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Protein extract of kenaf seed exhibits anticoagulant, antiplatelet and antioxidant activities

Sujatha M. Hanumegowda[1], Chandramma Srinivasa[2], Ashwini Shivaiah[2], Manjula M. Venkatappa[1], Ramesha Hanumanthappa[2], Rajesh Rangappa[3], Ramesh K. Laxmaiah[4], Sathisha J. Gonchigar[1]✉, Devaraja Sannaningaiah[2]✉

[1]*Department of Biochemistry Jnansahydri, Kuvempu University, Shankarghatta−577451, Shivamogga, Karnataka, India*

[2]*Department of Studies and Research in Biochemistry and Centre for Bioscience and Innovation, Tumkur University, Tumkur, Karnataka, India*

[3]*Liveon Biolabs Private Limited, Tumkur−572106, India*

[4]*Department of Food Science and Nutrition, Maharani's Science College for Women, Mysore, India*

ABSTRACT

Objective: To explore the anticoagulant, antiplatelet and antioxidant activities of protein extract of kenaf seed (PEKS).

Methods: Sodium dodecyl sulphate polyacrylamide gel electrophoresis and reverse-phase high-performance liquid chromatography techniques were employed for protein characterization. Antioxidant activity of PEKS was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The protective effect of PEKS on sodium nitrite ($NaNO_2$) induced oxidative stress was evaluated using the *in vitro* red blood cell model, while the effect of PEKS on diclofenac-induced oxidative stress was examined *in vivo* in rats. Platelet-rich plasma and platelet-poor plasma were used for anticoagulant and antiplatelet activities of PEKS.

Results: PEKS revealed similar protein bands on SDS-PAGE under reduced and non-reduced conditions. Several acidic proteins were present in native PAGE. PEKS showed antioxidant properties by scavenging DPPH with an IC_{50} of 24.58 μg. PEKS exhibited a protective effect on $NaNO_2$ induced oxidative stress in red blood cells by restoring the activity of stress markers. In addition, PEKS alleviated diclofenac-induced tissue damage of the liver, kidney, and small intestine. PEKS showed an anticoagulant effect in both *in vivo* and *in vitro* experiments by enhancing normal clotting time. PEKS did not affect prothrombin time but increase activated partial thromboplastin time. Furthermore, PEKS inhibited adenosine diphosphate and epinephrine-induced platelet aggregation.

Conclusions: PEKS protects tissues from oxidative stress and exhibits antithrombotic activity.

KEYWORDS: Protein; Extract; Kenaf seed; Oxidative stress; Antioxidant; Anticoagulant; Antiplatelet

1. Introduction

Kenaf (*Hibiscus cannabinus* L.) is a raw fiber plant that belongs to the family Malvaceae and the genus *Hibiscus*. It is a widely distributed annual crop containing more than 200 species[1]. Kenaf is native to central Africa and is also found in China, India, USA, and Thailand. Kenaf plant is valuable globally for its broad spectrum of utilities. Malaysia and Africa have been growing kenaf plants on a large scale for horticulture and commercial applications. Stalk, leaves, and seeds of the kenaf plant have been widely used for a long time due to their various medicinal

Significance

Although kenaf seed stores the highest amount of proteins, only a few studies documented their nutritional benefits. So far, none of the studies claims the protective role of kenaf seed proteins on oxidative stress and its associated complications such as thrombosis and tissue damage. This study shows the antioxidant, anticoagulant, and antiplatelet activities of the protein extract of kenaf seed.

✉To whom correspondence may be addressed. E-mail: sdevbiochem@gmail.com (Sannaningaiah D); satishlec@gmail.com (Gonchigar SJ)

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properties. The therapeutic potency of kenaf is attributed to dietary fibers, proteins, unsaturated fatty acids, and edible oils[2]. Kenaf seeds are brown with 6 mm long and 4 mm wide. Compared to other parts of kenaf, the seed contains a higher amount of proteins and other phytoconstituents. Therefore, kenaf seeds have been consumed in many parts of the world. Most importantly, seeds have been widely exploited commercially as functional food products, livestock feed, and for medicinal purposes[3]. Edible oil of kenaf seeds exhibits antioxidant, anticancer, anti-inflammatory, antithrombotic, antimicrobial, and chemopreventive activities[4]. Several research groups have characterized its phytochemicals such as tannins, saponins, polyphenols, alkaloids, essential oils, phytosterols, tocopherols, phenolic acids, and fatty acids from kenaf seeds[5]. Although kenaf seeds contain 21.4% to 30% proteins, their pharmacological properties are least characterized to date. Pshenichnov *et al*. isolated biocidal peptide from the kenaf seed for the first time[6]. In general, enzymatic/non-enzymatic proteins from plants possess immense therapeutic benefits. For instance, enzymatic proteins such as actinidin from kiwifruit, bromelain from pineapple, cardosin from cardoon, cucumisin from melon, ficin from fig, oryzasin from rice, papain from papaya, phytesin from barley, and gingipains from ginger were widely studied. Non-enzymatic proteins such as B-conglycinin from soybean, glycinin from pea, hypogin from peanuts, glutenin from rice, thaumatin-like proteins from peach, amandin from almonds, gluten from wheat were extensively studied[7]. Even though kenaf seeds are rich in proteins with numerous health benefits, none of the studies validate their therapeutic efficacy so far. Therefore, in this study, we attempted to examine the protective role of the protein extract of kenaf seed (PEKS) against oxidative stress and thrombosis.

2. Materials and methods

2.1. Chemicals and reagents

Molecular weight markers were procured from Bangalore Genei Private Limited, India. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) reagents were acquired from AGAPPE diagnostic Private Limited, Ernakulum, Kerala, India. Ethylene diamine tetra acetic acid (EDTA), trichloro acetic acid, adenosine diphosphate (ADP), and epinephrine were purchased from Sigma Chemical Company (St. Louis, USA). 2,4-Dinitrophenylhydrazine, sodium nitrite ($NaNO_2$), sodium dodecyl sulfate (SDS), acetic acid, thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), tetra methyl ethylene diamine (TEMED), quercetin, and hydrogen peroxide were purchased from local suppliers. All other chemicals used were of analytical grade. The blood was drawn from healthy volunteers of age 18-25 years, who were not under medications for 3 weeks, non-smokers, and non-alcoholic. The blood was instantly mixed with tri-sodium citrate tubes. A complete blood count was done before the experiments to ensure normal blood cell count. Blood samples were centrifuged at 800 and 3 000 rpm for 15 min to get platelet-rich plasma (PRP) and platelet-poor plasma (PPP). Before aggregation, the platelet count was adjusted to 3.1×10^5 platelets/mL.

2.2. Animals

Sprague Dawley rats weighing 100-120 g were procured from Liveon Bio Labs, Limited, Institutional Animal Ethical Committee (Tumkur, India) and kept in polypropylene cages for acclimatization for 6 d in groups. A standard laboratory pellet diet was provided for the rats along with the ordinary tap water and cattle feed *ad libitum*. The animals were kept in the animal house at a temperature of (27±2) ℃, 55%-65% humidity, under a 12 h light/12 h dark cycle.

2.3. Preparation of PEKS and protein estimation

Kenaf seeds were collected from Tumkur local market. About 50 g of seeds were washed thoroughly, ground into a powder with a grinder (SHARP, Japan), and added with 500 mL of double-distilled water. The sample was thoroughly mixed and centrifugated for 20 min at 1 500×*g* for 15 ℃. Finally, the supernatant protein was estimated by Lowry *et al*[8].

2.4. Ammonium sulphate precipitation

The obtained protein sample was subjected to ammonium sulphate precipitation using 40% ammonium sulphate, and centrifuged at 1 500 rpm for 20 min; the pellet was collected, dissolved in double distilled water, and dialyzed overnight. Then the obtained protein sample was concentrated using a vacuum evaporator. The yield of the protein extract was approximately 50% (*w/w*). The dried PEKS was stored at 4 ℃. The required amount of protein powder was weighed and dissolved in double distilled water for further studies.

2.5. SDS–PAGE and Periodic Acid–Schiff (PAS) staining

The method of Laemmli[9] was employed. The crude PEKS (100 μg) was prepared under reducing and non-reducing conditions. At room temperature, electrophoresis was carried out for 2 h, using running buffer tris (25 mmol/L), glycine (192 mmol/L), and SDS (0.1%). After electrophoresis, the gel was stained using 0.1% Coomassie brilliant blue R-250 and de-stained with 40% ethanol in 10% acetic acid and water (40:10:50 *v/v*). Standard molecular weight markers (15 to 125 kDa) were used. For the PAS

staining, the method of Leach *et al*.[10] was employed. Briefly, the gel obtained from electrophoresis was placed in 7.5% acetic acid solution for 1 h at room temperature. Then the gel was thoroughly washed using hydrochloric acid (1%) solution placed in periodic acid (0.2%) solution for 45 min at 4 ℃. Later, the gel was placed in Schiff's reagent at 4 ℃ for 24 h. The pink color band was visualized by de-staining with acetic acid (10%).

2.6. Determination of antioxidant activity by DPPH assay

The DPPH radical scavenging activity was measured by following the method of Okoh *et al*[11]. DPPH (0.04 mg/100 mL) radical solution was prepared using 95% ethanol. PEKS (10-50 μg) was dissolved in water mixed with 150 μL of DPPH radical solution and the final volume was made up to 600 μL using 95% ethanol incubated for 30 min in dark at room temperature. The absorbance was measured at 517 nm. Ethanol was used as a blank and the ascorbic acid was used as a positive control. The scavenging activity was calculated using the formula given below.

DPPH scavenging activity (%)=(Absorbance of blank-Absorbance of the sample)/(Absorbance of blank)×100

2.7. Animal grouping

Female Sprague Dawley rats were divided into five groups of six rats each. The dose selection of PEKS depended upon the weight of the experimental rats. The dose that showed fair activity was selected for further studies and the experiment was carried out as follows: Group 1: Control [saline 1 mL/kg body weight (b.w.)]; Group 2: Diclofenac (DFC) alone (50 mg/kg b.w./day, *i.p*. per day); Group 3: Silymarin (SLY) (25 mg/kg b.w./day, *i.p*.) was injected intraperitoneally. After 45 min, DFC (50 mg/kg b.w./day, *i.p*.) was injected; Group 4: PEKS (300 mg/kg b.w./day, *i.p*.) was injected intraperitoneally. After 45 min, DFC (50 mg/kg b.w./day, *i.p*.) was injected; Group 5: PEKS alone (300 mg/kg.b.w./day, *i.p*.). The above procedure was followed for 5 d. On the sixth day, rats were allowed to fast for 12 h after the last dosage. Then the rats were euthanized using diethyl ether and the blood samples were collected by cardiac puncture and the coagulation parameters such as PT and aPTT were measured.

2.8. In vitro and in vivo assessment of stress markers

The tissues of organs such as the liver, kidney, and small intestine were obtained from the rats and homogenized in 0.1 mol/L ice-cold phosphate-buffered saline (PBS). The homogenized samples were used to evaluate the stress markers such as lipid peroxidation (LPO), protein carbonyl content (PCC), superoxide dismutase (SOD), catalase (CAT) in the liver, kidney, and small intestine, respectively. Hepatoprotective activity of PEKS was also observed by measuring liver enzyme markers like albumin, globulin, total protein, total bilirubin, indirect bilirubin and direct bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT) alkaline phosphatase (ALP), and lactate dehydrogenase (LDH).

2.8.1. Evaluation of LPO

LPO was determined using the method of Ohkawa *et al*[12]. Briefly, each 100 μL of $NaNO_2$ (10 mmol/L) treated RBC lysate (2 mg) was treated with PEKS (50-150 μg/mL) and 2 mg of protein from tissue homogenate (liver, kidney and small intestine) from Sprague rats were mixed with 1.5 mL of acetic acid (pH 3.5, 20% *v/v*), SDS (8% *w/v*, 0.2 mL) and 1.5 mL thiobarbituric acid (0.8% *w/v*). The reaction mixture was boiled at 45-60 ℃ for 45 min and centrifuged at 2 000 rpm for 10 min. The formed adducts were extracted into 1-butanol (3 mL). Thiobarbituric acid-reactive substance was measured spectrophotometrically at 532 nm. Values were expressed in terms of malondialdehyde (MDA) equivalent as μmol MDA formed/mg of protein.

2.8.2. Determination of PCC

PCC was assessed according to the method of Levine *et al*[13]. Protein lysate (2 mg) from RBCs and the tissue homogenates (liver, kidney, and small intestine) of rats were taken in clean and dry test. Subsequently, an equal volume of 10 mmol/L 2,4-dinitrophenylhydrazine was added in a 2 mmol/L HCl mixed well and incubated for 1 h at room temperature. For blank, only 2 mmol/L HCl was used. Trichloroacetic acid (20%, *w/v*) was used for the precipitation of the mixture and centrifuged for 15 min at 1 200×*g*. The obtained precipitate was washed thoroughly using acetone again centrifuged for 15 min at 2 500×*g* to get the pellet. Finally, 1 mL of tris buffer [20 mmol/L pH 7.4 containing 0.14 mol/L NaCl, 2% SDS (*w/v*)] was used to dissolve the pellet and the supernatant was monitored at 360 nm and the results were expressed as μmol carbonyl groups/mg of protein.

2.8.3. SOD activity

SOD enzyme activity was determined according to the method of Sundaram *et al*[14]. The activity was assayed in samples (2% hematocrit) by monitoring the inhibition of quercetin (0.15% *w/v*) autoxidation. About 0.05 mg of protein from the lysate of PEKS (50-150 μg/mL) treated RBCs with an agonist $NaNO_2$ (10 mmol/L) and 2 mg tissue homogenate from the liver, kidney, and small intestine of rats were taken in clean and dry test tubes mixed with reaction mixture (1 mL) that consisted of phosphate buffer (16 mmol/L, pH 7.8) containing TEMED-EDTA (8 mmol/L/0.08 mmol/L) mixture. The decrease in absorbance was examined for 3 min at 406 nm. The amount of protein required to inhibit the autoxidation of quercetin (50%) is considered as one unit. The results were expressed as U/mg of protein.

2.8.4. CAT activity

The CAT enzyme activity was assayed by the method of Beers *et al*[15]. Protein (0.05 mg) lysate from PEKS (50-150 μg/mL) treated RBCs with an agonist $NaNO_2$ (10 mmol/L) and 2 mg tissue homogenate from the liver, kidney, and small intestine of rats were taken in clean and dry test tubes mixed with 1 mL of reaction mixture containing sodium phosphate buffer (100 mmol/L, pH 7.4) and H_2O_2 (8.8 mmol/L). The change in absorbance was measured at 240 nm for 3 min. The CAT activity was expressed as U/mg of protein.

2.9. PT and aPTT

aPTT and PT were measured as described by Gangaraju *et al*[16]. PEKS that was dissolved in double-distilled water (20-100 μg) was pre-incubated with 100 μL each of normal citrated human plasma and plasma obtained from rats for 1 min. For aPTT, 100 μL of LIQUICELINE (Cephaloplastin derived from rabbit brain with phospholipids and ellagic acid preparation) was activated for 3 min at 37 ℃. To induce the clot formation, 100 μL of 0.02 mmol/L $CaCl_2$ was added and clotting time was recorded. For PT, 200 μL of PT reagent (thromboplastin) was added to induce the clot. The time required to form the clot was measured in seconds. The ratio for aPTT and PT was calculated using the control plasma values incubated with the buffer concurrently. Heparin sodium (2.5 μg) was taken as a positive control.

2.10. Histopathological study

The organs such as liver, kidney, small intestine obtained from Sprague Dawley female rats were rinsed in PBS buffer and all preserved tissue samples from each group were fixed in 10% formal saline for at least 24 h. Before being dehydrated, the tissue samples were washed in ascending grades using ethanol cleared with xylene and embedded in paraffin wax. The samples were sectioned with a microtome for a thickness of 5 μm, stained with hematoxyline and eosin (H&E), and examined under a light microscope at ×40 magnification.

2.11. Preparation of PRP and PPP

The preparation of PRP and PPP was done according to the method of Ardlie and Han[17]. To prepare PRP and PPP, anticoagulated blood was centrifuged for 15 min at 800 rpm and 3 000 rpm, respectively. Platelet count was measured using a hematology analyzer and the count was adjusted to 3.1×10^5 platelets/mL. The aggregation experiments were carried out using plastic wares or siliconized glassware and completed within 2 h at 37 ℃.

2.12. Plasma recalcification time

The method of Quick *et al*[18] was followed. Crude PEKS (10-50 μg) was pre-treated with 0.2 mL of PRP in 10 mmol/L tris HCl (20 μL) buffer pH 7.4 for 1 min at 37 ℃. Then, 20 μL of 0.25 mol/L $CaCl_2$ was added to the pre-incubated mixture, and clotting time was monitored. Heparin sodium (2.5 μg) was taken as a positive control.

The bleeding time was done according to the method of Denis *et al*[19]. Briefly, a group of five mice were used in this study. PEKS (10-60 μg) in 30 μL of PBS was intravenously injected through the tail vein. After 10 min, mice were anesthetized using diethyl ether and a sharp cut was made at the tail tip of a mouse in 3 mm length. Immediately, the tail was vertically immersed in PBS at 37 ℃. The bleeding time was recorded.

2.13. Platelet aggregation

The effect of PEKS on platelet function was analyzed by the turbidiometric method of Born[20] using a chronology dual-channel whole blood/optical lumiaggregation system (Model700). PRP (0.25 mL) was pre-incubated with PEKS (10-30 μg), and aggregation was initiated by adding agonists such as ADP (10 μmol/L) and epinephrine (5 μmol/L). The aggregation was followed for 6 min. Aspirin (25 μg) was taken as a positive control. The percentage of platelet aggregation inhibition was calculated using the formula $[(X-Y)/X]\times100$, where X is the % aggregation of control (ADP), Y is the % aggregation of sample.

2.14. Direct hemolytic activity

Packed human erythrocytes (1 mL) and PBS (9 mL) were mixed thoroughly. Hematocrit (1 mL) suspension was incubated with various concentrations of PEKS (50-200 μg) independently for 1 h at 37 ℃. The reaction was terminated by adding 9 mL of ice-cold PBS, centrifuged at 1 000×*g* for 10 min at 37 ℃. The amount of hemoglobin released in the supernatant was monitored at 540 nm and expressed as a percentage of hemolysis against 100% lysis using water that served as a positive control and PBS served as a negative control.

2.15. Edema inducing activity

Edema inducing activity was done according to the method of Sannanaik Vishwanath *et al*[21]. Different doses (50-150 μg) of PEKS in 20 μL saline were injected separately into the footpads of mice (groups of five). The left footpads that received 20 μL saline alone served as control. After 1 h, the mice were anesthetized using diethyl ether inhalation. Hind limbs were cut at the ankle joint and weighed. Weight increase was calculated as the edema ratio, which

equals the weight of the edematous leg ×100/weight of the normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120%.

2.16. Hemorrhagic activity

The method of Kondo *et al*[22] was followed. Various doses of PEKS (50-200 µg) were intradermally injected into the groups of five mice. The mice that received saline (30 µL) alone were taken as the negative control group and the mice received venom (*Vipera russelli* venom) were the positive control. After 3 h, the mice were anesthetized by diethyl ether inhalation. Skin surface (dorsal patch) was removed carefully, and hemorrhagic spots were observed against saline-injected control experimental animals. The minimum hemorrhagic dose (MHD) was defined as the amount of protein that can cause hemorrhage in a 10 mm diameter.

2.17. Statistical analysis

Results are expressed as mean±SD. The significant difference among different groups was done using nonparametric one-way analysis of variance test by using graph pad Prism software version 5.0. $P<0.05$ was considered significantly different.

2.18. Ethical statement

For using human blood samples and experimental animals in the studies, ethical guidelines were approved by the Institutional Human Ethical Committee, Tumkur University, Tumkur and Institutional Animal Ethical Committee, Liveon Biolabs Private Limited, Tumkur with a protocol number LBPL-IAEC-47-05-2019. Experimental animals were handled according to the guidelines by the Committee for Monitoring and Supervision of Experiments on Animals (CPCSEA).

3. Results

3.1. Preliminary characterization of PEKS by SDS–PAGE and RP–HPLC

The PEKS revealed a similar type of protein bands under both reduced and non-reduced conditions on SDS-PAGE, only two prominent protein bands were noticed at 57 kDa and 31 kDa regions (Figure 1A). Basic PAGE of PEKS revealed both the proteins appeared to be acidic in nature (Figure 1B). Interestingly, both the proteins were positive for PAS staining, suggesting they possessed carbohydrate moieties (Figure 1C). RP-HPLC chromatogram of PEKS revealed two peaks at 280 nm supporting the observed protein bands on SDS-PAGE and native PAGE (Figure 1D).

3.2. PEKS exhibits antioxidant activity and protects the $NaNO_2$ induced oxidative stress in RBCs in vitro

PEKS exhibited antioxidant activity by scavenging DPPH free radicals and the observed percentage of radical scavenging was found to be more than 60% with an IC_{50} value of 24.58 µg (Figure 1E). As an indicator of LPO, the level of MDA was measured. There was a significant increase in the level of MDA in RBCs treated with $NaNO_2$ (10 mmol/L). Interestingly, PEKS reduced the level of MDA in RBCs in a dose-dependent manner ($P<0.05$) (Figure 2A). Furthermore, RBCs treated with $NaNO_2$ showed a high level of PCC compared with the control. However, PEKS significantly reduced the PCC dose-dependently in pretreated RBCs ($P<0.05$) (Figure 2B). The activity of antioxidant enzymes such as SOD and CAT was significantly decreased in $NaNO_2$ treated RBCs compared with the control ($P<0.01$), while PEKS restored the activities of SOD and CAT in a significant manner ($P<0.05$) (Figures 2C & 2D). These results indicated PEKS

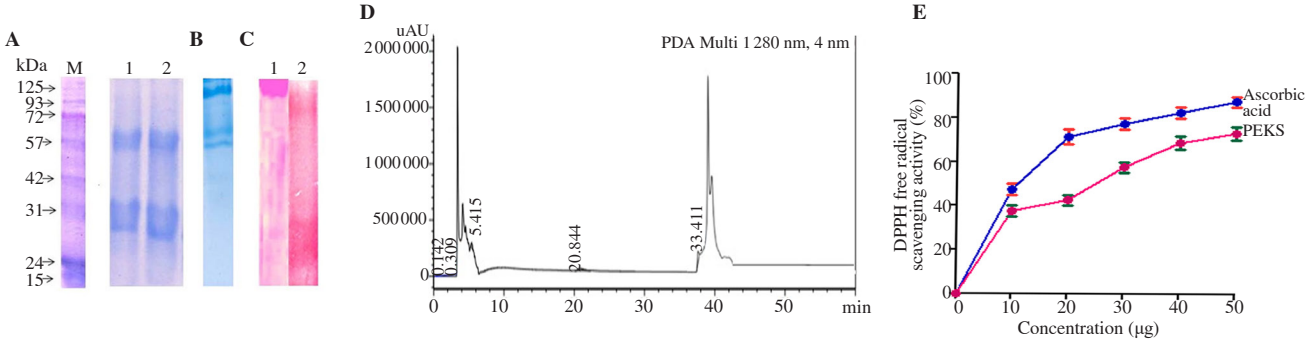

Figure 1. (A) PEKS shown in SDS-PAGE (10%): PEKS (100 µg) under non-reduced (A1) and reduced conditions (A2), (B) Native basic polyacrylamide gel electrophoresis of PEKS (100 µg) under non-reduced condition, (C) Periodic Acid-Schiff staining of PEKS: positive control fibrinogen (Lane 1) and PEKS (100 µg) (Lane 2). (D) RP-HPLC chromatogram of PEKS: PEKS (20 µg) was injected into RP-HPLC connected C18 column. The protein elution was monitored at the flow rate of 1 mL/min using a gradient buffer at 280 nm. (E) DPPH scavenging activity of PEKS: The antioxidant activity of PEKS was measured by DPPH method. Each value is presented as mean ± SD. PEKS: protein extract of kenaf seed. M: protein standard markers.

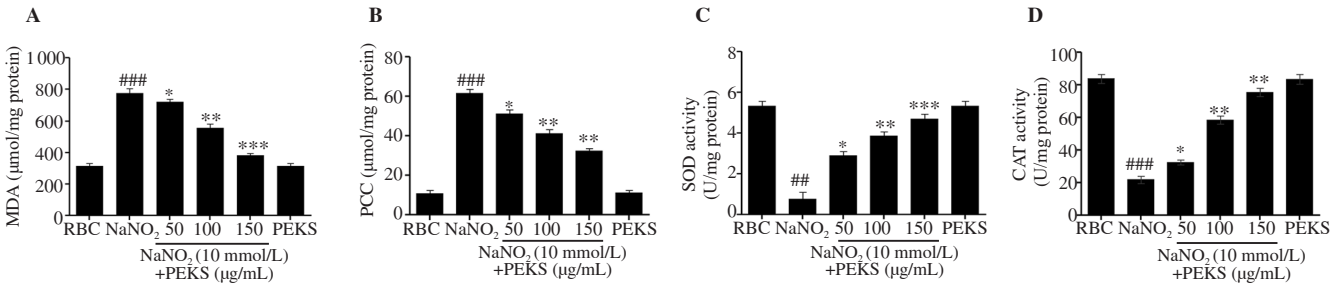

Figure 2. Effect of PEKS on (A) lipid peroxidation, (B) PCC, (C) SOD and (D) CAT in RBCs. For determination of oxidative stress markers, $NaNO_2$ (10 mmol/L) was used as an inducer. The studies were carried out by incubating RBCs with PEKS (50-150 μg/mL) for 10 min at 37 ℃ before $NaNO_2$ (10 mmol/L) administration. The data are expressed as mean ± SD (n=3). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with $NaNO_2$ (10 mmol/L) treated RBCs; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared with untreated RBCs. RBC: red blood cell; PCC: protein carbonyl content; SOD: superoxide dismutase; CAT: catalase.

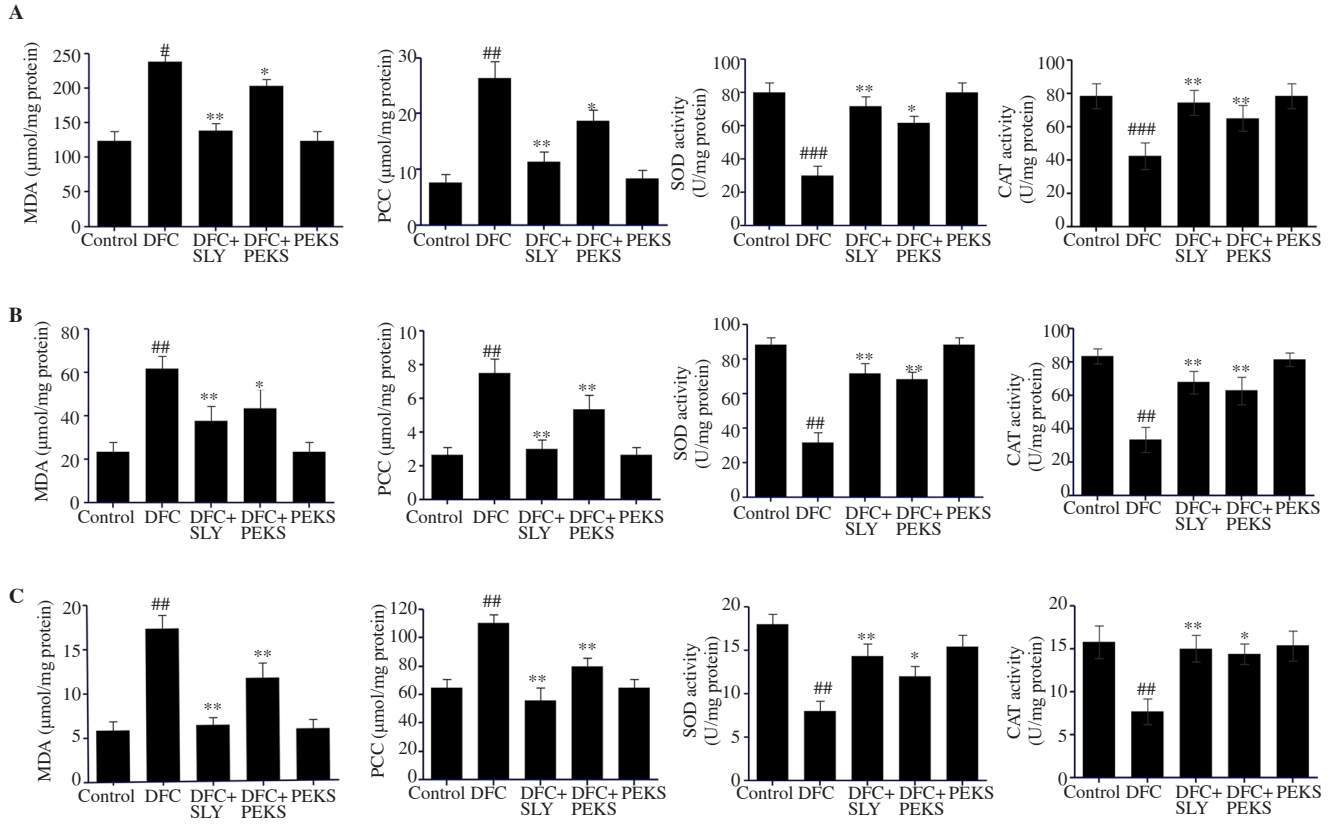

Figure 3. Effect of PEKS on oxidative stress caused by diclofenac (DFC) in the (A) liver, (B) kidney and (C) small intestine. The data are presented as mean ± SD (n=3). $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared with the normal control; $^{*}P$<0.05, $^{**}P$<0.01 compared with the DFC group.

protected the damaged RBCs induced by $NaNO_2$.

3.3. PEKS normalizes the DFC induced oxidative stress in vivo

PEKS normalized the DFC-induced oxidative stress in the liver, kidney, and small intestine by regulating the stress markers such as MDA, PCC, SOD, and CAT. DFC (50 mg/kg) elevated MDA and PCC significantly in the homogenates of the liver, kidney, and small intestine of rats (P<0.05). PEKS (300 mg/kg) markedly decreased the level of MDA and PCC (P<0.05), the activity of which was similar to SLY (25 mg/kg). In addition, the activities of SOD and CAT were significantly reduced in the homogenates of the liver, kidney, and small intestine of DFC-injected rats (P<0.05) in comparison with the normal control. Pretreatment with PEKS (300 mg/kg) restored antioxidant enzyme activity. Administration with PEKS alone did not cause any remarkable change in stress markers (Figure 3).

3.4. PEKS restores the biochemical parameters of the liver in DFC-injected rats

The DFC (50 mg/kg) injected rats showed decreased levels of albumin, globulin, and total protein (P<0.05) compared with

the normal control rats. On the other hand, the total, direct and indirect bilirubin levels were significantly higher in the DFC group (P<0.05). However, PEKS (300 mg/kg) normalized DFC-induced changes on these parameters (Figures 4A and 4B). Furthermore, the levels of liver marker enzymes such as ALT, AST and ALP were pronouncedly increased and LDH level was lowered in DFC injected rats. PEKS pretreatment normalized the liver marker enzymes and LDH (P<0.05) (Figure 4C). In addition, a remarkable rise in PT was seen as a result of DFC-induced hepatic damage. In contrast to the DFC group, DFC rats treated with PEKS showed normal PT (Figure 4D). However, aPTT did not alter in the DFC groups compared with the control group, However, aPTT was increased in the PEKS group compare with the DFC group.

3.5. PEKS recuperates the normal morphology of the liver, kidney, and small intestine from oxidative damage

Saline-injected rats exhibited normal liver histology (Figure 5A1). Whereas, massive necrosis, lymphocyte invasion, and lack of cell borders were seen in the liver section of DFC (50 mg/kg) exposed rats (Figure 5A2). PEKS alleviated necrosis, comparable to the SLY (25 mg/kg) treated tissue section (Figure 5A3 & 5A4). The section of the kidney treated with DFC showed massive tissue necrosis compared with the normal control group (Figure 5B1 & 5B2). Rats treated with PEKS displayed regeneration of tubular epithelial cells without necrosis (Figure 5B3 & 5B4), similar to the SLY group. Moreover, PEKS protected the small intestine from oxidative damage. The small intestine section of the normal control group showed characteristic mucosal villi (Figure 5C1). The DFC treated small intestine section demonstrated massive necrosis of the tips of intestinal villi (Figure 5C2). In contrast, PEKS and SLY restored normal intestinal structure (Figure 5C3 & 5C4).

3.6. PEKS exhibits anticoagulant effect via the intrinsic pathway of blood coagulation

PEKS showed an anticoagulant effect by postponing the clotting time of both PRP and PPP from control 200 s to 800 s and 220 s to 900 s, respectively (Figures 6A & 6B). In addition, PEKS delayed the *in vivo* tail bleeding time of mice as well. The delay in *in vivo* clotting time was above 400 s at the dose of 60 µg (Figure 6C). PEKS only prolonged the clotting time of aPTT without affecting PT which revealed its anticoagulant effect was due to the interference in the intrinsic pathway of the blood coagulation cascade (Figure 6D).

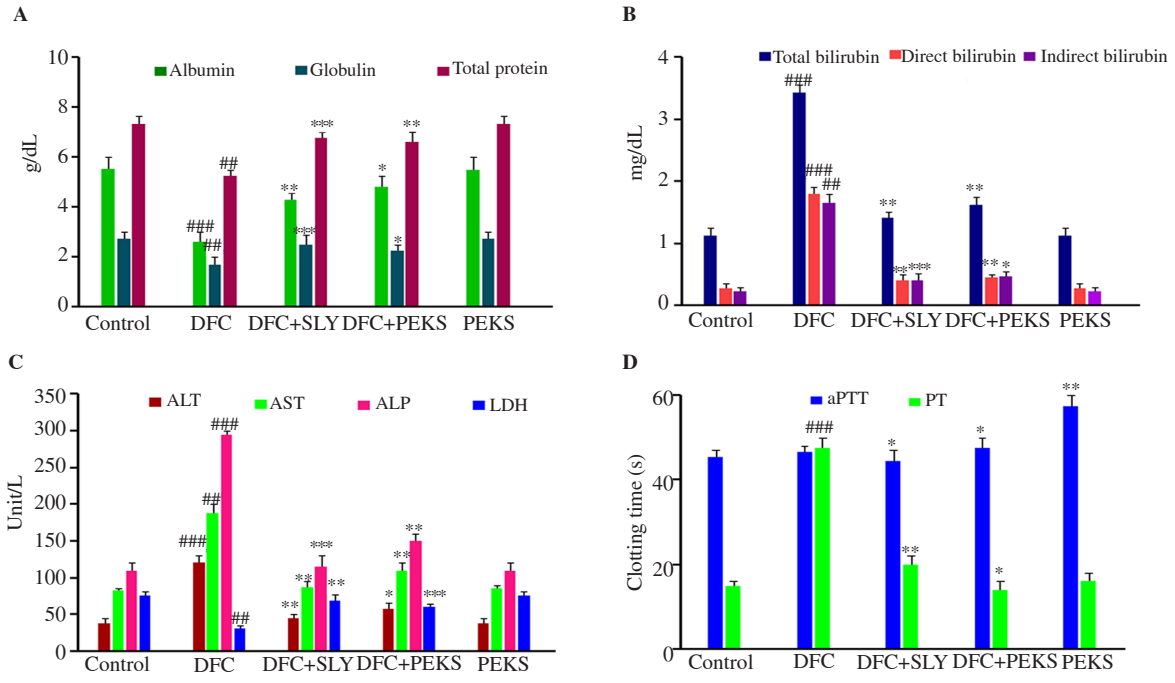

Figure 4. Effect of PEKS on biochemical parameters in DFC-induced oxidative stress. The data are expressed as mean ± SD (n=3). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the DFC treated groups $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared with the untreated control groups. ALT: alanine transaminase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; PT: prothrombin time; aPTT: activated partial thromboplastin time.

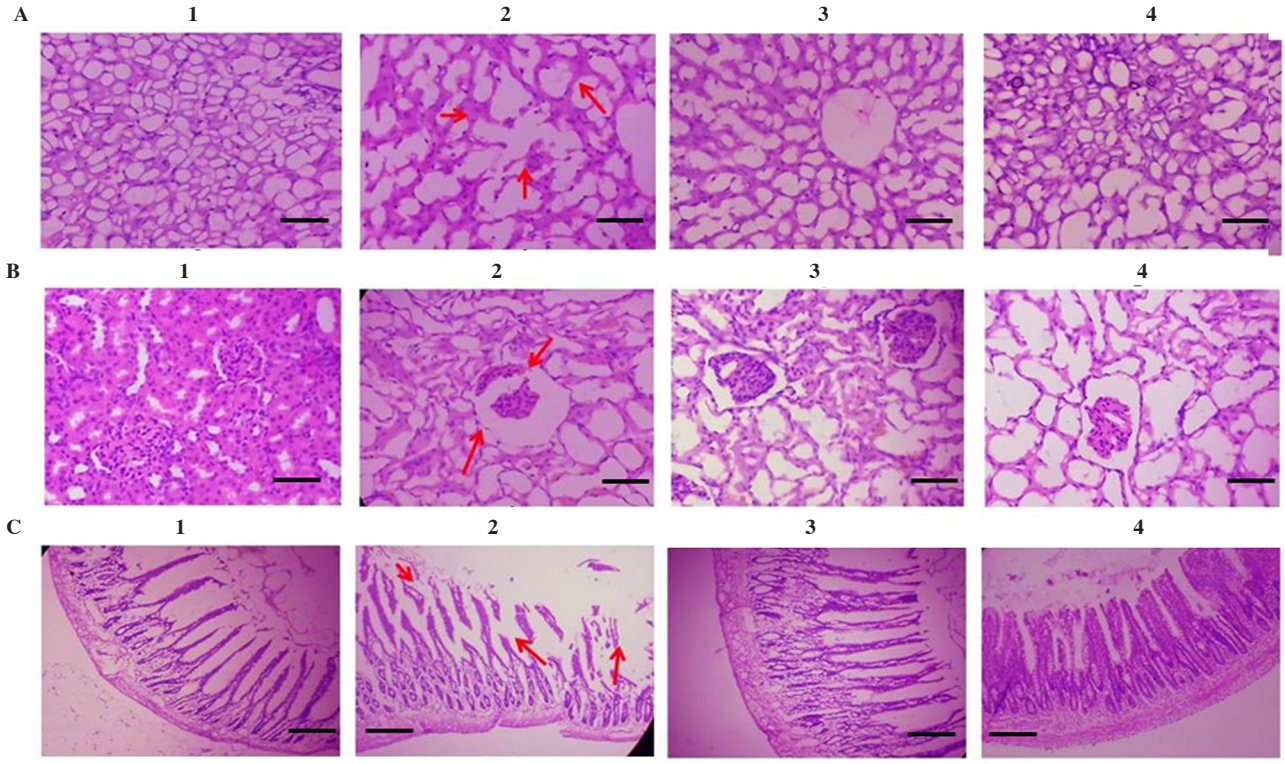

Figure 5. Effect of PEKS on DFC-induced histopathological changes of (A) the liver, (B) kidney, and (C) small intestine. (A1) The control group shows normal liver tissues. (A2) The DFC (50 mg/kg b.w./day, *i.p.*) group shows massive necrosis of the liver. (A3) The SLY (25 mg/kg b.w./day, *i.p.*) group shows normal liver structure. (A4) The PEKS (300 mg/kg b.w./day, *i.p.*) group restores liver structure and reduces DFC-induced damages. Red arrows indicate dilatation of sinusoid and nuclear changes. (B1) The control group shows normal kidney tissues. (B2) The DFC (50 mg/kg b.w./day, *i.p.*) group shows necrosis in tubular cells. (B3) The SLY (25 mg/kg b.w./day, *i.p.*) group alleviates tubular cell degeneration. (B4) The PEKS (300 mg/kg b.w./day, *i.p.*) group shows normal kidney structure. Red arrows indicate dilatation of tubules and necrosis of glomerulus. (C1) The control group shows normal epithelial villi. (C2) The DFC (50 mg/kg b.w./day, *i.p.*) group shows necrosis of the lamina propria and inflammation. (C3) The SLY (25 mg/kg b.w./day, *i.p.*) group shows normal intestine villi. (C4) The PEKS (300 mg/kg b.w./day, *i.p.*) group shows normal intestine villi. Red arrows indicate massive necrosis of the tips of intestinal villi. Magnification: ×40, H & E. Scale bars: 100 μm.

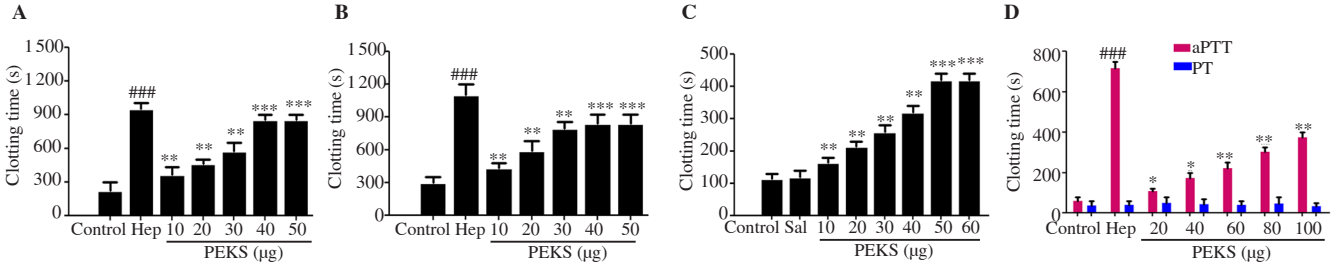

Figure 6. Anticoagulant activity of PEKS. Plasma recalcification time: (A) Platelet rich plasma (PRP) and (B) platelet poor plasma (PPP). PEKS (10-50 μg) was pre-incubated with PRP/PPP (0.2 mL) having 20 μL (10 mM) Tris-HCl buffer pH 7.4 for 1 min at 37℃. To initiate the clot, 20 μL of $CaCl_2$ (0.25 mol/L) was added and the clotting time was recorded. The data are presented as mean ± SD (n=3). $^{###}P$<0.001 compared with the control; $^{**}P$<0.01, $^{***}P$<0.001 compared with the positive control [heparin (Hep) (2.5 μg)]. (C) Tail bleeding time: the bleeding time was recorded 10 min after the intravenous injection of PBS and different doses of PEKS. The data are presented as mean ± SD (n=3). $^{**}P$<0.01, $^{***}P$<0.001 compared with the control. (D) aPTT and PT. The data are presented as mean ± SD (n=3). $^{###}P$<0.001 compared with the control; $^{*}P$<0.05, $^{**}P$<0.01 compared with the positive control (heparin). Sal: saline.

3.7. PEKS shows no toxicity on RBCs and inhibits platelet aggregation

The PEKS inhibited ADP and epinephrine-induced platelet aggregation in a concentration-dependent manner. The platelet aggregation inhibition was found to be 56% (ADP) and 34% (epinephrine) at the concentration of 30 μg (Figures 7A & 7B). The IC_{50} values were 13.05 μg (ADP) and 22.0 μg (epinephrine), respectively. PEKS did not damage RBCs at 200 μg compared with the positive control water (Figures 8A & 8B). PEKS cause no hemorrhagic and edematic effect even at the concentration of 200 μg (Supplementary Figure).

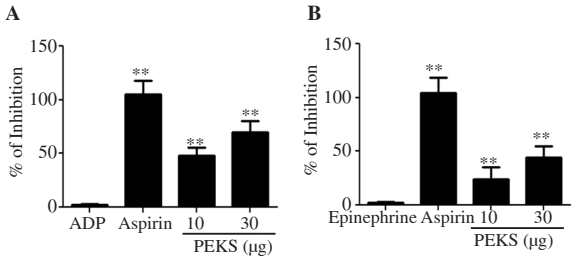

Figure 7. Effect of PEKS on (A) ADP and (B) epinephrine induced platelet aggregation. The data are presented as mean ± SD (n=3). $^{**}P$<0.01 compared with the control (ADP or epinephrine).

4. Discussion

The extracts and isolated compounds from the medicinal plants have been widely used in the management of both lifestyle and infectious diseases since ancient times. Kenaf is one of the most important fiber plants with its root, stalk, and leaves having immense therapeutic applications[23]. The kenaf fruit is about 2 cm in diameter bearing several seeds which were previously considered as waste materials. However, many research groups documented that the kenaf seeds are the protein factories having 32% of proteins and edible oils with antioxidant properties[24]. Pshenichnov *et al.*[6] for the first time isolated the biocidal peptide/protein from kenaf seed but its therapeutic function is not yet reported. Therefore, in the current study, an effort was made to understand the role of kenaf seed protein extract on oxidative stress-related issues such as thrombosis and tissue destruction. Higher levels of reactive oxygen species (ROS) and reactive nitrogen species are harmful as they elicit a mutagenic and carcinogenic effect. Importantly, ROS that developed due to RBCs eryptosis is the key culprit for causing thrombosis and damage of vital organs such as the liver, kidney, heart, brain, and small intestine[25]. Antioxidant loads may help to manage stress-related complications including thrombosis and tissue damage. In this connection, the antioxidant and antiplatelet activity of kenaf seed could be considered vital in the management of oxidative stress-related complications. PEKS revealed a couple of monomeric proteins under both reduced and non-reduced conditions. All of them were acidic in nature bearing carbohydrate moieties in them. The protective role of PEKS based on its antioxidant potential was evaluated using the *in vitro* RBCs model. PEKS did scavenge DPPH *in vitro* and protected RBCs by normalizing stress markers such as LPO, PCC, SOD, and CAT. RBCs are vital as they engage in several physiological functions of their transportation. However, most of the time, RBCs face a lot of challenges for their survival till 120 d. Microbes enter inside the human body, antibiotics, chemotherapeutic agents, steroids, antiinflammatory and endogenous ROS hampers the normal shape of the RBCs which results in eryptosis. Eryptosis not only destroys the RBCs morphology but also generates RBCs mediated ROS which is the key elicitor of blood coagulation cascade and platelets which end up in the formation of unusual clots in the arteries and veins leading to thrombosis[26]. In addition, uncontrolled eryptosis leads to tissue destruction and hemoglobin autoxidation resulting in necrotic heart formation and the development of a variety of cardiovascular diseases such as atherosclerosis, hypertension, heart disease, and damage of myocardial ischemic reperfusion injury[27]. The antioxidant property of PEKS was further verified by *in vivo* studies using DFC as an inducer of oxidative stress. DFC is a widely used non-steroidal anti-inflammatory drug; its prolonged use damages the digestive system, kidney, liver, and other vital organs. At a higher dose, DFC is even more toxic to vital organs rather than the therapeutic benefits as it does generate free radicals. Interestingly, PEKS exhibited a protective effect on DFC-induced damage in the liver, kidney, and small intestine by restoring the levels of LPO, PCC, SOD, and CAT. Antioxidant enzymes may stabilize or deactivate free radicals before the cell components get

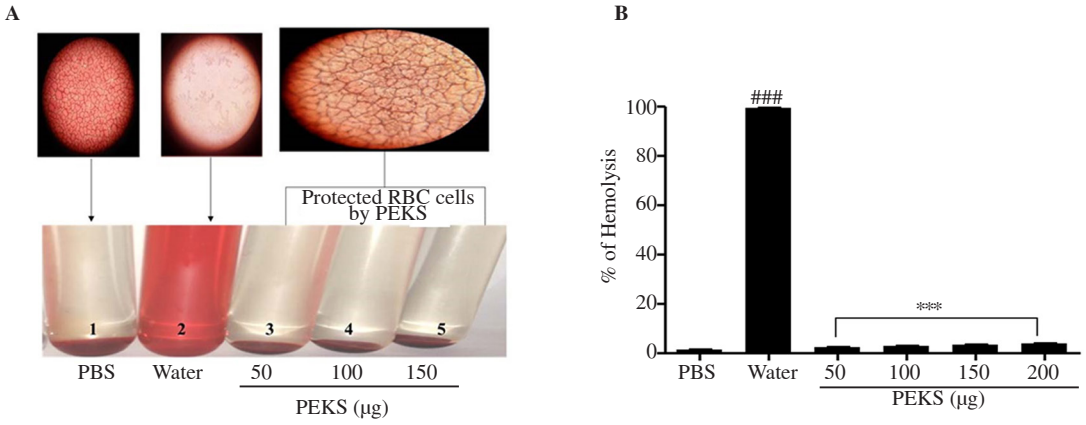

Figure 8. Direct hemolytic activity of PEKS. (A) Microscopic image of protected RBC from PEKS; (B) Percentage of hemolysis was calculated by measuring the amount of hemoglobin released in the supernatant at 540 nm. The data are presented as mean ± SD (n=3). $^{###}P$<0.001 compared with the PBS; $^{***}P$<0.001 compared with the positive control (water).

oxidized. Several studies demonstrated the ability of proteins to inhibit lipid oxidation in foods. Proteins originating from milk and soy exhibit antioxidant activity[28]. The antioxidant activity of proteins could be due to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate pro-oxidative transition metals, reduce hydroperoxides and enzymatically eliminate specific oxidants[29]. A number of antioxidant compounds have been isolated and characterized from dietary sources which have effective radical scavenging potential. Antioxidant proteins from *Olanum torrum* seeds (32 kDa) and curry leaves (35 kDa) were isolated and characterized[30,31]. Furthermore, the toxicity of DFC results in decreased levels of albumin, globulin, LDH and total protein, and increased concentration of total/direct/indirect bilirubin and ALT, AST and ALP, indicating liver damage. Compared to the normal rat, PEKS efficiently normalized the said biochemical parameters in the serum sample, the effect of which was comparable to the positive control SLY. In addition, PT, a measure of hepatic synthetic capacity, has a predictive value in both acute and chronic liver damage by acute hepatocellular necrosis with PT prolongation[32]. Treatment with PEKS reduced DFC induced PT prolongation in rats. aPTT increases only in chronic liver damage but remains unaltered in acute liver damage[33]. In case of DFC treated rats, aPTT was not altered but PT was increased, revealing its acute hepato-toxicity. In case of PEKS treated rats, PT was normalized while aPTT was increased, which indicates the observed *in vitro* anticoagulant effect in both plasma recalcification time and aPTT. This could be the reason why the PEKS and DFC treated group as well as the PEKS alone treated group showed higher aPTT than the DFC group. Histopathological pictures of the liver, kidney, and small intestine confirm the favorable effect of PEKS. Their activity exemplifies the protective impact against DFC toxicity. They can correct portal inflammation in liver tissues, vascular tumor necrosis in kidney tissues, necrosis in the lamina propria, and destruction in the small intestinal villi. The PEKS reformed the tissue configuration of the normal tissue structure due to DFC-induced toxicity. Protein extract of *Pennisetum glaucum* protects RBCs, liver, kidney, and small intestine from oxidative stress and exhibits anticoagulant and antiplatelet activity[34]. Aqueous extract of cortex dictamni exhibits *in vitro* and *in vivo* antioxidant activity and protection of the liver from oxidative damage[35]. Peptides from leaves of dill *Anethum graveolens* L. and celandine *Chelidonium majus* L exhibited *in vitro* hepatoprotective activity[36]. Most importantly, PEKS exhibited anticoagulant properties by prolonging the clot formation process of both PRP and PPP. The results in *in vivo* mouse tail bleeding assay were similar to the *in vitro* clotting time. Several anticoagulant proteases have been characterized from seeds such as flax seeds, and *Momordica charantia*[37,38]. The anticoagulant activity of PEKS interferes in the intrinsic pathway of blood coagulation as it specifically prolonged the clotting time of only aPTT. Platelets are essential in the maintenance of cardiovascular integrity and in the control of bleeding through the formation of a blood clot. However, they are also implicated in the pathological progression of thrombotic disorders. Uncontrolled platelet aggregation is critical in arterial thrombosis. Antiplatelet agents are therefore considered as a key tool in the treatment and/or prevention of thrombotic disorders. PEKS also showed the potential antiplatelet activity by inhibiting agonist such as ADP and epinephrine-induced platelet aggregation of human PRP. Furthermore, PEKS did not damage the RBCs relative to the positive control water suggesting its protective effect on the RBCs membrane. As it showed non-hemorrhagic activity, it did not damage the blood vessels either. Additionally, PEKS showed a non-toxic effect and did not lead to edema in the experimental mice. The observed anticoagulant, antiplatelet and antioxidant activity of PEKS could be due to the presence of enzymatic (protease) and non-enzymatic (peptides/proteins) agents. Proteases (serine, metallo, aspartate, and cysteine) were extensively studied for their anticoagulant, antiplatelet and antioxidant activities from animal and plant sources[39,40]. *In vitro* and *in vivo* studies of tamarind seed edible extract reveal anti-oxidative, anticoagulant, antiplatelet activities and protect RBCs from oxidative damage[41]. Proteins from finger millet and *Macrotyloma uniflorum* exhibit anticoagulant, antiplatelet and non-toxic effects on RBCs[42,43].

In conclusion, PEKS protected the liver, kidney, and small intestine from oxidative damage by its antioxidant property. In addition, PEKS showed anticoagulant and antiplatelet activities. Thus, it could be a better therapeutic agent for several oxidative stress-related pathologies and cardiovascular complications. Although, this piece of work evaluates the therapeutic potency of the PEKS, the mechanism by which it intereferes in coagulation cascade, platelet function and tissue protection must be identified. Moreover, purification and charaterization of active compounds from the PEKS may provide the better insight into understanding structure function relationship and the exact molecular mechanism.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

SH and DS together planned and designed the research work. CS, AS, MV, and RH assisted in the laboratory work. RKL, RR and SG reviewed the article.

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