



Sorghum Protein Extract Protects RBC from Sodium Nitrite-Induced Oxidative Stress and Exhibits Anticoagulant and Antiplatelet Activity

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Received: 18 Aug 2020 ♦ **Accepted:** 11 Mar 2021 ♦ **Published:** 31 Dec 2021

Citation: Sannanigaiah D, Shivaiah A, Kengaiah J, Srinivasa C, Nandish SKM, Ramachandraiah C, Hanumegowda S, Manjappa B, Martin SS, Laxmaiah RK, Shinde M. Sorghum protein extract protects RBC from sodium nitrite-induced oxidative stress and exhibits anticoagulant and antiplatelet activity. *Folia Med (Plovdiv)* 2021;63(6):884-94. doi: 10.3897/folmed.63.e57713.

Abstract

Introduction: Oxidative stress plays a critical role in the progression of diabetes, arthritis, cancer, eryptosis, cardiovascular disease, and thrombosis. Currently, antioxidants from natural sources are in high demand due to their beneficial role in the management of said diseases.

Aim: The purpose of the study was to evaluate the protective effect of sorghum protein buffer extract (SBE) on sodium nitrite-induced oxidative stress and thrombosis.

Materials and methods: Protein characterization of SBE was done using SDS-PAGE. Oxidative stress in RBC was induced using sodium nitrite (NaNO₂) and the key stress markers such as lipid peroxidation (LPO), protein carbonyl content (PCC), and the level of antioxidant enzymes (SOD and CAT) were measured. The anticoagulant effect of SBE was identified by employing in-vitro plasma recalcification time, activated partial thromboplastin time (APTT), prothrombin time (PT), and in-vivo mouse tail bleeding time. SBE antiplatelet activity was examined using agonist adenosine diphosphate (ADP) and epinephrine-induced platelet aggregation. Non-toxic property of SBE was identified using in-vitro direct haemolytic, haemorrhagic, and edema forming activities using experimental mice.

Results: SBE revealed similar protein banding pattern under both reduced and non-reduced conditions on SDS-PAGE. Interestingly, SBE normalized the level of LPO, PCC, SOD, and CAT in stress-induced RBCs. Furthermore, SBE showed anticoagulant effect in platelet rich plasma by enhancing the clotting time from the control 250 s to 610 s and bleeding time from the control 200 s to more than 500 s ($p < 0.01$) in a dose dependent manner. In addition, SBE prolonged the clot formation process of only APTT but not PT. SBE inhibited the agonists ADP and epinephrine induced platelet aggregation. SBE did not hydrolyze RBC cells, devoid of edema and haemorrhage properties.

Conclusions: This study demonstrates for the first time the anticoagulant, antiplatelet, and antioxidant properties of SBE. Thus, the observed results validate consumption of sorghum as good for health and well-being.

Keywords

anticoagulant, antioxidant, antiplatelet, oxidative stress, protein, thrombosis

INTRODUCTION

The reactive oxygen species (ROS) generated during oxidative stress at normal levels are involved in numerous physiological functions, but at higher concentrations they exert adverse effects by altering the structure of DNA, proteins, and lipids.¹ Thus, reduced antioxidant defence mechanism leads to diabetes, cancer, atherosclerosis, severe anaemia, stroke, myocardial infarction, and thrombosis.² Most importantly, ROS modulates blood coagulation, fibrinolysis, proteolysis, platelets, the complement system, endothelial cells, erythrocytes, neutrophils, mast cells, monocytes, and fibroblasts.³ The thrombus formation and resolution is greatly influenced by the damaged RBCs caused by oxidative stress. Several researchers documented the potential role of damaged RBCs in thrombosis. The RBC-ROS mediated accumulation of fibrin clot (thrombus) in blood vessels often breaks into pieces, and the free floating thrombi (emboli) may lodge anywhere in the vascular system, including the lungs and brain, leading to cardio/cerebrovascular diseases.⁴ Hence, thrombosis accounts for millions of death every year worldwide. Thus, inhibition of RBC-ROS generation from natural antioxidant agents aids in the prevalence of thrombotic disorders. Generally, anticoagulant, thrombolytic, and antiplatelet agents are widely employed in the treatment of stroke and myocardial infarction caused by thrombosis.⁵ However, currently available anticoagulant, thrombolytic, and antiplatelet agents have been found to cause severe bleeding, miscarriage, headache, and nausea.⁶ Thus, development of novel therapeutic agents having multiple therapeutic efficacy (antioxidant, anticoagulant, and antiplatelet) with least side effects could be the better alternative for the management of oxidative stress-induced eryptosis and thrombosis. Pharmacological and dietary antioxidants can curb ROS-induced thrombus formation but their overall effect is not yet identified.⁷ Therefore, there are no recommendations regarding their usage in medical practice. Importantly, the establishment of novel antioxidant treatment remedies that target to avoid the thrombus formation and permits ROS modulation constrained to their physiological redox mechanisms.⁸ Sorghum belongs to the family *Poaceae*, commonly called great millet/Indian millet.⁹ Sorghum grain is the fifth most important cereal crop in the world after wheat, rice, corn, and barley.¹⁰ It is the third most widely grown crop in India after rice and wheat. It is the most important staple food for a large proportion of the population as well.¹¹ Sorghum is generally more economical to produce and used in a variety of foods.¹² The white food sorghums are processed into flour and other products including expanded snacks, cookies and ethnic foods, and are gaining popularity in Japan.¹³ Whole grain consumption has been associated with a decreased incidence of cancer, cardiovascular disease (CVD), diabetes and obesity.¹⁴ Sorghum is the richest source of macro (proteins, lipids and carbohydrates) and micro molecules (vitamins and minerals). The therapeutic usage of proteins from sorghum has been least explored.

AIM

In this perspective, sorghum protein extract was evaluated for antioxidant, anticoagulant, and antiplatelet properties and the results were presented herein.

MATERIALS AND METHODS

Trichloroacetic acid (TCA), 95% ethanol, sodium nitrite (NaNO_2), sodium dodecyl sulfate (SDS), acetic acid, thiobarbituric acid, dinitrophenyl hydrazine (DNPH), tetramethyl ethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA), quercetin, and hydrogen peroxide were purchased from Sigma Chemicals Company (St. Louis, USA). All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the platelet rich plasma (PRP) & platelet poor plasma (PPP). Adenosine diphosphate (ADP) and epinephrine were purchased from Sigma chemicals company (St. Louis, USA). All experimentations were conducted in accordance with the ethical guidelines and were approved by the Institutional Human Ethical Committee, Tumkur University, Tumkur. Conducting animal experiments were permitted by the Institutional Animal Ethical Committee, Liveon Biolabs Private Limited, Tumkur. Animal handling was carried out in accordance with the guidelines of the Committee for the Purpose of Monitoring and Supervision of Experiments on Animals (CPCSEA).

Preparation of sorghum buffer extract (SBE) and protein estimation

Sorghum was purchased from the local market in Tumkur. The dried seeds were powdered, dissolved in phosphate buffer pH 7.5, and centrifuged at 8000 g for 15 min. Ammonium sulphate (30%) precipitation was done, centrifuged at 5000 g, discarded the supernatant, and dissolved the pellet with phosphate buffer pH 7.5. Protein concentration was determined as described by Bradford¹⁵ using bovine serum albumin (BSA) as standard and the sample was stored at -20°C until further usage.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% was carried out according to the method of Laemmli.¹⁶ The crude SBE (100 μg) prepared under reducing and non-reducing conditions was used for SDS-PAGE. The electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 2 hr at room temperature. After electrophoresis, the SDS-PAGE gel was stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and de-stained with 40% ethanol in 10% acetic acid and water (40:10:50 v/v). Molecular weight standards from 200 kDa to 14.3 kDa were used.

Anticoagulant assay

Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick et al.¹⁷ Briefly, the SBE (20-80 µg) was pre-incubated with 0.2 ml of citrated human platelet rich plasma (PRP) and platelet poor plasma (PPP) in the presence of 10 mM Tris HCl (20 µl) buffer pH 7.4 for 1 min at 37°C, 20 µl of 0.25 M CaCl₂ was added to the pre-incubated mixture and clotting time was recorded.

Activated partial thromboplastin time (APTT) and prothrombin time (PT)

Briefly, 100 µl of normal citrated human plasma and SBE (0-12 µg) were pre-incubated for 1 min. For APTT, 100 µl of LIQUICELIN-E phospholipids preparation derived from rabbit brain with ellagic acid (which was activated for 3 min at 37°C) was added. The clotting was initiated by adding 100 µl of 0.02 M CaCl₂ and the clotting time was measured. For PT, the clotting was initiated by adding 200 µl of PT reagent (UNIPLASTIN-rabbit brain thromboplastin). The time taken for the visible clot was recorded in seconds. The APTT ratio and the international normalized ratio (INR) for PT at each point were calculated from the values of control plasma incubated with the buffer for identical period of time.

Preparation of platelet-rich plasma and platelet-poor plasma

The method of Ardlie et al.¹⁸ was employed for the preparation of human platelet rich plasma (PRP) and platelet poor plasma (PPP). The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/ml with PPP. The PRP maintained at 37°C was used within 2 hr for the aggregation process. All the above preparations were carried out using plastic wares or siliconized glass wares.

Platelet aggregation

The turbid metric method of Born et al.¹⁹ was followed using a Chronology Dual Channel Whole Blood/Optical Lumi Aggregation System (Model-700). Aliquots of PRP were pre-incubated with various concentrations of SBE (0-100 µg) in 0.25 ml reaction volume. The aggregation was initiated independently by the addition of agonists, such as ADP and epinephrine and followed for 6 min.

Lipid peroxidation

Lipid peroxidation was done according to the method of Ohakawa et al.²⁰ approximately 1.0-2.0 mg of protein from lysate of RBC treated with an agonist NaNO₂ (1 mM), and SBE (0-150 µg/mL), was taken in dry test tubes, 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) was added, the reaction

mixture was boiled at 45-60°C for 45 min and centrifuged at 2000 rpm for 10 min. The formed adducts were extracted into 1-butanol (3 ml) and the thiobarbituric acid reactive substance (TBARS) and read photometrically at 532 nm and quantified using TMP as the standard.

Protein carbonyl content

The protein carbonyl content was measured using DNPH according to the method described by Levine et al.²¹ 1 mg of protein from lysate of RBC was treated with an agonist NaNO₂ (10 mM), and SBE (0-150 µg/ml) was taken and an equal volume of 10 mM DNPH in 2N HCl was added, incubated for 1 hour shaking intermittently at room temperature. Corresponding blank was carried out by adding only 2N HCl to the sample. After incubation, the mixture was precipitated with 20% trichloroacetate (TCA) and centrifuged at 5000 rpm for 15 min. The precipitate was washed twice with acetone by centrifuging at 10 000 rpm, for 15 min and finally dissolved in 1 ml of Tris buffer (20 mM, pH 7.4 containing 0.14 M NaCl, 2% SDS) and the supernatant was recorded at 360 nm. The difference in absorbance was expressed as nmols of carbonyl groups/mg protein using molar extinction coefficient of 22 mM⁻¹cm⁻¹ and was expressed as l mol H₂O₂ decomposed/min/mg protein (€-43.6/mM/cm).

Superoxide dismutase (SOD) and catalase (CAT) activity

SOD and CAT activities in erythrocyte lysates were assessed as described by Kostyuk et al.²² In brief, RBCs (2% hematocrit) in PBS were treated with or without (control) 10 mM NaNO₂. For the inhibition studies, erythrocytes exposed to NaNO₂ were incubated with SBE (0-150 µg/ml) for 2 hours. Following the incubation, erythrocytes were lysed with distilled water, and the lysates were assayed for SOD and CAT activities. To determine SOD activity, 0.1 ml of erythrocyte lysate (0.05 mg protein) was added to the reaction mixture (1 ml) containing 16 mM phosphate buffer, pH 7.8, TEMED/EDTA (8 mM/0.08 mM), and quercetin (0.15% w/v). The decrease in absorbance was monitored for 1 min at 406 nm. The amount of protein that inhibited quercetin autoxidation by 50% was defined as one unit of activity. Similarly, CAT activity in the erythrocyte lysate was determined by measuring the rate of H₂O₂ hydrolysis at 240 nm. Erythrocyte lysate (0.05 mg protein) was added to the reaction mixture (1 ml) containing 100 mM of sodium phosphate buffer, pH 7.4, and 8.8 mM H₂O₂. The decrease in absorbance at 240 nm was monitored for 3 min, and the activity was expressed as mmol H₂O₂ decomposed/min per mg protein.

Direct haemolytic activity

Direct haemolytic activity was determined by using washed human erythrocytes. Briefly, packed human erythrocytes

and phosphate buffered saline (PBS) (1:9 v/v) were mixed; 1 ml of this suspension was incubated independently with the various concentration of SBE (0-100 µg) for 1 hour at 37°C. The reaction was stopped by adding 9 ml of ice cold PBS and centrifuged at 1000 g for 10 min at 37°C. The amount of haemoglobin released in the supernatant was measured at 540 nm. Activity was expressed as percentage of haemolysis against 100% lysis of cells due to addition of water that served as positive control and phosphate buffered saline served as negative control.

Edema inducing activity

The procedure of Vishwanath et al.²³ was followed. Groups of five mice were injected separately into the right foot pads with different doses (10 to 200 µg) of SBE in 20 µl saline. The left foot pads received 20 µl saline alone served as control. After 1 hour, the mice were anaesthetized by diethyl ether inhalation. Hind limbs were removed at the ankle joint and weighed. The increased weight was calculated as the edema ratio, which equals the weight of edematous leg × 100/weight of normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120%.

Haemorrhagic activity

Haemorrhagic activity was assayed as described by Kondo et al.²⁴ Different concentration of SBE (0-200 µg) was injected (intradermal) independently into the groups of five mice in 30 µl saline. The group receiving saline alone served as negative control and the group receiving venom (2 MHD) – as positive control. After 3 hours, the mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for haemorrhage against saline injected control mice. The diameter of haemorrhagic spot on the inner surface of the skin was measured. The minimum haemorrhagic dose (MHD) was defined as the amount of the protein producing 10 mm of haemorrhage in diameter.

RESULTS

In order to eliminate probable contamination other than proteins from SBE, ammonium sulphate precipitation was done and desalted using dialysis. Protein characterization of SBE was done using SDS-PAGE under reduced and non-reduced conditions. SBE showed similar kind of protein bands from the range of 200 kDa to 18.4 kDa, suggesting the proteins present are monomeric in nature (Fig. 1). To evaluate its protective effect on oxidative stress, red blood cells (RBC) were used as model. Oxidative stress in RBC was induced using sodium nitrite (NaNO₂) and the key stress markers such as lipid peroxidation (LPO), protein carbonyl content (PCC) and antioxidant enzymes (SOD and CAT) level was measured.

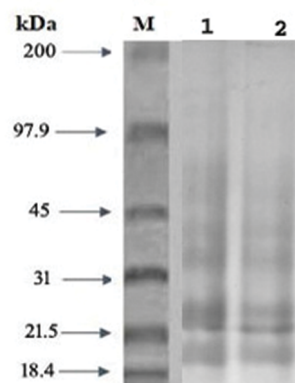


Figure 1. SDS-PAGE 10%: SBE (100 µg) prepared under reducing and non-reducing conditions was used for SDS-PAGE. M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), phosphorylase (97.2), ovalbumin (44.2), carbonic anhydrase (29), trypsin inhibitor (21.5), and lactoglobulin (18.4).

The level of malondialdehyde (MDA) was measured as a marker of lipid peroxidation (Fig. 2A). In case of NaNO₂ treated RBCs, the level of MDA was increased due to oxidative stress. Interestingly, SBE significantly ($p < 0.01$) normalized the MDA level in a dose dependent manner that was compared with the control. However, in SBE alone treated group there was no significant alteration of MDA. Furthermore, a significant elevation of PCC was observed in the sodium nitrite treated hemolysates compared to the control group (Fig. 2B). While in SBE pre-treated group, the altered PCC was significantly ($p < 0.01$) restored. In case of SBE alone treated group, the level of PCC remained unaltered. To substantiate the observed protective role of SBE in NaNO₂-induced oxidative stress in RBCs, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) activities were assayed in NaNO₂-treated RBC cells (Figs 2C, 2D). In case of NaNO₂-treated RBC hemolysate, there was a significant ($p < 0.01$) decrease in the activity of SOD and CAT. In contrast, in the SBE pre-treated groups, the activity of SOD and CAT increased significantly ($p < 0.01$) in a dose dependent manner that was compared with NaNO₂-treated cells. ROS and RBC-ROS were found to alter the normal function of coagulation factors and platelets. Hence, oxidative stress has been considered as the major contributor in the pathophysiology of thrombosis. Therefore, to check the protective role of SBE in the stress-induced thrombotic complications, anticoagulant and antiplatelet activities were carried out. Curiously, SBE showed anticoagulant effect in both PRP and PPP (Fig. 3A). In case of platelet rich plasma, SBE delayed the clotting time from the control 198 s to 535 s. However, in case of platelet rich plasma the delay in clotting time by SBE was found to be 250 s to 610 s. The anticoagulant effect of SBE was also authenticated by in vivo mouse tail bleeding assay. SBE significantly enhanced the bleeding time from the control 200 s to more than 500 s ($p < 0.01$) in a dose dependent manner (Fig. 3B). To decipher the observed anticoagulation effect

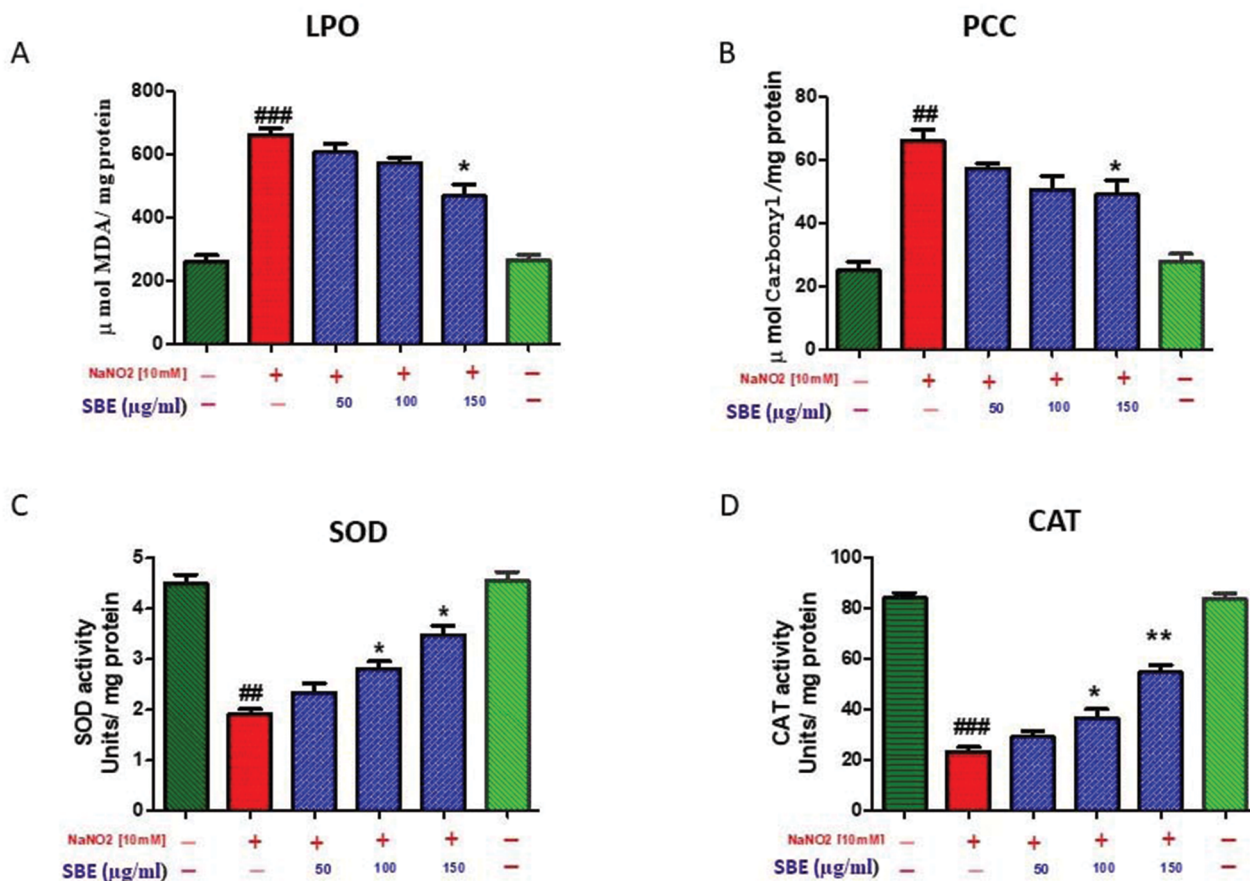


Figure 2. Effect of SBE on oxidative stress-induced erythrocytes: (A) Malondialdehyde (MDA); (B) Protein carbonyl content; (C) Activity of superoxide dismutase; (D) Activity of catalase activity: NaNO₂ (10 mM) was used as an agonist to induce oxidative stress in erythrocytes. Later, stress markers such as malondialdehyde (MDA), protein carbonyl content, SOD and CAT activities were measured after 1 hour of incubation period. For inhibition studies, RBC was pre-incubated with different doses (50-150 µg/ml) of SBE for 10 min at 37°C prior to NaNO₂ (10 mM) treatment. Data are presented as mean ± SEM (n=3). [n=3, *p*<0.05 (*), *p*<0.01 (**), *p*<0.001 (***/###); #: Significant compared to untreated RBC, *: significant compared to NaNO₂ (10 mM) treated RBC.

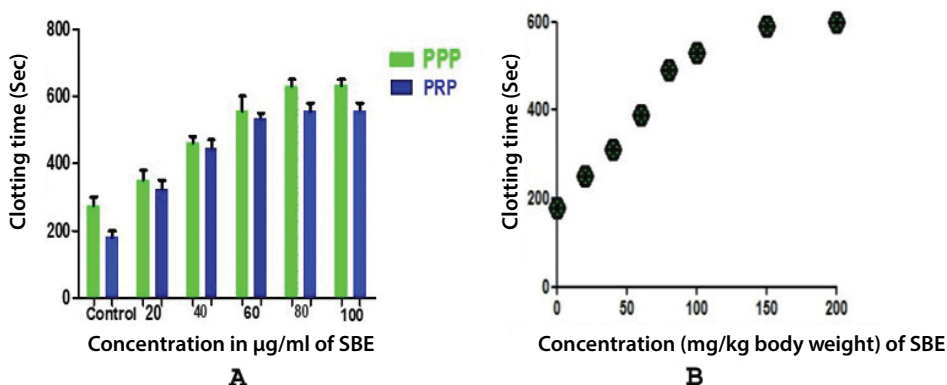


Figure 3. Plasma re-calcification time: (A) SBE (0–10 µg) was pre-incubated with 0.2 ml of citrated human plasma PRP/PPP in the presence of 20 µl 10 mM Tris–HCl buffer (pH 7.4) for 1 min at 37°C. 20 µl of 0.25 M CaCl₂ was added to the pre-incubated mixture and clotting time was recorded. Tail bleeding time: (B) Tail bleeding time was measured 10 min after intravenous administration of PBS or various doses of SBE. Each point represents the mean ± SD of three independent experiments (*p*<0.01). Bleeding time was longer than 500 s.

of SBE due to interference in intrinsic or extrinsic pathway of blood coagulation cascade activated partial thromboplastin time (APTT) and prothrombin time (PT) assays were carried out. Curiously, SBE significantly ($p < 0.01$) prolonged only APTT without altering PT (Fig. 4). These results suggested that the anticoagulation effect by SBE could be due to its involvement in intrinsic pathway of blood coagulation cascade. In addition, SBE inhibited agonists ADP and epinephrine induced platelet aggregation in platelet rich plasma (Figs 5, 6). The percentage of platelet aggregation inhibition was found to be 48% and 60% at the concentration of 100 μg , respectively. To investigate its probable toxic property haemolytic, haemorrhagic, and edema inducing activities were done using experimental mice. Surprisingly, SBE did not lyse RBCs even at the dose of 200 μg (Fig. 7). SBE was unable to cause haemorrhage and foot edema in the experimental mice up to the concentration of 10 mg/kg body weight, whereas *Daboia resseli* venom a positive control caused haemorrhage and foot edema in the experimental mice (Fig. 8).

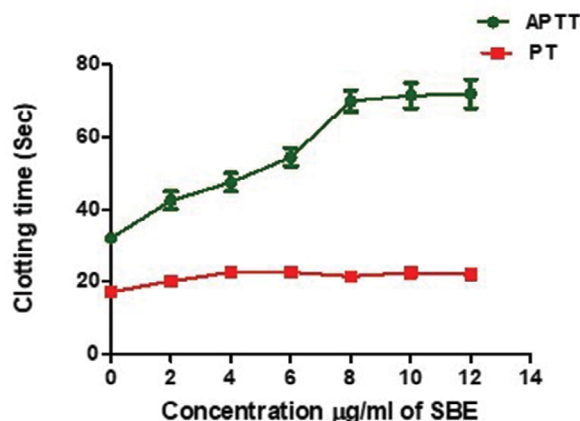
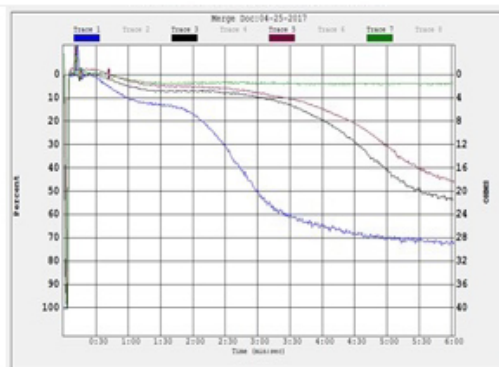
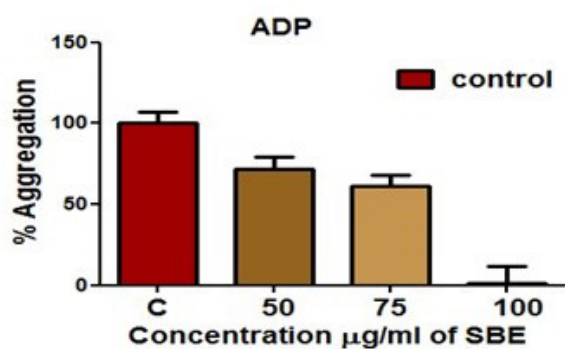


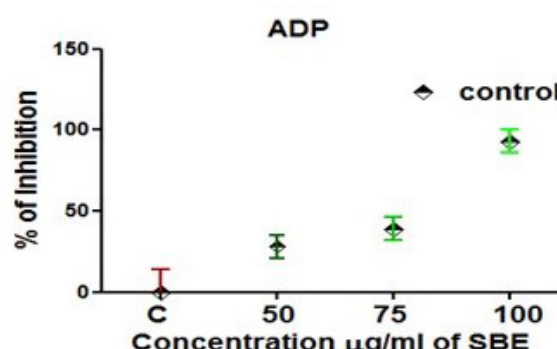
Figure 4. Effect of SBE on clotting time of APTT and PT tests: APTT test was done by pre-incubating plasma (100 μl) with SBE (0–12 μg) for 1 min. 100 μl of APTT reagent was added and activated for 3 min at 37°C, then clotting was initiated by adding 100 μl of 0.02 M CaCl_2 . For PT, the clotting was initiated by adding 200 μl of PT reagent. The time taken for the visible clot was recorded in seconds. Each point represents the mean \pm SD of three independent experiments ($p < 0.01$).



A



B



C

Figure 5. Platelet aggregation was initiated by adding ADP as an agonist: (A) Traces of platelet aggregation: Trace 1 (ADP 10 μM), Trace 3 (ADP 10 μM + 50 μg of SBE), Trace 5 (ADP 10 μM + 75 μg of SBE), Trace 7 (ADP 10 μM + 100 μg of SBE); (B) Dose dependent platelet aggregation inhibition %; (C) Dose dependent platelet aggregation %. The values represent the mean \pm SD of three independent experiments. Each point represents the mean \pm SD of three independent experiments ($p < 0.01$).

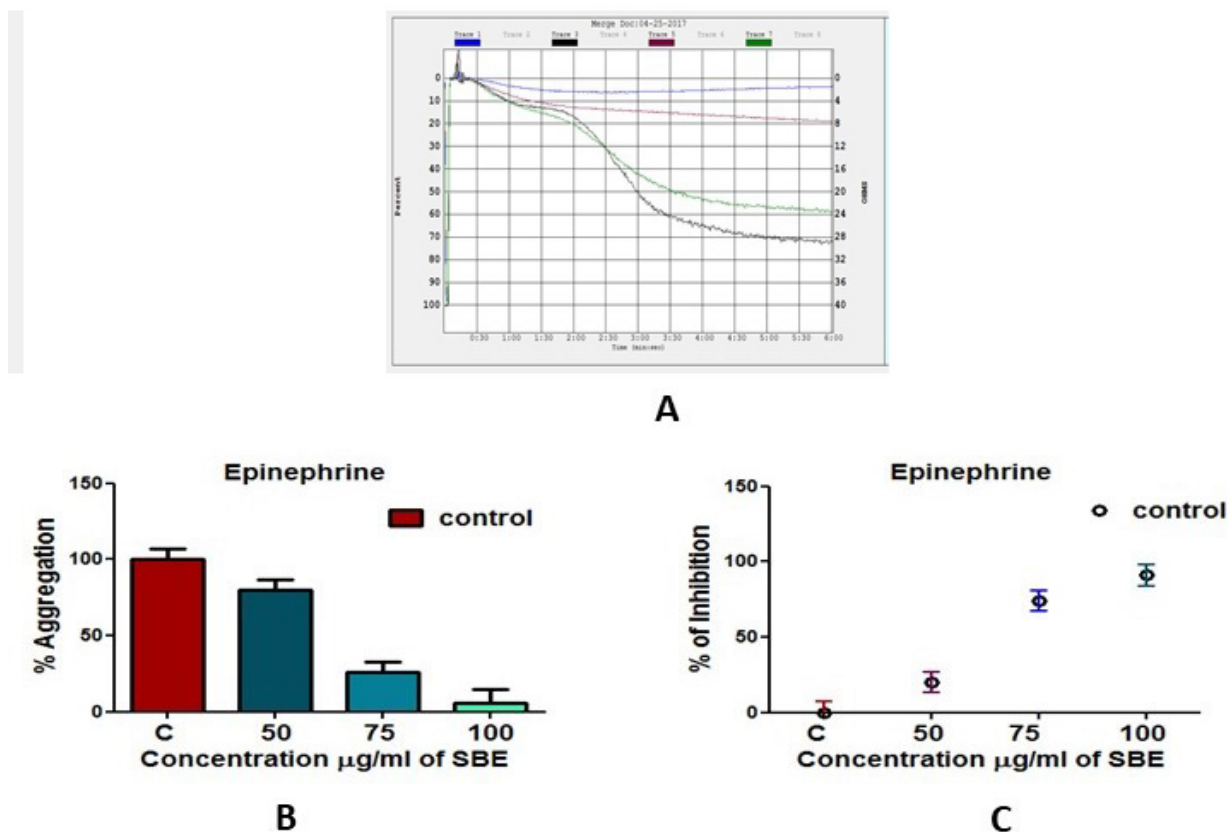


Figure 6. Platelet aggregation was initiated by adding Epinephrine as an agonist: (A) Traces of platelet aggregation: Trace 1 (epinephrine 5 µM); Trace 3 (epinephrine 5 µM + 50 µg of SBE); Trace 5 (epinephrine 5 µM + 75 µg of SBE); Trace 7 (epinephrine 5 µM + 100 µg of SBE); (B) Dose dependent platelet aggregation inhibition %; (C) Dose dependent platelet aggregation. The values represent the mean ± SD of three independent experiments. Each point represents the mean ± SD of three independent experiments ($p < 0.01$).

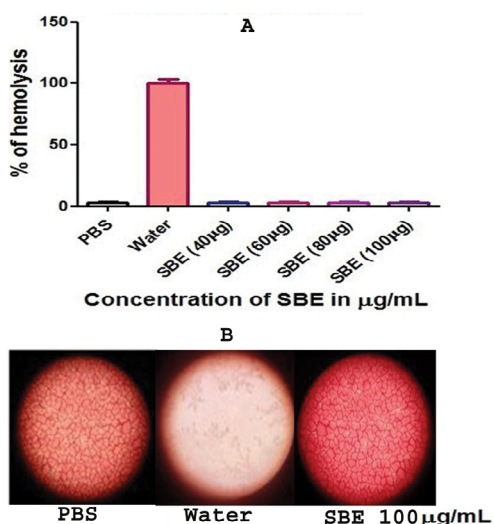


Figure 7. Direct hemolytic assay: (A) The different concentration of SBE (40-100 µg) was incubated independently for 1 hour at 37°C with the 1 ml of suspension made with packed human erythrocytes and phosphate buffered saline (PBS) 1:9 v/v. The reaction was stopped by adding 9 ml of ice cold PBS and centrifuged at 1000 g for 10 min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540 nm; (B) Microscopic image of RBC. The values represent the mean ± SD of three independent experiments.

DISCUSSION

Natural products/extracts from medicinal plants are in growing demand due to their immense therapeutic potential and the least side effects as compared to synthetic drugs. Large amount of drugs used in modern medicines are either directly purified from the plants or chemically modified from a lead molecule from natural origin. In recent times, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds.²⁵ Currently, about 25 drugs derived from plants are in high demand worldwide.²⁶ Sorghum is once such medicinally/nutritionally important ancient grain which stores large quantities of macro- and micronutrients. Studies have suggested that sorghum based diets can be used in the prevention of chronic diseases such as diabetes, obesity, cancer, and heart disease. Yet sorghum grains are considered to have a low value to humans and often used in feeding animals.²⁷

Oxidative stress is the key contributor in the pathophysiology of several diseases such as diabetes, cancer, arthritis, thrombosis, and cardiovascular diseases.²⁸ Free radicals formed during oxidative stress modify the essential functions of the erythrocytes by provoking intracellular calcium and phosphatidyl serine (PS) exposure, that

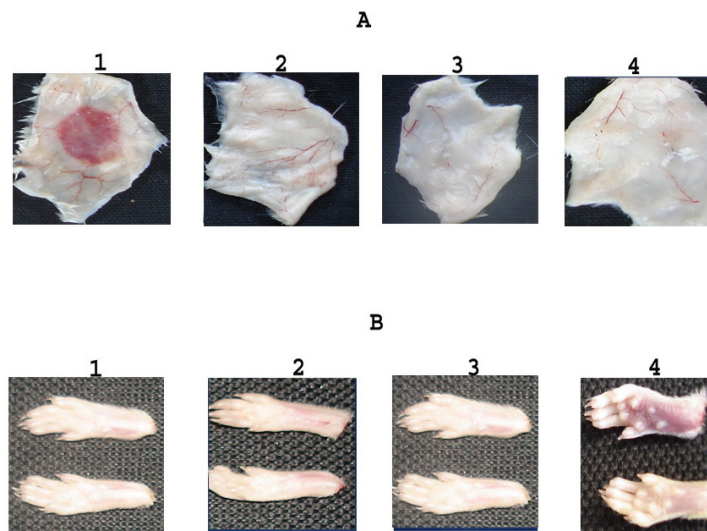


Figure 8. (A) Effect of SBE haemorrhagic activity: (A1) Positive control *Daboia ressellii* venom; (A2) Saline; (A3) 200 mg/kg body weight of SBE; (A4) 400 mg/kg body weight of SBE was injected independently into mice in a total volume of 50 μ l intradermally; (B) Effect of SBE on edema: (B1) 200 mg/kg body weight of SBE; (B2) 400 mg/kg body weight of SBE (B3) Saline; (B4) Positive control *Daboia ressellii* venom was injected independently into the mice foot pad in a total volume of 50 μ l. The values represent the mean \pm SD of three independent experiments.

eventually lead to eryptosis (cell death). Eryptosis is categorized by erythrocyte shrinkage, cell membrane blebbing and cell membrane scrambling with PS translocation to the erythrocyte surface triggering the phagocytosis of the cells.²⁹ Augmented eryptosis may subsist in numerous clinical conditions like malaria, sickle cell anaemia, diabetes, sepsis, renal insufficiency, Wilson's disease, hemolytic uremic syndrome, hypophosphatemia, glucose-6-phosphate dehydrogenase-deficiency, chronic kidney disease, and cardiovascular diseases.³⁰ Moreover, certain drugs used in the treatment of diabetes, cancer, and thrombosis are found to induce eryptosis.³¹ Thus, controlling oxidative stress could be a key concern in combating anemia associated with said diseases. Several antioxidant molecules derived from plants are found to offer protection against the eryptosis induced by oxidative stress.³² Therapeutic applications of *Caesalpinia crista* seed was well documented in folk medicine.³³

SBE ameliorated the altered level of MDA, PCC, and antioxidant enzymes (SOD and CAT) evidenced erythrocyte protective role on sodium nitrite induced oxidative stress.³⁴ ROS and RBC-ROS generated during oxidative stress alters blood coagulation cascade by activating coagulation factors and platelets.³⁵ Thus, ROS/RBC-ROS mediated activation of coagulation factors and platelets is associated with thrombosis and cardiovascular diseases.³⁵ Interestingly, SBE exhibited anticoagulant effect by significantly ($p < 0.01$) increasing the plasma recalcification time of citrated human plasma in vitro and bleeding time in vivo revealed, it could inhibit the function of clotting factors. Moreover, SBE did not interfere in the clotting time of PT but altered the clotting time of only APTT suggesting its anticoagulant effect could be due to its inhibitory effect on intrinsic pathway of blood coagulation cascade. Several studies

reported the participation of proteins from seeds/latex on blood coagulation cascade by triggering pro/anticoagulant activity.³⁶⁻³⁹

Platelets are vulnerable to numerous stimuli including oxidative stress and undergo apoptosis. They are the main contributor in the development of thrombosis, arthritis, Alzheimer, cancer, and chronic inflammatory responses.⁴⁰ Perhaps, platelet aggregation inhibitors are commercially available in the market, the secondary complications (hemolysis, internal bleeding, and miscarriage) associated with them limits their use.³⁹ Therefore, the agent that protects the platelet shape and inhibit platelet aggregation could be a better therapeutic agent in managing stress persuaded thrombosis. SBE inhibited agonist ADP and epinephrine induced platelet aggregation, supports its therapeutic ability. The observed antiplatelet activity primarily attributed to its inherited antioxidant potential that scavenge free radicals by ameliorating the oxidative stress. Protein extract of jackfruit seed, bitter gourd seed, flax seed inhibited platelet aggregation.³⁸⁻⁴⁰ Moreover, SBE was nontoxic to experimental mice as it did not show hemolytic activity, hemorrhage, and edema in experimental mice. Red blood cell haemolysis is characterized by the breakdown of cell membrane leading to the release of haemoglobin into the surrounding plasma. The anemia and hypoxia conditions are caused by these deleterious events.²⁹

CONCLUSIONS

This study for the first time demonstrates the anticoagulant, antiplatelet, and antioxidant properties of SBE. Thus, it could be a better candidate in the management of stress related life style diseases.

Limitation of the study

This study reports on the antioxidant, anticoagulant, and antiplatelet activity of sorghum protein extract. The observed medicinal property of sorghum could be due to its enzymatic or non-enzymatic proteins have to be authenticated. Thus, purification and characterization of proteins/peptide/enzyme from sorghum helps in the better understanding the structure function relationship.

Acknowledgments

D.S. thanks the Department of Science and Technology, Government of India, New Delhi and Vision Group on Science and Technology, Government of Karnataka, Bangalore for financial assistance.

Funding

This study was funded by Department of Science and Technology Government of India New Delhi (SB/YS/LS-87/2014).

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of Interest

The authors declared no potential conflict of interest with respect to the authorship and publication.

REFERENCES

- Goode HF, Webster NR. Free radicals and antioxidants in sepsis. *Crit Care Med* 1993; 21:1770–6.
- Bateman RM, Jagger JE, Sharpe MD, et al. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am J Physiol Heart Circ Physiol* 2001; 280:2848–56.
- Brown AS, Moro MA, Masse JM, et al. Nitric oxide-dependent and independent effects on human platelets treated with peroxynitrite. *Cardiovasc Res* 1998; 40:380–8.
- Cooper D, Stokes KY, Tailor A, et al. Oxidative stress promotes blood cell-endothelial cell interactions in the microcirculation. *Cardiovasc Toxicol* 2002; 2:165–80.
- Herkert O, Diebold I, Brandes RP, et al. NADPH oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation* 2002; 105:2030–6.
- Hu W, Jin R, Zhang J, et al. The critical roles of platelet activation and reduced NO bioavailability in fatal pulmonary arterial hypertension in a murine hemolysis model. *Blood* 2010; 116:1613–22.
- Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol* 2004; 24:1988–96.
- Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med* 1999; 341:586–92.
- Flight I, Clifton P. Cereal grains and legumes in the prevention of coronary heart disease and stroke: A review of the literature. *Eur J Clin Nutr* 2006; 60:1145–59.
- Taylor JRN, Schober TJ, Bean SR. Novel food and non-food uses for sorghum and millets. *J Cereal Sci* 2006; 44: 252–71.
- Awika JM, Rooney LW. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* 2004; 65:1199–221.
- Bach Knudsen KE, Munck L. Dietary fibre contents and compositions of sorghum and sorghum-based foods. *J Cereal Sci* 1985; 3:153–64.
- Niba LL, Hoffman J. Resistant starch and b-glucan levels in grain sorghum (*Sorghum bicolor* M.) are influenced by soaking and autoclaving. *Food Chem* 2003; 81:113–8.
- Kayitesi E, Duodu KG, Minnaar A, et al. Sensory quality of marama/sorghum composite porridges. *J Sci Food Agric* 2010; 90:2124–32.
- Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976; 72:248–54.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Quick AJ, Stanley-Brown M, Bancroft FW. A study of the coagulation defect in haemophilia and in jaundice. *Am J Med Sci* 1935; 90:501–11.
- Ardlie NG, Han P. Enzymatic basis for platelet aggregation and release: the significance of the 'platelet atmosphere' and the relationship between platelet function and blood coagulation. *J Haematol* 1974; 26:331–56.
- Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962; 194:27–9.
- Ohakawa H, Oshishi N, Yagi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 1979; 75:351–8.
- Levine RL, Garland D, Oliver CN, et al. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1991; 186:464–78.
- KostyukVA, Potapovich AI, Kovaleva JI. Simple and sensitive method of definition of superoxide dismutase, based on reaction of oxidation of kvercetine. *Questions Med Chem* 1990; 2:88–91.
- Vishwanath BS, Kini RM, Gowda TV. Characterization of three edema-inducing phospholipase A2 enzymes from habu (*Trimeresurus flavoviridis*) venom and their interaction with the alkaloid aristolochic acid. *Toxicon* 1987; 25(5):501–15.
- Kondo H, Kondo S, Itezawa H, et al. A study on the quantitative method from determination of hemorrhagic activity of Habu snake venom. *Jpn J Med Sci Biol* 1969; 13:43–51.
- Joseph B, Raj SJ. Pharmacognostic and phytochemical properties of Aloe Vera Linn – an overview. *Int J Pharm Sci Rev Res* 2010; 4(2):106–10.
- Cordier W, Steenkamp V. Herbal remedies affecting coagulation: a review. *Pharm Biol* 2012; 50(4):443–52.
- Ohigashi H, Ishikawa O, Eguchi H, et al. A simple and safe anastomosis in pancreatico gastrotomy using mattress sutures. *Am J Surg* 2008; 196(1):130–4.
- Fenster C. White food sorghum in the American diet. In: US Grains Council 43rd Board of Delegates' Meeting. 2003.
- Jayanna K, Nandish M, Chethana R, et al. Protective effect of tamarind seed coat ethanol extract on eryptosis induced by oxidative

- stress. *Biochemistry (Moscow)* 2020; 85(1):119–29.
30. Lang F, Lang KS, Lang PA, et al. Mechanisms and significance of eryptosis. *Antioxid Redox Signal* 2006; 8:1183–92.
 31. Andrisic L, Dudzik D, Barbas C, et al. Short overview on metabolomics approach to study pathophysiology of oxidative stress in cancer. *Redox Biol* 2018; 14:47–58.
 32. Grinberg L, Newmark H, Kitrossky N, et al. Protective effects of tea polyphenols against oxidative damage to red blood cells. *Bioche Pharma* 1997; 54:973–8.
 33. Srinivasa C, Kengaiah J, Nandish SK, et al. *Caesalpinia crista* coat extract protects red blood cell from sodium nitrite-induced oxidative stress and exhibits antiplatelet activity. *Blood Coagulation & Fibrinolysis* 2020; 31(5):293–302.
 34. Nagababu E, Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. *Biochem Biophys Res Commun* 1998; 247:592–6.
 35. Tysl K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. *Microcirculation* 2011; 18(2):152–62.
 36. Nandish SK, Kengaiah J, Ramachandraiah C, et al. Anticoagulant, antiplatelet and fibrin clot hydrolysing activities of flax seed buffer extract. *Phcog Mag* 2018; 14:175–83.
 37. Sowmyashree G, Bhagyalakshmi M, Girish KS, et al. Jackfruit (*Artocarpusheterophyllus*) seed extract exhibits fibrino(geno)lytic activity. *Phcog J* 2015; 7:171–7.
 38. Manjappa B, Gangaraju S, Girish KS, et al. *Momordica charantia* seed extract exhibits strong anticoagulant effect by specifically interfering in intrinsic pathway of blood coagulation and dissolves fibrin clot. *Blood Coagulation and Fibrinolysis* 2015; 26(2):191–9.
 39. Nandish SK, Kengaiah J, Ramachandraiah C, et al. Purification and characterization of non-enzymatic glycoprotein (NEGp) from flax seed buffer extract that exhibits anticoagulant and antiplatelet activity. *Int J Biol Macromol* 2020; 163(3):317–26.
 40. Devaraja S, Girish KS, Devaraj VR, et al. Factor Xa-like and fibrin(o-ogen)olytic activities of a serine protease from *Hippasa agelenoides* spider venom gland extract. *J Thromb Thrombolysis* 2010; 29(1):119–26.

Экстракт протеина сорго защищает эритроциты от окислительного стресса, вызванного нитритом натрия, и проявляет антикоагулянтную и антитромбоцитарную активность

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Дата получения: 18 августа 2020 ♦ **Дата приемки:** 11 марта 2021 ♦ **Дата публикации:** 31 декабря 2021

Образец цитирования: Sannanigaiah D, Shivaiah A, Kengaiyah J, Srinivasa C, Nandish SKM, Ramachandraiah C, Hanumegowda S, Manjappa B, Martin SS, Laxmaiah RK, Shinde M. Sorghum protein extract protects RBC from sodium nitrite-induced oxidative stress and exhibits anticoagulant and antiplatelet activity. *Folia Med (Plovdiv)* 2021;63(6):884-94. doi: 10.3897/folmed.63.e57713.

Резюме

Введение: Окислительный стресс играет решающую роль в развитии диабета, артрита, рака, эриптоза, сердечно-сосудистых заболеваний и тромбозов. В настоящее время существует большой спрос на антиоксиданты из природных источников из-за их полезной роли в борьбе с вышеупомянутыми заболеваниями.

Цель: Целью исследования было оценить защитный эффект буферного белкового экстракта сорго (БЭС) на окислительный стресс, вызванный нитритом натрия и тромбозом.

Материалы и методы: Характеристику белка БЭС проводили с помощью SDS-PAGE. Окислительный стресс эритроцитов был вызван нитритом натрия (NaNO_2), и были измерены ключевые маркеры стресса, такие как перекисное окисление липидов (LPO), содержание карбонила белка (PCC) и уровни антиоксидантных ферментов (SOD и CAT). Антикоагулянтный эффект БЭС был установлен с применением времени рекальцификации плазмы *in vitro*, активированного частичного тромбопластинового времени (АРТТ), протромбинового времени (PT), времени *in vivo* для кровотечения из хвоста мыши. Антитромбоцитарную активность БЭС изучали с использованием агониста аденозиндифосфата (ААД) и агрегации тромбоцитов, индуцированной адреналином. Нетоксичные свойства БЭС были идентифицированы с использованием *in vitro* прямых активностей, индуцирующих гемолитическую, геморрагическую и отечную активность у экспериментальных мышей.

Результаты: БЭС показал сходные показатели прикрепления белка как в редуцированных, так и в не-редуцированных условиях SDS-PAGE. Интересно, что БЭС нормализовал уровни LPO, PCC, SOD и CAT, вызванных окислительным стрессом эритроцитов. Кроме того, БЭС оказывал антикоагулянтный эффект в богатой тромбоцитами плазме, улучшая время свёртывания с контрольных 250 с до 610 с и время кровотечения с контрольных 200 с до более чем 500 с ($p < 0.01$) в зависимости от дозы. Кроме того, БЭС распространил процесс образования сгустка только на АРТТ, но не на PT. БЭС подавляет агонист ADP и агрегацию тромбоцитов, вызванную адреналином. БЭС не гидролизует клетки эритроцитов, лишённые отечных и геморрагических свойств.

Заключение: Это исследование впервые раскрывает антикоагулянтные, антиагрегантные и антиоксидантные свойства БЭС. Таким образом, наблюдаемые результаты подтверждают, что потребление сорго полезно для здоровья и благополучия.

Ключевые слова

антикоагулянт, антиоксидант, антиагрегант, окислительный стресс, белок, тромбоз