



Effect of Selected Fruit Wastes on Hematological Parameters in DEN-Induced Hepatic Carcinoma in Rats

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| Article History | Abstract |
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| Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 21 Nov 2023 | <p>Fruits and processed food demand has greatly increased due to which a huge amount of fruit waste is generated. In this study, the effect of the ethanolic extract of waste material <i>Punica granatum</i> (pomegranate) peel and <i>Vitis vinifera</i> (grapes) seeds was evaluated on hematological parameters in Wistar rats with diethylnitrosamine (DEN) induced hepatocellular carcinoma. The treatments were divided into four groups: Group 1 was untreated while Groups 2-4 received 200 mg/kg body weight of DEN by single intraperitoneal administration. Groups 3 and 4 received DEN and co-treated with 400 mg/kg pomegranate peel extract and 400 mg/kg grape seed extract, respectively. There was a significant decrease in the body weight of animals in Group 2, while Group 3 and 4 animals were found to have a significant rise in body weight. The weight of the liver was significantly increased in the cancer-bearing group and its size was significantly reverted in the treated groups. The kidney and spleen showed a significant decrease in size of cancer-induced groups, and these organs significantly increased in treated groups. Hemoglobin, red blood cells (RBC), neutrophil, packed cell volume (PCV), Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were significantly reduced in Group 2 animals compared to Group 1. The levels of white blood cells (WBC) and erythrocyte sedimentation rate were increased significantly in Group 2 animals relating to Group 1. Both in Group 3 and 4 animals, these changes were reversed. Results reveal that selected fruit wastes alleviate vital hematological parameters in hepatocellular carcinoma-induced rats.</p> |
| CC License CC-BY-NC-SA 4.0 | Keywords: <i>Punica granatum</i> ; Neutrophil; Packed cell Volume; Hemoglobin; Ethanol extract |

1. Introduction

Fruit wastes are the reservoir of renewable and environmentally friendly resources that may be utilised to create a variety of high-value products in line with the “circular economy” (Yoong, & Jo-Shu, 2022). Annually, more than 887 million metric tonnes of fruits are produced each year, there is considerable growth in the demand for fruits and processed goods. Nevertheless, due to the inedible portion of fruits (Peel, seeds, core, pomace, rind) are generated during the process of food processing (Solangi et al., 2021) have provided a list of typical fruit waste and their corresponding trash volumes, producing between 25 to 57 million tonnes of garbage annually. Fruit peel waste represents 15-60% of total fruit waste produced and is often thrown away into the garbage (Rifna et al., 2023). Fruit waste causes significant environmental burdens and is a major source of pollution that, if not managed properly, may cause health issues in humans and animals. Therefore, waste recycling and recovery of resources are essential for the effective valorization of fruit wastes. Hepatocellular carcinoma (HCC) is one of the most common malignancies across the globe, accounting for more than 80% of cases with an estimated five million deaths (WHO, 2016) and also ranks third place among all cancers including cancer-associated mortality (Kang et al., 2022). The causes of hepatic carcinoma include chronic viral

hepatitis, toxins such as aflatoxin, alcohol, α -1-antitrypsin deficiency, hemochromatosis, and non-alcoholic fatty liver disease (Kar, 2014). The primary cause of HCC includes Hepatic B and C viral infections. Patients with hepatic cirrhosis account for 80% of hepatic cancer cases (Llovet et al., 2018). The minor factors that contribute towards HCC include exposure to chemical carcinogens, fatty liver diseases, an overload of iron, and alcohol abuse (Park et al., 2009). In many animal species, nitrosamines like Diethylnitrosamine are acute hepatotoxins. DEN increases free radicals' production, leading to oxidative stress and cellular damage (Ramakrishnan et al., 2006).

High amounts of DEN given orally or intravenously to rats over a long period are very successful at causing hepatic tumours (Schmaehl et al., 1960). DEN a hepatocarcinogen is used to induce hepatic carcinoma in experimental rats by a single intraperitoneal injection of 200mg/kg body weight (Zhang et al., 2023)

Herbal-based medicine is the oldest form of health care known to humans. Due to their anti-tumour properties, plant-based products have recently attracted a lot of interest in the scientific community (Murthy et al, 2021). Plants are the major source of potential anti-cancer drugs, and they are currently used in 60% of anti-cancer drugs, including plants, marine organisms, and microorganisms (Khalifa et al., 2019).

Phytochemicals are naturally occurring bioactive compounds found in plants and play a major role in defence mechanisms against many diseases. The secondary metabolites of plants comprise alkaloids, terpenoids, and phenolic compounds (Khalifa et al., 2019). Saponins, tannins, terpenoids, steroids, flavonoids, and alkaloids possess anti-inflammatory potential (Lee et al., 2013). Glycosides, tannins, alkaloids, and flavonoids show potential hypoglycemic effects (Ota & Ulrich, N.P., 2017). The potent phytoconstituent terpenoids decrease blood glucose levels in animal studies (Nazaruk & Borzym-Kluczyk., 2016). The secondary metabolites, especially steroids and saponins, are known to play a major role in central nervous system activities (Argal, A & Pathak, A.K., 2006). *Punica granatum* L. is a shrub native to the Mediterranean region. It's commonly known as pomegranate and its peel is rich in tannins, flavonoids, polyphenols, and some anthocyanins as delphinidins, cyanidins, and also exerts various biological activities (Rahmani, A.H., et al., 2017). *Vitis vinifera* commonly known as grapes, a popular fruit cultivated across the globe, is the reservoir of many phytochemicals including anthocyanins and resveratrol, which possess many health benefits and pharmacological effects (Nassiri-Asl & Hosseinzadeh., 2009). The *Punica granatum* juice and Punicalagin possess chemopreventive effects against Hepatic carcinoma. Both these plant components down-regulate miR-21 expression and trigger apoptosis with *Punica granatum* juice shows superior activity by improving the hepatic antioxidant and inflammatory status. The grape seed extract possesses antioxidative, anti-inflammatory, antimicrobial, cardioprotective, hepatoprotective, and neuroprotective activities (Rameshrad et al., 2019; Kumar & Vijayalakshmi, 2015). Many inflammatory cells in tumour tissue and peripheral blood promote tumour development by altering the homeostasis of the immune microenvironment (Arvanitakis et al., 2021). Due to its routine nature and ease of detection, the complete blood count (CBC) is a frequent parameter employed as a predictor of tumour prognosis due to its high reproducibility and usefulness in healthcare situations (Fang et al., 2022). However, the effect of the extracts of pomegranate peel (PGPE) and grape seed (VVSE) on hematological parameters in HCC-induced rats is not fully understood and requires to be addressed to provide an insight into the beneficial role of PGPE and VVSE in alleviating HCC in rats. Both fruit wastes selected for the study contain more bioactive compounds and this study is carried out to provide a rationale for the hematological changes associated with experimentally induced HCC in rats, and also to recover and recycle the wastes into the pharmacologically active product.

2. Materials And Methods

Plant material

The two fruits used for this investigation were procured from the same cultivar at the Koyambedu Fruit Market in Chennai, India. Dr.P. Jayaraman, Director, NIHS, Plant Anatomy Research Centre, Chennai, identified the authenticity of both fruits.

Drugs and Chemicals

Diethylnitrosamine, Phenobarbital, Drabkin's Reagent, potassium ferricyanide, Cyanomethemoglobin, formalin, trisodium citrate, and Leishman stain. All chemicals used were of analytical grade.

Preparation of Extract

The ethanolic extracts of pomegranate peel and grape seeds were prepared for the study using the method followed by (Kumar & Vijayalakshmi, 2015). The obtained ethanol extracts were stored in air-tight screw-cap glass containers, under refrigerated conditions until use.

Selection of Animals

Male Wistar rats weighing 150-200 g used for this study were procured from Kings Institute, Chennai, India, and maintained at BRULAC, Saveetha University, Chennai, under standard conditions. The animals used for the study were fed and maintained as per the standard guidelines provided by CPCSEA (IAEC No. Biochem BWC.004/2009).

Experimental Design

The carcinogen diethylnitrosamine (DEN) was used to induce hepatic carcinoma in rats (200mg/kg body weight by i.p.) and after two weeks of induction, 0.05% phenobarbital was used to promote the hepatic carcinoma by supplementing for 20 successive weeks. The dosage and the timeline of this study were done following the procedure of [23]. The Wistar rats used for this study were divided into four groups of seven each as detailed below:

Group 1: Animals were normal control and were given normal saline only.

Group 2: Animals were hepatic carcinoma-induced positive control and were given DEN (200mg/kg body weight by i.p.).

Group 3: Animals were induced cancer as in Group 2 and were co-treated with *Punica granatum* fruit peel extract (400 mg/kg body weight orally) for 12 weeks.

Group 4: Animals were co-treated with *Vitis vinifera* seed extract (400 mg/kg body weight orally) for 12 weeks after cancer induction as in Group 2.

The experimental design of the study is represented in pictorial form (figure 1)

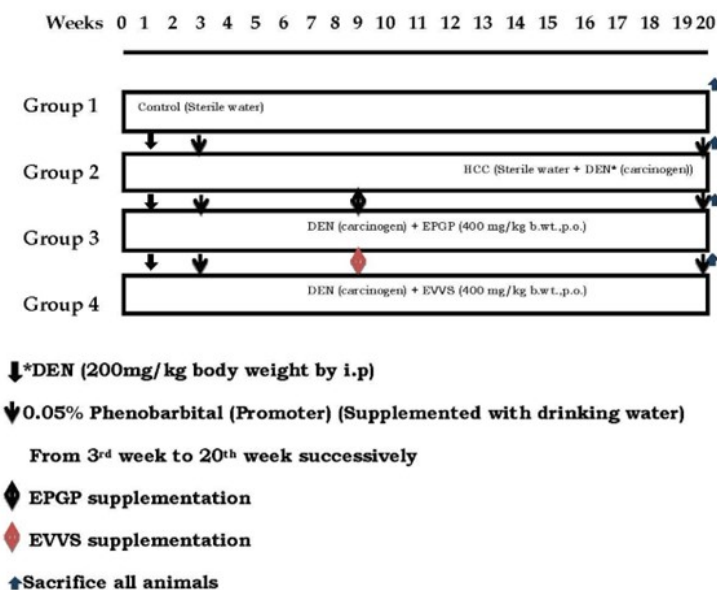


Fig 1: Experimental design of the Animal studies

Hematological parameters

All the animals after the experimental period were sacrificed by cervical decapitation. The blood samples were collected by the Cardiac puncture method to determine the constituents of blood and used for further analysis.

The level of hemoglobin (Hb) (Drabkin & Austin., 1932)

5 ml of Drabkin's Reagent was used to dilute 0.2 ml of blood, mixed well, and allowed to stand for 10 minutes until the completion of the reaction. The solution was read at 540 nm along with 0.2 ml of standard solution Cyanomethemoglobin diluted with 5 ml of Drabkin's reagent.

Red Blood cells (RBC) and White Blood cells (WBC) were estimated by the standard procedure. (Cheesbrough & McArthur, 1976)

Enumeration of RBC

In the RBC pipette, blood was taken until it reached the 0.5 mark and then dilution fluid was drawn up to the 101-mark followed by shaking for 3 minutes. The Neubaur chamber was charged and the RBC was counted.

Enumeration of WBC

In the WBC pipette, blood was taken at 0.5 points. Then, diluting fluid was prepared up to the 11 markings followed by 3 minutes of shaking and then the Neubaur chamber was charged and the WBC was counted.

The level of Packed cell volume (PCV) using a wintrobe tube and mean corpuscular volume (MCV) were determined by the method of (Sood., 2015).

Differential count of white blood corpuscles was done by the Leishman stain method.

The whole blood was taken on the slide and spread with a pair of micro haematocrit capillary tubes. The slide was covered with undiluted Leishman's stain for 2 minutes. Then add the stain on the slide. The staining was allowed to continue for 5 to 7 minutes. The stain was then washed in a stream of buffered water until it acquired a pinkish tinge. The blood film was then dried in a drying rack. The cells were then identified based on size and shape.

Standard laboratory methods were adopted for the determination of MCH (Mean corpuscular hemoglobin) and MCHC (Mean corpuscular hemoglobin concentration). Apart from the hematological parameters, the weight of the kidney, liver, heart, spleen and whole body of experimental animals was recorded using a digital balance.

Statistical Analysis

Statistical analyses for the studies were performed by one-way analysis of variance (ANOVA) using SPSS version 20 (IBM, USA) with a statistical significance of $p < 0.05$. The graph was plotted by Graph Pad Prism version 6.02.

3. Results and Discussion

The rats selected for the study were randomly separated into four groups. Bodyweight and tumour incidences of all four groups are represented in Fig. 2. There was a significant decrease ($p < 0.001$) (a) in the body weight of animals induced with hepatic cancer (Group 2) in comparison with Group 1 animals. PGPE ($p < 0.01$) (b) and VVSE ($p < 0.01$) (b) treated animals (Group 3 and Group 4) showed a significant increase in body weight when compared with Group 2. All seven animals of Group 2 treated with DEN developed tumours but all the animals survived till the study was completed. The group co-treated with selected extracts showed a significant reduction in the tumour incidence with one animal from PGPE treated (Group 3) and two animals from VVSE treated (Group 4) developing a tumour with all seven animals of both the groups survived till the end of the study.

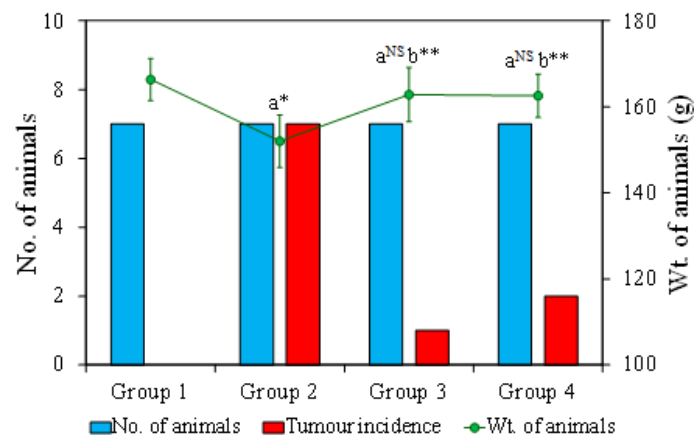


Fig. 2: Tumour incidence and weight of animals. Results expressed as Mean \pm SD (error bars); n = 7 animals.

Group 1: Control rats; Group 2: HCC induced animals; Group 3: Co-treated with PGPE (400 mg/kg b.w); Group

4: Co-treated with VVSE (400 mg/kg b.w). Statistical significance: * = $p < 0.001$; ** = $p < 0.01$; NS - Non-

significant; a - comparison with Group 1; b - comparison with Group 2.

There was a considerable decrease in tumour incidence in the extract-treated groups compared to DEN induced group. The cancer cachexia may be the reason behind decreased body weight in Group 2 and is the reason for more than 20% of cancer mortality (Tisdale., 2002). Cachexia refers to a complex metabolic process with progressive loss of weight and lessening of host adipose tissue and skeletal muscle reserves. The decreased body weight is due to less food intake and poor absorption, which may cause muscle wasting and weight loss in tumour cachexia (Petruzzelli *et al.*, 2016). Due to cachexia, Group 2 animals significantly dropped their body weights. On co-treatment with both extracts in Group 3 and Group 4, the body weight shows a significant increase, and this may be due to the antineoplastic potential of PGPE and VVSE.

In the cancer-induced Group, 2 animals, the high proliferation of the cancer cells may be the reason for significant tumour progression. The treatment with the selected extracts may show significant tumour regression due to the inhibition of tumour growth which shows that selected extracts may be potential anti-cancer agents. The weight change of essential organs of animals in different treatment groups is shown in Fig. 3. The weight of the liver is significantly increased ($p < 0.001$) (a) in Group 2 animals and its size is reverted towards the control group in the PGPE and VVSE treated groups with a significance of $p < 0.001$ (b) (Fig. 3A). This result was supported by the findings of Nermin and co-workers (Sadik *et al.*, 2008), where blueberries supplementation decreased the size of the liver in DEN induced hepatocarcinogenesis towards the normal. In the present study, the size of the heart showed no significant changes in the experimental animals (Fig. 3C). The kidney ($p < 0.05$) (a) and spleen ($p < 0.01$) (a) showed a significant decrease in their size in cancer-induced groups and the size of these organs showed improvement with a significant increase in size when treated with PGPE and VVSE. Both the kidney and spleen showed a significant increase ($p < 0.01$) (b) with PGPE and VVSE (Fig. 3B, 3D)

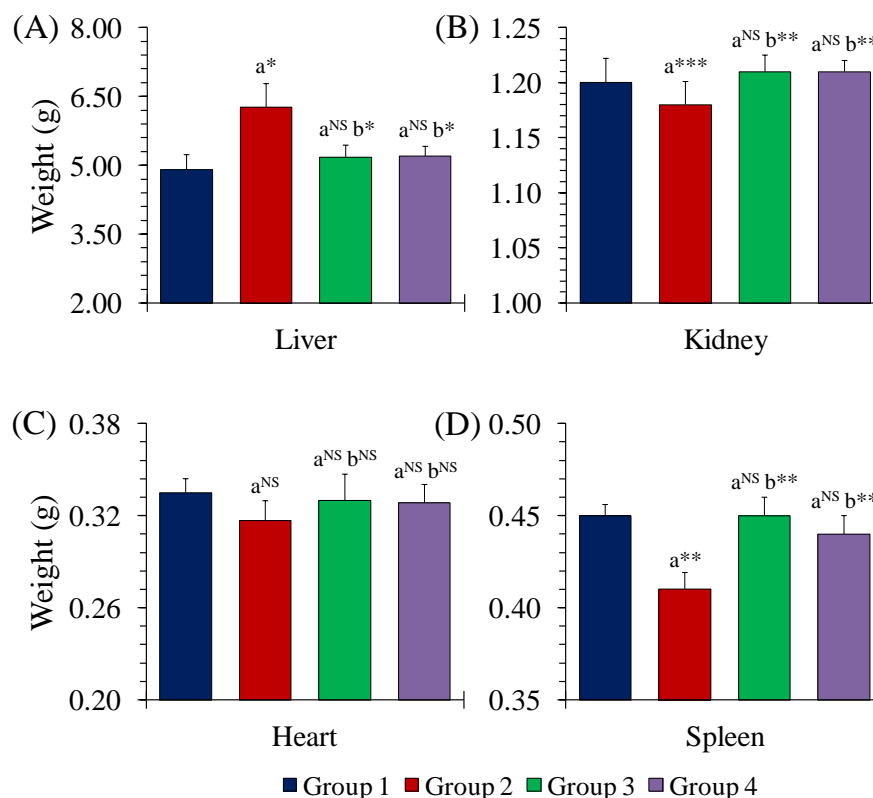


Fig. 3: Weight of essential organs of animals: (A) Liver, (B) Kidney, (C) Heart and (D) Spleen. Results expressed as Mean \pm SD (error bars); $n = 7$ animals. Group 1: Control rats; Group 2: HCC induced animals; Group 3: Co-treated with PGPE (400 mg/kg b.w); Group 4: Co-treated with VVSE (400 mg/kg b.w). Statistical significance: * = $p < 0.001$; ** = $p < 0.01$; *** = $p < 0.05$; ^{NS} - Non-significant. a- comparison with Group 1; b - comparison with Group 2.

The results shown in Fig. 4, Tables 1 and 2 represent the changes in hematological parameters of blood samples of control and treated animals. The Hb, RBC, neutrophil, PCV, and lymphocytes were significantly reduced ($p < 0.001$) (a) in Group 2 animals when compared with Group 1 animals. The MCV, MCH, and MCHC were also significantly decreased ($p < 0.001$) (a) in Group 2 when compared with Group 1. The WBC and erythrocyte sedimentation rate (ESR) ($p < 0.001$) (a) levels were increased significantly in Group 2 when compared with Group 1. In both Group 3 and Group 4 animals, these changes were brought back towards the normal range. Blood is a significant index of physiological and

pathological conditions in humans and animals. The parameters usually measured are hemoglobin, RBC, WBC count, PCV, neutrophils, lymphocytes, eosinophils, and ESR. In the present study, the blood cells were significantly altered in Group 2 as compared to Group 1.

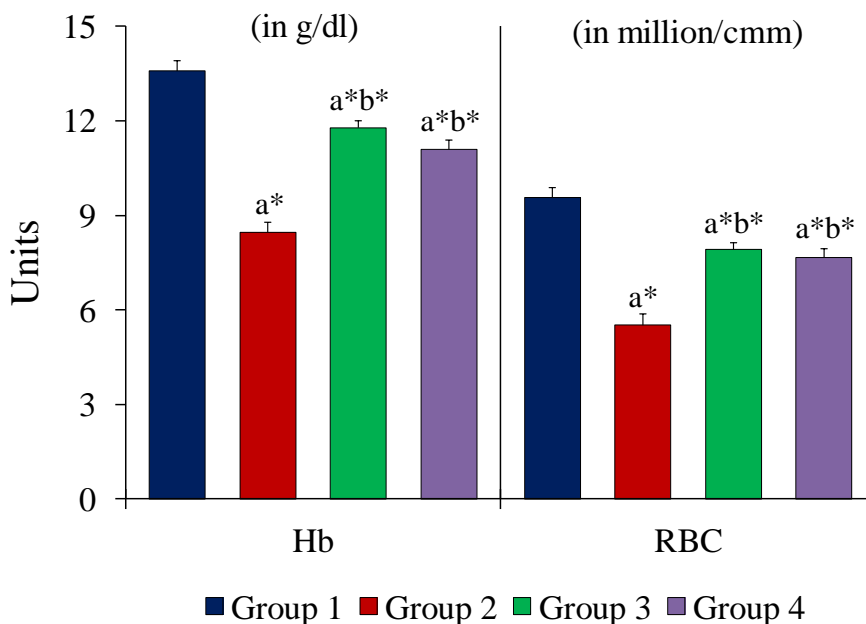


Fig. 4: Hemoglobin level and RBC count in experimental animals. Results expressed as Mean ± SD (error bars); n= 7 animals. Group 1: Control rats; Group 2: HCC induced animals; Group 3: Co-treated with PGPE (400 mg/kg b.w); Group 4: Co-treated with VVSE (400 mg/kg b.w). Statistical significance: * p<0.001: a-comparison with Group 1; b - comparison with Group 2.

Table 1. PCV, ESR, MCV, MCH And MCHC In Experimental Animals.

| Groups | PCV (%) | ESR (mm/hr) | MCV(C.Microns) | MCH (Picogram) | MCHC (%) |
|---------|---------------------|-------------------|-----------------------|----------------------|---------------------|
| Group 1 | 47.36 ± 0.54 | 3.53 ± 0.13 | 34.05 ± 1.68 | 17.1 ± 1.06 | 37.44 ± 1.27 |
| Group 2 | 37.63 ± 0.94 a* | 5.36 ± 0.2 a* | 23.13 ± 1.77 a* | 12.5 ± 0.97 a* | 24.19 ± 1.06 a* |
| Group 3 | 43.89 ± 0.69 a*, b* | 2.8 ± 0.09 a*, b* | 26.58 ± 1.1 a*, b* | 14.51 ± 1.19 a*, b** | 29.04 ± 1.71 a*, b* |
| Group 4 | 43.18 ± 0.68 a*, b* | 2.9 ± 0.2 a*, b* | 25.38 ± 1.56 a*, b*** | 14.1 ± 1.00 a*, b*** | 27.1 ± 1.53 a*, b** |

Results expressed as Mean ± SD; n= 7 animals; Group 1: Control rats; Group 2: HCC induced animals; Group 3: Co-treated with PGPE (400 mg/kg b.w); Group 4: Co-treated with VVSE (400 mg/kg b.w); Statistical significance: * = p<0.001; ** = p<0.01; *** = p<0.05; a – comparison with Group 1; b – comparison with Group 2. Packed cell volume (PCV), ESR (Erythrocyte sedimentation rate), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean corpuscular Hemoglobin concentration)

Table 2. Hematological Parameters in Experimental Animals.

| Groups | WBC (1000/cmm) | Neutrophil (%) | Lymphocytes (%) | Monocytes (%) |
|---------|---------------------|---------------------|---------------------|--------------------|
| Group 1 | 8.35 ± 0.18 | 32.54 ± 0.55 | 67.55 ± 1.19 | 0.97 ± 0.06 |
| Group 2 | 15.03 ± 0.20 a* | 24.61 ± 0.66 a* | 43.10 ± 1.33 a* | 0.33 ± 0.03 a* |
| Group 3 | 11.51 ± 0.27 a*, b* | 28.46 ± 0.82 a*, b* | 55.65 ± 1.28 a*, b* | 0.76 ± 0.03 a*, b* |
| Group 4 | 11.40 ± 0.29 a*, b* | 29.11 ± 0.95 a*, b* | 53.98 ± 2.03 a*, b* | 0.75 ± 0.03 a*, b* |

Results expressed as Mean ± SD, n= 7 animals; Group 1: Control rats; Group 2: HCC induced animals; Group 3: Co-treated with PGPE (400 mg/kg b.w); Group 4: Co-treated with VVSE (400 mg/kg b.w). Statistical significance: * = p<0.001 comparison with Group 1; b - comparison with Group 2. WBC (White blood cells)

Anemia is frequently found to occur in cancer patients and the reduction in RBC or hemoglobin percentage results in anemia in tumour-bearing rats which can cause fatigue, shortness of breath, and light-headedness in cancer-bearing animals and this can also occur due to the deficiency of iron as a result of the reduction in hemoglobin or due to hemolytic conditions (Busti *et al.*, 2018; Ballard, 1997). Low hemoglobin/ RBC distribution width ratio levels are potential tumour prognostic markers and are linked to poor patient prognosis. One of the components of RBC is hemoglobin and in the present investigation, the hemoglobin levels are significantly reduced which may be one of the reasons for reduced RBC levels in tumour-bearing animals. It was observed that there was a significant reduction ($p < 0.001$) in MCV, MCH, and MCHC levels of Group 2 when compared to Group 1 which may be due to the reduction in hemoglobin content. The decrease in MCV and MCH observed in iron-deficient anemia was due to the absolute reduction of the iron store or reduction in Hb percentage Nairz *et al.*, 2016; Yenilmez & Tuli, 2018). In the tumour-bearing group, it was found that there was an increase in WBC and ESR count following cancer cell proliferation. In the differential count of WBC, the percentage of neutrophils and lymphocyte count were significantly decreased in the cancer-induced group. The PGPE and VVSE treated group restored all the altered hematological parameters to normal. The PGPE and VVSE treated groups significantly reversed the hemoglobin, RBC, WBC, PCV, neutrophils, lymphocytes, and ESR to normal values. The present study indicates both the selected extracts might have possessed the protective action on the hemopoietic system.

4. Conclusion

P. granatum peel and *V. vinifera* seeds are non-edible parts of common fruits consumed worldwide. They are rich in many nutrients and phytochemicals. The present study shows that both the selected plant extracts, PGPE, and VVPE significantly reversed the effect of the weight of animals, vital organs, and hematological parameters due to DEN-induced HCC. There were significant changes in RBC, WBC and other hematological parameters in PGPE and VVSE-treated groups of rats respectively compared to DEN-induced cancer-treated groups. The study shows that the selected fruit materials are having anti-cancer potential against DEN-induced HCC and improve hematological parameters in rats. The study results show that *Punica granatum* peel extract shows better activity than the *Vitis vinifera* seed extract. The combined use of both the plant waste is not analyzed in the study and also the combined extract might have a synergetic effect which might affect the overall effect. Formulating the drugs from these fruit wastes will help utilize the bioactive compounds in the nonedible waste material as pharmacologically active drugs and recycle them for waste management.

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Conflict of Interest: The authors declare no conflicts of interest related to this research.

Ethical Approval: Ethical approval for this study was obtained for Animal Studies from the Institutional Animal Ethical Committee.

Data Availability: Not applicable.

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