable variation in platelet count at the beginning of the study across the groups. In the preventive study, on day 20, the platelet count was found to be significantly decreased in the busulfan group ($p<0.001$), in comparison with normal controls. In treatment Groups III and IV (PUF-150 and PUF 300 mg/kg), platelet counts were considerably reduced ($p<0.001$) compared to the normal control. The platelet counts in the busulfan control and PUF-treated groups exhibited no significant differences at any of the tested dosage levels.

In the curative study, the platelet counts of the busulfan control group were substantially lower ($p<0.05$) than the normal control group. In treatment groups V and VI (PUF-150 and PUF 300 mg/kg), the dose-dependent elevation in platelet count was observed and values were comparable to normal control group. A dose of 300 mg/kg of PUF resulted in a more significant rise in platelet count compared to the low dose group of 150 mg/kg (Table 2).

4.1.4 Haematological and Biochemical Investigation

In the preventive study, total WBC count in the busulfan control and treatment groups was decreased ($p<0.001$) compared to the normal control. There was no difference in total WBC counts between the busulfan control and treatment groups. Similarly, no difference was found in Hb%, RBC value, HCT value, and differential WBC counts when compared to normal and busulfan control (Table 3).

In the curative study, no difference was observed in total WBC counts in the busulfan control and treatment groups, compared to normal and busulfan control. While no difference was found in Hb%, RBC value, HCT value and differential WBC count when compared to normal and busulfan control. The RBC value was found to decrease ($p<0.05$) in the busulfan group vs. normal group (Table 4).

In the preventive study, no significant change was seen in AST value, serum protein, serum albumin,

Table 2. Effect of PUF on platelet count in busulfan-induced thrombocytopenia

Groups	Platelet Count (lakh/mm^3) (percentage change in platelet counts)		
	Day 0	Day 20	Day 40
Group I (Normal Control), (0.9% w/v)	5.067±0.217 (100%)	4.870±0.246 (-4%)	--
Group II (busulfan control) (5mg/kg)	5.400±0.179 (100%)	1.703± 0.210*** (-68%)	3.217±0.455* (-40%)
Group III (Preventive) (PUF-150 mg/kg)	5.467±0.24 (100%	0.933± 0.131*** (-83%)	--
Group IV (Preventive) (PUF-300 mg/kg)	5.767±0.255 (100%)	1.153 ± 0.1834*** (-80%)	--
Group V (Curative) (PUF-150 mg/kg)	5.367±0.189 (100%)	1.333 ±0.115 (-75%)	4.250±0.578 (-21%)
Group VI (Curative) (PUF-300 mg/kg)	5.367±0.189 (100%)	1.292±0.058 (-76%)	4.533±0.256 (-16%)

* = $p<0.05$; **= $p<0.01$; ***= $p<0.001$ verses normal control

Table 3. Haematology of preventive study on (20th day)

Parameter	"Group I" (NC)	"Group II" (BC)	"Group III" (PUF-150)	"Group IV" (PUF-300)
Haemoglobin (gm% %)	15.033 ±0.316	14.067±0.502	14.467±0.251	13.667±0.599
RBC (million/mm^3)	7.467±0.143	6.900±0.235	6.933±0.161	6.600±0.322
WBC (/mm^3)	5533.333 ±711.181	2566.667 ±311.627***	1633.333 ±174.483***	1833.333 ±140.633***
Platelets (lakhs/mm^3)	4.870±0.246	1.703±0.210***	0.933±0.131***	1.153±0.1834***
HCT (%)	38.567±0.738	36.200±1.229	37.133±0.682	34.733±1.666
Neutrophils (%)	1±0	1±0	1.167±0.167	1.333±0.211
Lymphocytes (%)	96.33±0.211	96.17±0.401	95.50±0.500	95.33±0.422
Eosinophils (%)	1.667±0.211	1.500±0.224	2.167±0.167	1.833±0.307
Monocytes (%)	1±0	1.333±0.211	1.167±0.167	1.5±0.224

* = $p<0.05$; **= $p<0.01$; ***= $p<0.001$

serum globulin, serum Alb. and Glob. The ratio and serum creatinine between any group. There was a significant decrease in serum ALT value ($p<0.05$), serum ALP value ($p<0.01$), and serum bilirubin ($p<0.05$) in the PUF-150 group compared to the busulfan group. The serum uric acid was decreased ($p<0.05$) in the busulfan group and treatment groups ($p<0.001$) vs. the normal control. The serum BUN was increased ($p<0.001$) in the busulfan group vs. the normal control, while reduction was seen in PUF-150 and PUF-300 ($p<0.05$) treated groups vs. the busulfan control (Table 5).

In the curative study, no significant difference was found in serum ALT, serum ALP, serum bilirubin and serum protein between any group. Serum albumin was decreased ($p<0.01$) in the busulfan group vs the normal control and increased ($p<0.001$) in the PUF-150 and PUF-300 groups vs. the busulfan control. The AST value was increased in the busulfan group ($p<0.05$) vs. normal control, while it decreased ($p<0.05$) in the treatment groups vs the busulfan group. Serum globulin was increased ($p<0.01$) in the busulfan group vs normal control and decreased in the PUF-300 group ($p<0.001$) as compared to the busulfan control. The serum BUN was increased ($p<0.05$) in the busulfan group vs. the normal control. The serum creatinine was increased ($p<0.01$) in the busulfan group vs. the normal control. There was no difference in serum uric acid for the busulfan group, while a reduction ($p<0.01$) in serum uric acid was seen in the PUF-300 group vs. the busulfan and normal control groups (Table 6).

Table 4. Haematology of the curative study on (40th day)

Parameter	"Group I" (NC)	"Group II" (BC)	"Group V" (PUF-150)	"Group VI" (PUF-300)
Haemoglobin (gm% %)	15.033 ±0.316	14.6±0.268	14.933±0.431	14.767±0.275
RBC (million/mm^3)	7.467±0.143	6.600±0.137*	7.267±0.143	6.800±0.231
WBC (/mm^3)	4960±515.3639	4680±358.701	3760±254.296	4080±187.972
Platelets (lakhs/mm^3)	4.867±0.246	3.217±0.455*	4.250±0.578	4.533±0.256
HCT (%)	38.567±0.738	37.333±0.843	38.667±1.116	38.333±0.955
Neutrophils (%)	1±0	1±0	1.167±0.167	1±0
Lymphocytes (%)	96.33±0.211	95.333±0.919	96.000±0.632	96.000±0.258
Eosinophils (%)	1.667±0.211	2.333±0.715	1.500±0.224	1.000±0.000
Monocytes (%)	1±0	1.333±0.211	1.167±0.167	1.167±0.167

* = p<0.05; **= p<0.01; ***= p<0.001 verses normal control

Table 5. Biochemistry of preventive study (20th day)

Parameter	"Group I" (NC)	"Group II" (BC)	"Group III" (PUF-150)	"Group IV" (PUF-300)
AST(IU/L)	159.50±6.443	158.17±8.905	137.17±4.813	152.17±8.014
ALT(IU/L)	90.000±4.374	98.333±3.480	83.167±2.414#	92.833±4.061
ALP (IU/L)	192.167±14.64	230.500±5.766	166.833±11.42##	198.833±14.94
Total Bilirubin (mg/dl)	0.1617±0.011	0.2067±0.019	0.1483±0.009#	0.1667±0.014
Total protein (g/dl)	6.417±0.070	6.500±0.113	6.400±0.115	6.550±0.081
Albumin (g/dl)	3.433±0.049	3.433±0.088	3.417±0.083	3.433±0.061
Globulin (g/dl)	2.983±0.070	3.067±0.056	2.983±0.091	3.117±0.091
Albumin Globulin Ratio	1.1±0.037	1.05±0.043	1.117±0.048	1.067±0.042
BUN (mg/dl)	33.667±13.744	43.167±17.623***	36.333±14.833#	36.333±14.83#
Creatinine mg/dl)	0.722±0.014	0.757±0.019	0.708±0.016	0.740±0.015
Uric acid (mg/dl)	1.867±0.095	1.617±0.048*	1.417±0.048***	1.417±0.048***

* = p<0.05; **= p<0.01; ***= p<0.001 vs. normal control, ≠ = p<0.05; ## = p<0.01 vs. busulfan control

4.1.5 Bleeding Time

At the baseline, no significant difference in bleeding time was found between any groups. In the preventive study, on day 20, bleeding time in Group II (BC) was significantly increased ($p<0.05$) vs. Group I (NC). In the treatment group III (**PUF-150**) and IV (**PUF-300**), bleeding time was found to be markedly elevated ($p<0.05$) vs. normal control (Table 7).

In the curative study on day 40, bleeding time in Group II (BC) was found to be significantly increased ($p<0.05$) as compared with Group I (NC). In treatment Groups V (PUF-150) and VI (PUF-300), no statistically significant difference was seen in bleeding time compared to normal control group (Table 8).

4.1.6 Clotting Time

In the preventive study, on day 20, clotting time in Group II (BC), statistically significant increase ($p<0.05$) was seen vs. normal control. In the treatment Groups III (PUF-150) and IV (PUF-300), clotting time in busulfan control group was elevated significantly ($p<0.05$) vs. the normal control. No significant difference was found in PUF-treated groups compared to the busulfan control (Table 9).

In the curative study on day 40, clotting time in busulfan control group was found to significantly increase ($p<0.05$) vs. normal control. In treatment groups V (PUF-150) and VI (PUF-300), no significant difference was found in clotting time compared to normal control groups (Table 10).

Table 6. Biochemistry of curative study (40th day)

Parameter	"Group I" (NC)	"Group II" (BC)	"Group V" (PUF-150)	"Group VI" (PUF-300)
AST/SGOT (IU/L)	171.200±4.974	285.600±56.842*	167.200±6.304#	172.8±6.741#
ALT/SGPT (IU/L)	92.600±3.933	84.333±10.184	64.167±4.622	73.833±3.240
ALP (IU/L)	150.6±13.725	110.6±8.730	117.2±8.710	125.2±7.976
Total Bilirubin (mg/dl)	0.1617±0.011	0.162±0.026	0.137±0.019	0.142±0.018
Total protein (g/dl)	6.417±0.070	6.517±0.060	6.600±0.037	6.483±0.075
Albumin (g/dl)	3.433±0.049	3.1±0.077**	3.45±0.043###	3.517±0.031###
Globulin (g/dl)	2.983±0.070	3.417±0.101**	3.150±0.056	2.967±0.076##
Albumin Globulin Ratio	1.1±0.037	0.883±0.048**	1.033±0.033#	1.150±0.034###
BUN (mg/dl)	33.667±13.744	49.667±5.637*	43.833±2.798	49.833±3.005
Creatinine (mg/dl)	0.722±0.014	0.813±0.011**	0.760±0.008	0.77±0.026
Uric acid (mg/dl)	1.867±0.095	1.633±0.088	1.300±0.052	1.067±0.061** and ##

* = p<0.05; **= p<0.01; ***= p<0.001 vs. normal control, # = p<0.05; ## = p<0.01; ### = p<0.001 vs. busulfan control

Table 7. Bleeding times of preventive study (20th day)

Bleeding time (sec)	Group I (NC)	Group II (BC)	Group III (PUF-150)	Group IV (PUF-300)
Bleeding Time Preventive study (20 days)	32.167±5.856	80.667±7.517*	87.833±14.554*	79.667±16.409*

* = p<0.05 vs. normal control.

Table 8. Bleeding times of the curative study (40th day)

Bleeding time (sec)	Group I (NC)	Group II (BC)	Group V (PUF-150)	Group VI (PUF-300)
Bleeding Time Curative study (40 days)	32.167±5.856	46.83±5.69*	40.00±4.63	37.33±2.17

* = p<0.05 vs. normal control.

Table 9. Clotting times of preventive study (20th day)

Clotting time (sec)	Group I (NC)	Group II (BC)	Group III (PUF-150)	Group IV (PUF-300)
Clotting time preventive study (20 days)	163.33±26.362	261.83±16.995*	245.33±20.333*	246.67±11.644*

* = p<0.05 vs. normal control.

4.2 Histopathological Examination

4.2.1 Liver

Based on histopathological observation, no sinusoidal haemorrhages were observed in the normal control; foci of inflammation in the periportal/ centrilobular region were observed only in one animal of group I. In Group-II, sinusoidal haemorrhages in the liver of three animals and foci of inflammation in peri portal/ centrilobular region in the liver of one animal were observed. Group III showed sinusoidal haemorrhages in the liver of 2 animals, while Group IV showed sinusoidal haemorrhages only in one animal. No foci of inflammation in the periportal/centrilobular region were observed in the treatment groups. Neither treatment group showed any inflammatory changes in the liver (Table 11).

In the curative group, PUF-150 (Group V) showed no sinusoidal haemorrhages, while PUF-300 (Group VI) showed sinusoidal haemorrhages only in one animal. PUF-150 (Group V) showed no foci of inflammation in the periportal/centrilobular region. Only one animal in PUF-300 (group VI) showed foci of inflammation in the periportal/centrilobular region (Tables 11 and 12).

4.2.2 Spleen

Congestion and haemorrhages of the spleen were observed in one animal from "Group I", three animals from "Group II", four animals from "Group IV", four animals from "Group V" and two animals from "Group VI". Extramedullary hematopoiesis was observed in two animals from "Group II, one animal from "Group III", and two animals from "Group VI". Lymphoid follicular hyperplasia was observed in two animals from "Group III" and one from "Group IV".

Based on the above discussion, PUF-150 (Group III) showed less reactive changes and a positive response versus negative control and normal animals (Table 11).

Table 10. Clotting times of the curative study (40th days)

Clotting time (sec)	Group I (NC)	Group II (BC)	Group V (PUF-150)	Group VI (PUF-300)
Clotting time curative study (40 days)	163.33±26.362	276.17±54.13*	208.00±25.97	192.17±24.64

* = p<0.05 vs. normal control.

Table 11. Summary of histopathology findings

Group / Organ	"Group I"	"Group II"	"Group III"	"Group IV"
Liver	Foci of inflammation in periportal/ centrilobular region/1 NAD/5	Sinusoidal hemorrhages/3, Foci of inflammation in periportal/centrilobular region/1 NAD/3	Sinusoidal haemorrhages/2, NAD/4	Sinusoidal haemorrhages/1, NAD/5
Spleen	Congestion/ hemorrhages/1 NAD/5	Congestion/ hemorrhages/3 Extra medullary hematopoiesis/2 NAD/1	Extra medullary hematopoiesis/1 Lymphoid follicular hyperplasia/2 NAD/3	Congestion/ hemorrhages/4 Lymphoid follicular hyperplasia/1 NAD/2
Liver	Foci of inflammation in periportal/ centrilobular region/1 NAD/5	Sinusoidal hemorrhages/3, Foci of inflammation in periportal/centrilobular region/1 NAD/3	NAD/6	Sinusoidal haemorrhages/1, Foci of inflammation in periportal/centrilobular region/1 NAD/4
Spleen	Congestion/ hemorrhages/1 NAD/5	Congestion/ hemorrhages/3 Extra medullary hematopoiesis/2 NAD/1	Congestion/ hemorrhages/4 NAD/2	Congestion/ hemorrhages/2 Extra medullary hematopoiesis/2 NAD/3

5. Discussion

Thrombotic microangiopathies, hemolysis, and low platelet count syndrome, thrombotic thrombocytopenic purpura, *etc*., are associated with anaemia and thrombocytopenia, and many populations are affected by these ailments[27,28]. Many virus infections cause thrombocytopenia, like rubella, influenza, measles, infectious hepatitis, epidemic hemorrhagic fever, dengue fever, *etc*.[2]. Dengue and chikungunya have surfaced as global health hazards in recent decades. The global incidence of dengue has significantly increased; current estimates suggest approximately 39 million infections annually, with 9.6 million cases exhibiting clinical symptoms[29,30]. The occurrence of dengue cases in India has escalated during two decades. In 2013, the 74,454 cases of dengue were documented, resulting in 167 deaths. Additionally, there were 18639 cases of chikungunya reported. In 2019, the number of dengue infections in India rose to 136422, resulting in 132 deaths. Additionally, there were 65,217 reported cases of chikungunya[31,32].

The examination of herbal medications identified the existence of chemical components, including alkaloids, glycosides, tannins, carbohydrates, and protein, *etc*[33]. Alkaloids have demonstrated efficacy in combating anti-platelet macrophages in individuals diagnosed with Idiopathic Thrombocytopenic Purpura (ITP)[34]. A preclinical study revealed that alkaloids, particularly *Carpaine* (*Carica papaya*), have shown anti-thrombocytopenic activity, and the validity of the popular ethnopharmacological usage was also proven in a clinical study later[22]. Studies have demonstrated that saponins can decrease the tendency to adhere together and aggregate platelets, prevent the formation of blood clots, and enhance the flow of blood in small blood vessels[35].

To evaluate the anti-thrombocytopenic activity of PUF, the busulfan-induced thrombocytopenia model was used in SD rats. PUF-150 mg/kg and PUF-300 mg/kg were administered as a preventive and curative regimen. To assess the anti-thrombocytopenic activity of PUF on busulfan-induced thrombocytopenia, the platelet count, bleeding time, and clotting time of animals were estimated along with other haematological parameters. Biochemical parameters such as LFT and KFT were also evaluated to assess the effect of PUF on liver and kidney function. Busulfan is an alkylating drug that has the ability to destroy bone marrow cells that are not actively dividing, as well as potentially targeting non-dividing cancer cells[36,37].

The rats treated with busulfan showed no significant changes in body weight, indicating that the treatment did not impact the animals' metabolism. No significant difference in the relative weight of the liver and spleen was seen between preventative and curative studies. This indicates that the body tolerated the test medication without any adverse effects.

In the preventative experiment, the platelet counts markedly diminished in the busulfan control group relative to the normal group. The test medicines PUF, administered at doses of 150 and 300 mg/kg, showed no anti-thrombocytopenic action in the subjects. No significant difference was observed in either group as compared to the busulfan control. Haematological indicators, including haemoglobin (Hb)%, RBC count, haematocrit value, and differential WBC counts, exhibited no significant differences, with the exception of WBC count, between the treatment and control groups. A notable reduction in WBC count was observed in the busulfan control, PUF-150 mg/kg, and PUF-300 mg/kg relative to the normal control. The administration of test medications PUF at doses of 150 and 300 mg/kg did not elevate the white blood cell counts in the animals.

Bleeding time is a measure utilised to evaluate the functionality of haemostasis. The heightened duration of bleeding increases the likelihood of prolonged haemorrhaging and significant loss of blood. This is directly associated with the reduction in the quantity of platelets in circulation[14]. In the preventive study, after induction of thrombocytopenia, the bleeding time of the busulfan control group exhibited a significant elevation versus the normal control group. Also, test drugs PUF at doses of 150 and 300 mg/kg showed increased bleeding time compared to normal control. In both groups, bleeding time did not significantly differ from the busulfan control, which can be due to the low platelet count.

Clotting time evaluates coagulation and the efficacy of clotting agents by quantifying the activation level of the coagulation pathways[13]. In the preventive research, upon the induction of thrombocytopenia, a substantial increase in clotting time was observed in busulfan

compared to the normal control. Furthermore, the administration of Test medicines PUF at dosages of 150 and 300 mg/kg resulted in an extended clotting time relative to the normal control, maybe attributable to a reduced platelet count. No significant change in clotting time was seen in either group when compared to the busulfan control.

The preventative study revealed no significant change in liver function tests among the busulfan control, PUF-150 mg/kg, and PUF-300 mg/kg treated groups when compared to the normal control. However, rats treated with PUF-150 mg/kg exhibited a notable decrease in ALT, ALP, and bilirubin levels in comparison to the busulfan control group. The data indicated that PUF at 150 mg/kg may possess hepatoprotective properties and show the ability to mitigate the adverse effects of busulfan. This conclusion is further corroborated by histological analysis. Both preventive groups, PUF-150 and PUF-300 mg/kg, did not show any inflammatory changes in the liver. PUF-150 mg/kg showed less reactive changes and a positive response when compared with busulfan control and normal control animals, and reactive change in the spleen suggests that it might be due to a trigger of the immune system and maybe a positive response (lymphoid hyperplasia).

KFT analysis showed interesting findings in a protective regimen. Serum BUN was markedly elevated ($p<0.001$) in the busulfan group relative to the normal control. However, the groups treated with test medicines PUF-150 and PUF-300 normalised the elevated BUN levels in rats compared to the busulfan control group. Serum creatinine was normal in all experimental groups. A notable decrease in uric acid levels was seen in the busulfan control, PUF-150, and PUF-300 mg/kg treated groups compared to the normal group, but values remained within the normal physiological range.

The finding suggested that PUF-150 and PUF-300 mg/kg, as preventive regimens, did not show anti-thrombocytopenic activity, but they have good potential as hepatoprotective and might be nephroprotective.

In the curative study, platelets decreased sharply in the busulfan control in comparison to the normal control. However, PUF-150 and PUF-300 both show a rise in platelet counts that is directly proportional to the dosage, but not significant in comparison to the busulfan control. At a high dose of 300 mg/kg of PUF, there was a greater increase in platelet count than low-dose group of 150 mg/kg. Except for RBC count, the remaining haematological parameters, like Hb, WBC count, hematocrit value, and differential WBC counts, showed no significant difference between any experimental groups. A notable reduction in RBC count was observed in the busulfan control relative to the normal control, although it remained within the normal physiological range. The test medications PUF-150 and PUF-300 mg/kg exhibited no significant variation in RBC count when compared to the normal or busulfan control groups.

In the curative study, after induction of thrombocytopenia, a significant increase in the bleeding time of busulfan control compared to the standard control was seen. But test drugs PUF-150 and PUF-300 mg/kg revealed no significant difference in bleeding time compared to normal or busulfan control, which may be due to increased platelet count. In the curative regimen, there was a significant increase in the clotting time of the busulfan control compared to the normal control. Test drugs PUF-150 and PUF-300 mg/kg showed no significant difference in clotting time compared to normal control and busulfan control. This could be due to increased platelet counts and liver enzymes, and proteins normalised by PUF treatment.

LFT analysis showed that in the curative study, a significant difference was observed in the serum AST of the busulfan control (more than physiological range: 63-175 U/L), which was found significantly increased ($p<0.05$) in comparison to the normal control group. AST values in PUF-treated animals were significantly reduced ($p<0.05$) as compared to the busulfan control group. It showed that PUF treatment normalised the increased AST level in animals. There was no significant difference between groups in other liver biomarkers such as ALT, ALP, and serum bilirubin.

In the total serum protein of the curative study, no significant difference was found between groups. Serum albumins in the busulfan group significantly decreased ($p<0.01$) as compared to normal control, but serum albumin in PUF-150 and PUF-300 mg/kg was significantly increased ($p<0.001$) in comparison to the busulfan group. Serum globulin in the busulfan control group (more than physiological range: 1.8-3.2) was significantly increased ($p<0.01$) as compared to a standard control, but in

the PUF-300 mg/kg serum globulin was significantly decreased ($p<0.01$) as compared to the busulfan control. Also, the Albumin and Globulin ratio in the busulfan control group was markedly reduced ($p<0.01$) compared to the normal control. But in the PUF-300 serum Albumin and Globulin ratio was significantly increased ($p<0.001$) compared to the busulfan control. Increased AST level, hypoalbuminemia, increased globulin, and decreased Albumin and Globulin ratio in busulfan control suggested hepatopathy developed in busulfan control. The treatment with PUF-150 PUF-300 mg/kg exhibited an ability to counteract the adverse effect of busulfan by normalising the level of these enzymes and increasing of albumin and globulin ratio.

In the curative study, serum BUN and creatinine in the busulfan group significantly increased in comparison to the standard control. No significant difference was observed in serum BUN and creatinine of PUF-150 and PUF-300 mg/kg treated groups as compared to normal and busulfan control. In case of serum Uric acid, no significant difference was found in the busulfan control group compared to the normal control. However, only the group treated with PUF-300 exhibited a significant decrease ($p<0.01$) in Uric acid level, in comparison to both the normal and busulfan control groups.

Preclinical studies have verified that certain herbal medications have the ability to enhance platelet count in animals. For instance, *Triticum aestivum* has been proven to be effective in cases of thrombocytopenia and pancytopenia by boosting platelet counts, and in another study, *Euphorbia hirta* exhibited a significant increase in the platelet count. *Asparagus racemosus* possessed anti-anaemic and anti-thrombocytopenic properties in an animal study. *Gilo* (*Tinospora cordifolia*) is one of the drugs in this formulation. An animal study of an aqueous extract of *Tinospora cordifolia* showed a significant improvement in platelet counts and suggests that it probably improves the production of platelets from bone marrow due to its antioxidant and immunomodulatory properties. In many studies, the administration of *Carica papaya* leaf extract resulted in an elevation in the platelet count[6,10,12-16].

Another clinical trial utilised Cap PMA, an Ayurvedic composition that includes *Swertia chirata, Tinospora cordifolia*, and *Carica papaya* extract, and was used to enhance platelet count in individuals suffering from endemic fevers. Every patient exhibited enhancement in both clinical and haematological factors, including a notable rise in platelet count. This suggests that the formulation has a preventive effect against endemic fevers[38]. Various phytochemicals, including alkaloids, flavonoids, phenolic compounds, terpenoids, saponins, sterols, and others, are accountable for inducing changes in immunomodulatory properties[17].

The above finding suggested that PUF-150 and PUF-300 mg/kg as curative regimens exhibited increased platelet count, much better than the negative control animals. No statistically significant change was noticed as compared to the control group. PUF showed anti-thrombocytopenic potential and could further be evaluated for longer-duration treatment to determine the promising effect on thrombocytopenia. Nevertheless, our findings showed that PUF has good hepatoprotective and nephroprotective potential.

6. Conclusion

PUF did not show anti-thrombocytopenic activity in a preventive study at both dose levels, PUF-150 and PUF-300 mg/kg. But in the curative regimen, PUF-150 and PUF-300 exhibited increased platelet count much better in animals than the busulfan control animals and showed mild anti-thrombocytopenic activity. Also, the hepatoprotective and nephroprotective potential of PUF was observed in preventive and as well as in curative regimens.

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8. Author Contribution

Conceptualization: MHK, MU, GMH, and MZ; Resources: SK, MU, GMH, MZ, and MHK; Methodology, Analysis and Visualization: SK, MU, GMH, MZ; Writing: MZ, MU and GMH; Review and Editing: All; Supervision: MU, MHK, MZ, GMH, and YIM; Project administration: MHK, MU, GMH, and YIM. All authors have read and agreed to the published version of the manuscript.

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