Article

Black Sorghum Phenolic Extract Modulates Platelet Activation and Platelet Microparticle Release

Borkwei Ed Nignpense [1] , Kenneth A Chinkwo [1,2] , Christopher L Blanchard [1,2] and Abishek B Santhakumar [1,2,*]

[1]   School of Biomedical Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia; bednignpense@csu.edu.au (B.E.D.); kchinkwo@csu.edu.au (K.A.C.); CBlanchard@csu.edu.au (C.L.B.)

[2]   Australian Research Council (ARC) Industrial Transformation Training Centre (ITTC) for Functional Grains, Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, NSW 2650, Australia

*   Correspondence: asanthakumar@csu.edu.au; Tel.: +61-2-6933-2678

check for updates

Abstract: Platelet hyper-activation and platelet microparticles (PMPs) play a key role in the pathogenesis of cardiovascular diseases. Dietary polyphenols are believed to mimic antiplatelet agents by blunting platelet activation receptors via its antioxidant phenomenon. However, there is limited information on the anti-platelet activity of grain-derived polyphenols. The aim of the study is to evaluate the effects of sorghum extract (Shawaya short black 1 variety), an extract previously characterised for its high antioxidant activity and reduction of oxidative stress-related endothelial dysfunction, on platelet aggregation, platelet activation and PMP release. Whole blood samples collected from 18 healthy volunteers were treated with varying non-cytotoxic concentrations of polyphenol-rich black sorghum extract (BSE). Platelet aggregation study utilised 5 μg/mL collagen to target the GPVI pathway of thrombus formation whereas adenine phosphate (ADP) was used to stimulate the P2Y1/P2Y12 pathway of platelet activation assessed by flow cytometry. Procaspase-activating compound 1 (PAC-1) and P-selectin/CD62P were used to evaluate platelet activation- related conformational changes and degranulation respectively. PMPs were isolated from unstimulated platelets and quantified by size distribution and binding to CD42b. BSE treatment significantly reduced both collagen-induced platelet aggregation and circulatory PMP release at 40 μg/mL ($p < 0.001$) when compared to control. However, there was no significant impact of BSE on ADP-induced activation-dependent conformational change and degranulation of platelets. Results of this study suggest that phenolic rich BSE may confer cardio-protection by modulating specific signalling pathways involved in platelet activation and PMP release.

Keywords: black sorghum; polyphenols; platelets; platelet microparticles; atherosclerosis

1. Introduction

According to a World Health Organisation report, cardiovascular diseases accounted for an estimated 31% of deaths globally with majority being a result of stroke or heart attack [1,2]. In clinical settings, treatment involves blunting the activity of platelets using antiplatelet drugs. These drugs interfere with the thrombotic pathophysiology—wherein a rupture of an atherosclerotic plaque triggers platelet hyper-activation resulting in unwanted clot formation and occlusion of the blood vessel. Macrovesicles referred to as platelet microparticles (PMPs) are released following platelet activation and can contribute to the thrombotic situation [3,4].

The several signalling pathways involved in platelet activation and thrombus formation include receptor-agonist pathways such as P2Y1/P2Y12-ADP, GPVI-collagen, PAR1-thrombin and the COX-1-thromboxane [5]. An agonist such as collagen when exposed by atherosclerotic plaque may

activate nearby platelets by binding to their GPVI receptor resulting in complex intracellular signalling that produce a conformational change (indicated by GPIIb/IIIa receptor expression), degranulation (indicated by P-selectin secretion) and subsequent formation of platelet aggregates [5]. In addition, PMP released upon activation possess adhesive and pro-coagulant platelet-derived receptors that further enhance thrombus formation, thereby acting as biomarkers of platelet activation [3]. The common antiplatelet agents, clopidogrel and aspirin, used in clinical treatments inhibit platelet activation and its circulating biomarkers by selectively targeting P2Y1/P2Y12-ADP and COX-1-thromboxane respectively [6]. Unfortunately, because of the unresponsiveness and side effects associated with administration there have been considerable research in dietary bioactive agents known as polyphenols [7].

One such example of a polyphenol-rich functional food is sorghum whole grain. Although mainly used as animal feed, studies have demonstrated that it possesses anti-inflammatory, anti-cancer and antioxidant properties which add value to it as a food for human consumption [8]. Sorghum of different types exist that are classified based on the pigmentation of the pericarp and vary in their phenolic content [9]. The polyphenols found in sorghum that contribute to its bioactivity include flavonoids, hydroxybenzoic acids and hydroxycinnamic acid [8]. Furthermore Francis et al. [10] recently demonstrated that black sorghum rich in catechins and their derivatives may confer cardioprotective properties. The treatment of human umbilical vein cells with flavonoid-rich extract was found to prevent oxidative stress-related endothelial dysfunction through the modulation of gene expression.

Furthermore, these cardio-protective benefits of polyphenols apply in the context of platelet function. Several studies have demonstrated that polyphenols may inhibit platelet activation, adhesion, degranulation and aggregation by targeting specific thrombogenic pathways for example $P2Y_1/P2Y_{12}$-ADP, GPVI-collagen, PAR1-thrombin and the COX-1-thromboxane. As reviewed by Ed Nignpense et al. [11] many of the studies that investigate the polyphenol impact on platelet function and PMP generation utilise aggregometry and flow cytometry. However there is limited research on sorghum-derived polyphenols in modulating biomarkers of platelet activation. This study aims to investigate the impact of black sorghum derived polyphenol extracts on collagen-induced platelet aggregation, ADP-induced platelet activation and PMP generation.

2. Materials and Methods

2.1. Research Ethics

The study protocol was approved by the Charles Sturt University Human Research Ethics Committee (HR17012) and the Institutional Biosafety Committee (19HB02). The study was performed in compliance with relevant laws and institutional guidelines.

2.2. Volunteer Recruitment

Eighteen healthy volunteers between 18–65 years of age (9 males and 9 females) were recruited from Charles Sturt University and the local community. Informed consent was obtained from all participants prior to commencement of the study. The criteria for recruitment involved normal health status with no history of conditions such as cardiovascular, metabolic, liver or lung disease. Other parameters that could affect the integrity of the analysis such as alcohol consumption, smoking, pregnancy, allergies or venepuncture difficulty were considered during the recruitment process. A health screening questionnaire was used to assess the already mentioned parameters. A dietary questionnaire (adapted from WINTEC and NZ academy of sport) was used to assess the usual dietary intake of volunteers and to avoid recruitment of participants on a high antioxidant diet. The cut-off figure for each type of food listed in the questionnaire was based on nutrient reference ranges for Australia and New Zealand—recommended daily nutrient intake values.

2.3. Blood Collection and Processing

After fasting for at least 8 h, whole blood was collected from each participant by a trained phlebotomist into a tri-potassium ethylene diamine tetra-acetic acid (EDTA-1.8 mg/mL concentration) anticoagulant tube (Vacuette Greiner Bio-one, Interpath Services, Heidelberg West, VIC, Australia) and a tri-sodium citrate (28.12 g/L concentration) anticoagulant tube (Becton, Dickson and Company, North Ryde, NSW, Australia). A 20-mL syringe (Becton, Dickson and Company, North Ryde, NSW, Australia) and 21-gauge 1.5-inch needle (Terumo Medical Corporation, Macquarie Park, Australia) were used to draw blood from the median cubital vein. The purpose of choosing a larger gauge was to avoid the activation of platelets while drawing or dispensing blood. Utmost care was taken to ensure samples were not obtained through a traumatic collection and that none contained obvious clots. In addition, care was taken to ensure minimal specimen handling and agitation in order to prevent artefactual platelet activation. The first 2 mL of blood was discarded before drawing into the tri-sodium citrate tubes in order to avoid the risk of collecting platelets activated by venepuncture. Tri-sodium tubes were used for aggregometry and flow cytometry assays whereas the EDTA was used to perform full blood examinations.

2.4. Full Blood Examination

Using an Abbott CELL-DYN Emerald 22 Haematology Analyser (Abbott Diagnostics, Illinois, USA), a full blood examination (FBE) was performed on all samples. The FBE results of volunteers indicated that the blood cell parameters were within normal reference ranges as determined by the Royal College of pathologists of Australia. Individuals with cell counts, especially platelet counts, outside of the reference range were excluded from the study. Quality control validation and maintenance were all performed according to the Abbott CELL-DYN Emerald 22 Haematology Analyser manual.

2.5. Extraction of Black Sorghum Polyphenols

Sorghum (*Sorghum bicolor*) samples of six different pericarp varieties were obtained from glasshouse trials conducted by Curtin University, Perth, Western Australia. Six pigmented varieties of sorghum were cultivated under the same conditions, grown in a glasshouse equipped with Lumisol Clear AF cover (200 μm thick, at a transparency of ca UV-A 94%, UV-B 84% and photosynthetic active radiation (PAR, 400-700 nm) 93% [8]. Extraction and analysis of phenolic composition and antioxidant activity were performed previously using methods described by Rao et al. [8]. Among the different sorghum varieties, the black pericarp variety (Shawaya short black 1) was selected for this study because of its relatively high antioxidant activity when analysed with ferric reducing antioxidant power (FRAP; 20.19 ± 2.69 mg/g TE) and 2,2-dipheny-1-picrylhydrazyl (DPPH; 18.04 ± 3.53 mg/g TE) antioxidant assays (Supplementary Figure S1, Tables S1 and S2). The highest level of polyphenols found in the BSE included catechin derivatives, catechins and pentahydroxyflavanone-(3->4)-catechin-7-O- glucoside (Supplementary Table S2). Stock concentrations of BSE (20 mg/mL in 50% DMSO) were diluted in phosphate buffered saline (PBS) to achieve desired concentrations (5 μg/mL, 20 μg/mL and 40 μg/mL) in whole blood. Desired concentrations were selected based on viability studies done by Francis et al. [10].

2.6. Whole Blood Platelet Aggregometry

Platelets in whole blood were stimulated for aggregation using 5 μg/mL collagen exogenous platelet agonists (DSKH Australia Pty. Ltd., Hallam, VIC, Australia) to investigate the effect of BSE treatment on the platelet aggregation. Five hundred microliters of citrated whole blood were added to 100 μL of 0.1% DMSO control (Sigma-Aldrich, Castle Hill, NSW, Australia) or BSE stock concentrations (5 μg/mL, 20 μg/mL and 40 μg/mL) and mixed with 400 μL of saline. The sample was then incubated at 37 °C for 20 min. Using a Chrono-log model 700 lumi-aggregometer (DKSH Australia Pty. Ltd., Hallam, VIC, Australia) the samples were analysed by means of electrical impedance (ohms) to determine the amount of platelet aggregation occurring in the sample over a 6-min time period (Supplementary Figure S2).

2.7. Flow Cytometry

2.7.1. Standardisation

Flow-check fluorospheres were run as quality control for optimal laser alignments. Antibody capture beads (Anti-Mouse Ig, K CompBeads, BD Biosciences, North Ryde, NSW, Australia) were used for single colour compensation controls in order to achieve optimal compensation. Megamix beads (0.1 µm, 0.3 µm, 0.5 µm, 1 µm) from Biocytex, Marseille, France were used as per manufacturer's instructions to set up an appropriate gating to detecting microparticles. They were run before each PMP analysis.

2.7.2. Measurement of Platelet Activation-Dependent Conformational Change and Degranulation

The effects of BSE on ADP-induced platelet activation were analysed and interpreted using a Gallios flow cytometer (Beckman Coulter, Inc., Lane Cove NSW, Australia). The protocols were adopted from the method described by Santhakumar et al. [12] with some modifications. Platelet activation and thrombogenic indicators were assessed via activation-dependent platelet monoclonal antibodies (mAbs) purchased from Becton, Dickinson and Company, North Ryde, NSW, Australia. Procaspase activating compound-1 (PAC-1)-fluorescein isothiocyanate-fluorescein isothiocyanate was used to detect platelet activation-related conformational change and P-selectin/CD62P-allophycocyanin highlighted activation dependent degranulation. CD42b-phycoerythrin identified the GPIb-IX-V receptor, a common receptor found on the surface of all platelets, activated and resting included. A decreased expression of mAb exhibits alleviation of thrombogenesis. Within 5 min of collection tri-sodium citrated whole blood was used for assay preparation to avoid artefactual activation of platelets. A volume of 40 µL of blood was incubated with DMSO control or the various BSE concentration for 20 min at 37 °C in the dark. A 10-µL mixture of all three monoclonal antibodies (3.33 µL each of PAC-1, CD62P and CD42b) was added to blood samples and incubated for 20 min at room temperature in the dark. To induce platelet activation, 10 µM ADP (Helena laboratories Pty. Ltd., Mt Waverly, VIC, Australia) was added, and samples were incubated for a further 15 min in the dark at room temperature, after which erythrocytes were lysed (575 µL of 10 % lysing solution). Samples were thoroughly vortexed to ensure homogeneity and incubated in the dark at room temperature for a further 15 min and then analysed. In all, 10,000 platelet events were acquired, gated based on light scatter and CD42b mAb expression and activated platelets were articulated as mean fluorescence intensity (MFI) (Supplementary Figure S3).

2.7.3. Measurement of Circulatory PMPs

Using the microparticle gating established with Megamix beads, PMPs were identified and quantified by size distribution and binding to CD42b (Supplementary Figure S4). The protocol for circulatory PMP analysis was adapted from Lu et al. [13]. A volume of 1 mL whole blood was added to micro-centrifuge tubes in the presence of PGE1 (120 nmol/L; Sigma-Aldrich, Castle Hill, NSW, Australia). PGE1 was added to prevent artefactual activation during centrifugation. The blood samples were incubated with the respective 100 µL BSE concentrations and DMSO control for 20 min at 37 °C in the dark. Each sample was then centrifuged for 15 min at a 1000 rpm and the resultant platelet rich plasma (PRP) was discarded. The remaining blood was spun a further 15 min at 3000 rpm. The supernatant rich in PMPs (40 µL) was collected into flow tubes and incubated with 4 µL of CD42b and 6 µL of stain buffer (Becton, Dickson and Company, North Ryde, NSW, Australia) in dark room for 15 min. Four percent formaldehyde was used to fix any activation of platelets left in the supernatant. After a 10-min incubation period the samples were run for PMP analysis on the flow cytometer.

2.8. Statistical Analysis

A two-way ANOVA following Tukey's post comparison test was performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, California, USA). A minimum sample size of 14 participants in total was required for 80% power to detect a 5% variation in the laboratory

parameters measured where a 3–5% standard deviation exists in the population, assuming an alpha error of 0.05. All the data were expressed as mean ± standard deviation (SD). Differences between the groups were significant when $p < 0.05$. Any significant statistical interactions were included in the analysis where applicable.

3. Results

The baseline parameters including full blood counts for all 18 participants were within normal reference ranges set by the Royal College of Pathologists of Australasia (Table 1) [14].

Table 1. Baseline full blood counts of participants.

Parameters	Mean ± SD
Age (years)	26 ± 8
WBC ($\times 10^9$/L)	5.5 ± 1.3
Neutrophil (%)	48.6 ± 9.5
Lymphocytes (%)	37.8 ± 9.2
Monocytes (%)	10.6 ± 2.3
Eosinophils (%)	2.8 ± 1.3
Basophils (%)	0.1 ± 0.1
RBC ($\times 10^{12}$/L)	4.6 ± 0.5
Haemoglobin (g/L)	147.9 ± 16.1
PCV (%)	0.41 ± 0.04
MCV (fL)	90.0 ± 3.5
MCH (pg)	35.8 ± 15.2
MCHC (g/dL)	360.1 ± 6.0
RDW (%)	14.9 ± 0.8
Platelet count ($\times 10^9$/L)	248.3 ± 50.0
MPV	8.41 ± 0.89

Values are represented as mean ± Standard deviation (SD). RBC, red blood cell, PCV, packed cell volume, MCV, mean cell volume, MCH, mean cell haemoglobin, MCHC, mean cell haemoglobin concentration, RDW, red cell distribution width, MPV, mean platelet volume.

3.1. Effect of BSE on Whole Blood Platelet Aggregation and Platelet Activation

BSE at 40 μg/mL concentration significantly reduced platelet aggregation stimulated by collagen by 19 % ($p = 0.0004$) (Figure 1). BSE at lower concentrations did not exhibit any significant reduction in aggregation.

It was observed that whole blood treatment with the varying concentrations of BSE did not significantly affect ADP-induced platelet conformational change and degranulation indicated by PAC-1 and P-selectin expression respectively (Supplementary Figures S5 and S6).

3.2. Effect of BSE on Circulatory PMPs

BSE at a concentration of 40 μg/mL significantly reduced the amount of circulatory PMPs in whole blood by 47% ($p = 0.0008$). Lower concentrations of BSE did not exhibit any significant reduction to the amount circulatory PMPs (Figure 2).
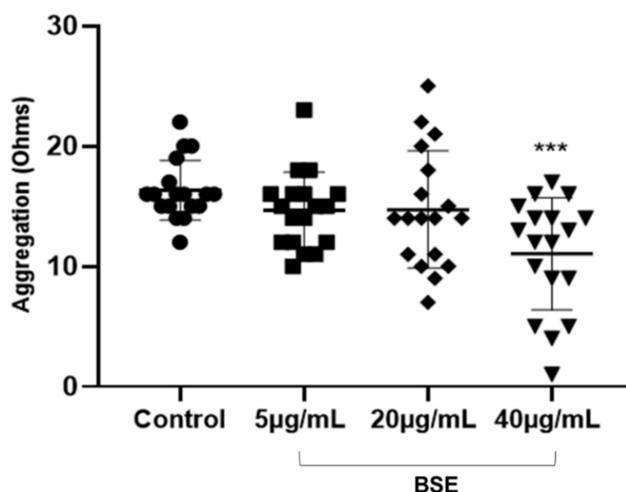
Figure 1. The effect of varying concentrations of BSE on collagen-induced aggregation. BSE at 40 μg/mL significantly reduced platelet aggregation (5.3 ± 1.3; *p* value = 0.0004). BSE at 5 μg/mL and 20 μg/mL did not reduce platelet aggregation when compared to control (*p* value > 0.1). N = 18 and data is represented in aggregation (Ohms) versus BSE concentrations. *** signifies statistical significance *p* < 0.001 compared to control. Error bars expressed as mean ± SD.
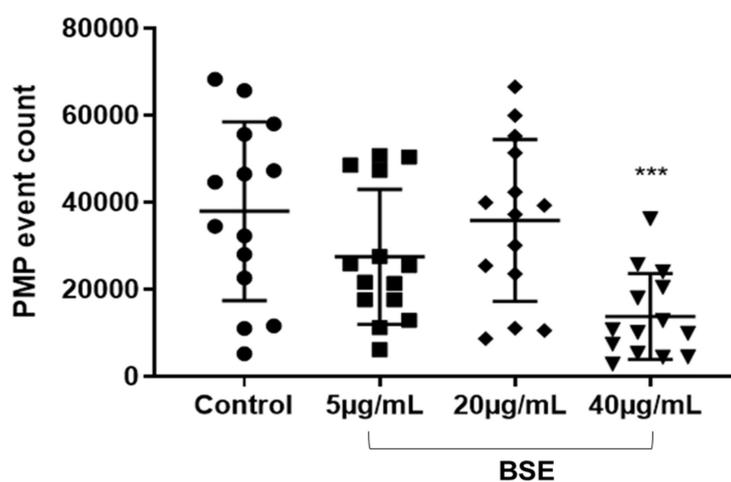

Figure 2. The effect of varying concentrations of BSE on circulatory PMP production in vitro. BSE at 40 μg/mL significantly reduced the amount of circulatory PMPs (<24190 ± 4935, *p* = 0.0008). BSE at 5 μg/mL and 20 μg/mL did not reduce platelet aggregation when compared to control (*p* value > 0.1). N = 14 and data are represented in number of PMP events versus BSE concentrations. *** signifies statistical significance *p* < 0.001 compared to control. Error bars expressed as mean ± SD.

4. Discussion

There is growing interest in understanding the therapeutic benefits of functional foods. Sorghum for example is one of the functional foods that is showing promise in this area. With sorghum-derived polyphenols already having demonstrated anti-inflammatory, anti-cancer and antioxidant properties, the current study aimed to evaluate the effects of polyphenol-rich BSE on platelet function in terms of aggregation, conformational change, degranulation and circulatory PMP production [8–10]. It was observed that BSE significantly inhibited collagen-induced platelet aggregation and decreased the release of circulatory PMPs but did not have a significant effect on ADP-induced platelet conformational change or degranulation. Although these results do not reflect a typical dose-dependent inhibition, they suggest a potential role of BSE polyphenols at optimum concentrations to interfere with pathways in the GPVI-collagen signalling and the release of circulatory PMPs but little or no effect on $P2Y_1$/$P2Y_{12}$-ADP pathway.

To the best of our knowledge only a few studies have investigated the antiplatelet effects of sorghum extracts. Li, Yu and Fan et al. [15] extracted alditols and monosaccharides from sorghum vinegar to evaluate their anti-aggregation activity using the turbidimetric method. Results from their study indicated a significant dose-dependent inhibition of aggregation via multiple agonists, arachidonic acid, collagen, ADP and thrombin. Furthermore, a different study by Fan et al. [16] reported in vitro inhibition of ADP- and thrombin- induced rabbit platelet aggregation by methanolic extracts of aged sorghum vinegar with the half maximal inhibitory concentrations (IC_{50}) of 1.7 ± 0.3 and 8.9 ± 1.9 mg/mL respectively. When rats were orally administered the extracts (>100 mg/kg), both collagen- and epinephrine-induced pulmonary thrombosis were inhibited. In comparison with the present study it is to be noted that these studies employed platelet-rich plasma rather than whole blood hence not accounting for the possible involvement of other blood cells and extracellular mediators involved in thrombus formation. In addition, sorghum vinegar extracts were used at higher concentrations; milligrams compared to micrograms used in this study. This raises the question of bioavailability and the importance of employing physiological concentrations of extracts.

Although the BSE concentration of 40 μg/mL at which antiplatelet effect were observed is relatively lesser in concentration than used in the other studies, the most bioactive compounds with respect to antioxidant activity were catechins and other flavonoids which are usually considered to have low bioavailability [8]. It has been suggested that the total plasma polyphenol concentration rarely exceeds 1 μM and that their antiplatelet effects are only found at high non-physiological concentrations (greater than 50 μM) [17,18]. However, it is likely that these plasma concentrations are underestimations due to the ability of polyphenols to bind to the surface of red blood cells and thereby exert their bioactivity [19]. Furthermore polyphenols (structurally related to catechin) and their metabolites have been shown to inhibit platelet function in vitro [20]. This highlights the possibility of sorghum catechins and their metabolites having antiplatelet effects in vivo despite bioavailability concern. Interestingly, an in vivo human dietary intervention trial compared consumption of red and white wholegrain sorghum-based pasta to a control pasta in order to investigate its acute effect on the total phenol content and antioxidant activity in the plasma of healthy subjects [21]. Results showed that when compared to the control pasta, the red sorghum pasta showed significantly increased net plasma phenolic content and antioxidant activity post consumption (from 216.90 ± 2.62 at baseline to 269.40 ± 2.33 at 2 h; *p* < 0.001), thus demonstrating a plausible correlation between antioxidant activity and sorghum polyphenol consumption—which in turn may contribute to antiplatelet effects.

The antiplatelet effects was observed with BSE-included inhibition of collagen-induced aggregation and circulatory PMP production but no effect on ADP-induced platelet activation. The absence of antiplatelet effects on the $P2Y_1$/ $P2Y_{12}$-ADP activation pathway suggests that BSE polyphenols are not mimicking the action of drugs such as clopidogrel that antagonise $P2Y_{12}$ receptor activation [22]. However, the inhibition of collagen-induced aggregation suggest that BSE polyphenols interfere with GPVI-collagen signalling pathways by either blunting the GPVI receptor directly or by other mechanisms [5]. Previous studies have demonstrated that flavonoids, specifically quercetin and catechin, can act synergistically to inhibit collagen-induced aggregation by blunting the associated burst of H_2O_2 and subsequent PLC activation [23,24]. Thus, a possible mechanism of inhibition BSE flavonoids may be a synergistic antagonism of the positive feedback activation of intracellular signals triggered by H_2O_2. Moreover, it has been shown that the phosphorylation cascade initiated by collagen can be inhibited by flavonoids [25,26]. Flavones, especially apigenin and luteolin, by virtue of a double bond in the C2-C3 and the keto group in C4 can also inhibit collagen-induced activation by antagonizing the TxA_2 receptor activation which is also involved in the positive feedback loop [27]. Besides inhibition of the GPVI-collagen signalling, BSE polyphenols showed inhibition of the circulatory PMP production.

To the best of our knowledge, this is the first study investigating the effect of sorghum-derived polyphenols on PMP production. In contrast to this study, other PMP studies have employed the use of Annexin V as well as the platelet specific antibody CD42b, to identify pro-coagulant PMPs

by their phosphatidylserine expression and to limit background noise [28]. However, because of the heterogeneity of PMPs, not all PMPs express phosphatidylserine [29]. Moreover, the measurement of CD42b-positive PMPs alone is significant as its increase has been associated with an increased risk of coronary heart disease [30]. From the current study, the significant inhibition of CD42b-positive circulatory PMPs observed in vitro may be attributed to the antioxidant properties of BSE polyphenols. It is believed that the inhibition of PMP generation may be the result of neutralisation of H_2O_2, scavenging of other free radicals or interaction with intracellular signalling leading to PMP release.

The juxtaposition of both the present study and that of an earlier study by Francis et al. [10] highlights the multifaceted role of BSE polyphenols in cardio-protection. The group investigated the effects of BSE polyphenols on the expression of antioxidant- and inflammatory-linked genes involved in endothelial dysfunction under oxidative stress. Results indicated that BSE polyphenols alleviate oxidative stress–induced damage to endothelial cells. Since vascular dysfunction is a precursor to cardiovascular diseases, the current study builds upon earlier findings by exhibiting the antiplatelet effects of BSE. In the context of endothelial dysfunction, platelet activation and circulatory PMPs play central roles in the pathogenesis of atherothrombosis. The disruption of the plaque exposes collagen that binds to the GPVI receptor resulting in platelet activation and subsequent thrombus formation [31]. Circulatory PMPs may contribute to thrombosis via GPIb-IX-V receptor binding and have a pro-inflammatory effect to promote the development of the plaque [32]. Therefore, by reducing collagen-induced platelet aggregation and circulatory PMP generation, BSE polyphenols may be displaying the potential to augment thrombosis.

5. Conclusion and Future Considerations

In summary, the present study contributes to the growing body of literature on bioactivity of sorghum polyphenols and highlights possible mechanisms of antiplatelet action that may result in cardiovascular health benefits. Because of the ability to reduce collagen-induced platelet activation and circulatory PMP generation, BSE polyphenols demonstrate the potential to interfere with pathological processes involved in vascular disorders and thrombotic complications. However, a larger panel of agonist for the flow cytometry and aggregometry studies will aid to further elucidate antiplatelet mechanisms. Because of the bioavailability concerns, well-controlled dietary intervention trials using larger sample sizes to evaluate the antiplatelet effects of sorghum consumption in healthy and pro-thrombotic populations are warranted to justify our findings. Because of the varied phenolic profiles of the different sorghum varieties, further research comparing the antiplatelet therapeutic potential of different grains is also warranted. Furthermore, this study attest to the measurement of circulatory PMPs as a biomarker of platelet activation to assess the bioactivity of functional foods.

and data is represented in mean fluorescence intensity (MFI) versus BSE concentrations Error bars expressed as mean ± SD, Table S1: Phenolic composition and antioxidant activity of sorghum varieties on as is basis, Table S2: List of top ten phenolic compounds identified in the black sorghum phenolic rich extracts by Q-TOF LC/MS and quantified using UHPLC-Online ABTS system (Adapted from Rao et al., 2018).

Author Contributions: B.E.N. conducted the experiments outlined in this study and drafted the manuscript. K.A.C., C.L.B. and A.B.S. were involved in the designing and critical reviewing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSE	Black sorghum extract
COX-1	Cyclooxygenase-1
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
FBE	Full blood examination
GAE	Gallic acid equivalents
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell haemoglobin
MFI	Mean fluorescence intensity
MPV	Mean platelet volume
PAR	Protease-activated receptor
PAC-1	Procaspase activating compound-1
PBS	Phosphate buffered saline
PCV	Packed cell volume
PGE1	Prostaglandin E1
PMP	Platelet microparticle
RBC	Red blood cell
SD	Standard deviation
WBC	White blood cell

References

1. Waters, A.M.; Trinh, T.; Chau, M.B.; Moon, L. Latest Statistics on Cardiovascular Disease in Australia. *Clin. Exp. Pharm. Physiol.* **2013**, *40*, 347–356. [CrossRef] [PubMed]

2. Organization World Health. *Cardiovascular Diseases (Cvds) Fact. Sheet*; Organization World Health: Geneva, Switzerland, 2017.

3. Zaldivia, M.T.K.; McFadyen, J.D.; Lim, B.; Wang, X.; Peter, K. Platelet-Derived Microvesicles in Cardiovascular Diseases. *Front. Cardiovasc. Med.* **2017**, *4*, 74. [CrossRef] [PubMed]

4. Wolf, P. The Nature and Significance of Platelet Products in Human Plasma. *Br. J. Haematol.* **1967**, *13*, 269–288. [CrossRef] [PubMed]

5. Santhakumar, B.A.; Bulmer, A.C.; Singh, I. A Review of the Mechanisms and Effectiveness of Dietary Polyphenols in Reducing Oxidative Stress and Thrombotic Risk. *J. Hum. Nutr. Diet.* **2014**, *27*, 1–21. [CrossRef]

6. Sambu, N.; Curzen, N. Monitoring the Effectiveness of Antiplatelet Therapy: Opportunities and Limitations. *Br. J. Clin. Pharmacol.* **2011**, *72*, 683–696. [CrossRef]

7. Sharma, R.; Reddy, V.; Singh, R.S.; Bhatt, G. Aspirin and Clopidogrel Hyporesponsiveness and Nonresponsiveness in Patients with Coronary Artery Stenting. *Vasc. Health Risk Manag.* **2009**, *5*, 965–972. [CrossRef]

8. Rao, S.; Santhakumar, A.B.; Chinkwo, K.A.; Wu, G.; Johnson, S.K.; Blanchard, C.L. Characterization of phenolic compounds and antioxidant activity in sorghum grains. *J. Cereal Sci.* **2018**, *84*, 103–111. [CrossRef]

9. Dykes, L.; Rooney, L.W.; Waniska, R.D.; Rooney, W.L. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. *J. Agric. Food Chem.* **2005**, *53*, 6813–6818. [CrossRef]

10. Francis, N.; Rao, S.; Blanchard, C.; Santhakumar, A. Black Sorghum Phenolic Extract Regulates Expression of Genes Associated with Oxidative Stress and Inflammation in Human Endothelial Cells. *Molecules* **2019**, *24*, 3321. [CrossRef]

11. Ed Nignpense, B.; Chinkwo, K.A.; Blanchard, C.L.; Santhakumar, A.B. Polyphenols: Modulators of Platelet Function and Platelet Microparticle Generation? *Int. J. Mol. Sci.* **2020**, *21*, 146. [CrossRef]

12. Santhakumar, A.B.; Stanley, R.; Singh, I. The ex vivo antiplatelet activation potential of fruit phenolic metabolite hippuric acid. *Food Funct.* **2015**, *6*, 2679–2683. [CrossRef] [PubMed]

13. Lu, G.Y.; Xu, R.J.; Zhang, S.H.; Qiao, Q.; Shen, L.; Li, M.; Xu, D.Y.; Wang, Z.Y. Alteration of circulatory platelet microparticles and endothelial microparticles in patients with chronic kidney disease. *Int. J. Clin. Exp. Med.* **2015**, *8*, 16704–16708. [PubMed]

14. Royal College of Pathologists of Australasia. RCPA manual. 2004.

15. Li, J.; Yu, G.; Fan, J. Alditols and monosaccharides from sorghum vinegar can attenuate platelet aggregation by inhibiting cyclooxygenase-1 and thromboxane-A2 synthase. *J. Ethnopharmacol.* **2014**, *155*, 285–292. [CrossRef]

16. Fan, J.; Zhang, Y.; Chang, X.; Bolin, Z.; Jiang, D.; Saito, M.; Zaigui, L. Antithrombotic and fibrinolytic activities of methanolic extract of aged sorghum vinegar. *J. Agric. Food Chem.* **2009**, *57*, 8683–8687. [CrossRef]

17. Scalbert, A.; Williamson, G. Dietary Intake and Bioavailability of Polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [CrossRef]

18. Ostertag, L.M.; O'Kennedy, N.G.W.; Horgan, P.A.; Kroon, G.G.D.; de Roos, B. In Vitro Anti-Platelet Effects of Simple Plant-Derived Phenolic Compounds Are Only Found at High, Non-Physiological Concentrations. *Mol. Nutr. Food Res.* **2011**, *55*, 1624–1636. [CrossRef] [PubMed]

19. Koren, E.; Kohen, R.; Ginsburg, I. Polyphenols enhance total oxidant-scavenging capacities of human blood by binding to red blood cells. *Exp. Biol. Med.* **2010**, *235*, 689–699. [CrossRef]

20. Wright, B.; Moraes, C.; Kemp, W.M.; Jonathan, M.G. A Structural Basis for the Inhibition of Collagen-Stimulated Platelet Function by Quercetin and Structurally Related Flavonoids. *Br. J. Pharmacol.* **2010**, *159*, 1312–1325. [CrossRef] [PubMed]

21. Khan, I.; Yousif, A.M.; Johnson, S.K.; Gamlath, S. Acute effect of sorghum flour-containing pasta on plasma total polyphenols, antioxidant capacity and oxidative stress markers in healthy subjects: A randomised controlled trial. *Clin. Nutr.* **2015**, *34*, 415–421. [CrossRef]

22. Damman, P.; Kuijt, R.; Stefan, K.J. P2y12 Platelet Inhibition in Clinical Practice. *J. Thromb. Thrombolysis* **2012**, *33*, 143–153. [CrossRef]

23. Pignatelli, P.; Pulcinelli, F.M.; Celestini, A.; Lenti, L.; Ghiselli, A.; Gazzaniga, P.P.; Violi, F. The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. *Am. J. Clin. Nutr.* **2000**, *72*, 1150–1155. [CrossRef] [PubMed]

24. Pignatelli, P.; Pulcinelli, F.M.; Lenti, L.; Gazzaniga, P.P.; Violi, F. Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood.* **1998**, *91*, 484–490. [CrossRef] [PubMed]

25. Jang, J.Y.; Min, J.; Chae, Y.H.; Baek, J.Y.; Wang, S.B.; Park, S.J.; Oh, G.T.; Lee, S.; Ho, Y.; Chang, T. Reactive oxygen species play a critical role in collagen-induced platelet activation via SHP-2 oxidation. *Antioxid. Redox Signal.* **2014**, *20*, 2528–2540. [CrossRef] [PubMed]

26. Yao, Y.; Chen, Y.; Adili, R.; McKeown, T.; Chen, P.; Zhu, G.; Li, D.; Ling, W.; Ni, H.; Yang, Y. Plant-Based Food Cyanidin-3-Glucoside Modulates Human Platelet Glycoprotein Vi Signaling and Inhibits Platelet Activation and Thrombus Formation. *J. Nutr.* **2017**, *147*, 1917–1925. [CrossRef]

27. Guerrero, J.A.; Lozano, M.L.; Castillo, J.; Benavente-Garcia, O.; Vicente, V.; Rivera, J. Flavonoids inhibit platelet function through binding to the thromboxane A2 receptor. *J. Thromb. Haemost.* **2005**, *3*, 369–376. [CrossRef]

28. Poncelet, P.; Robert, S.; Bailly, N.; Garnache-Ottou, F.; Bouriche, T.; Devalet, B.; Seghatchian, J.; Saas, P.; Mullier, F. Tips and tricks for flow cytometry-based analysis and counting of microparticles. *Transfus. Apher. Sci.* **2015**, *53*, 110–126. [CrossRef]

29. Connor, D.E.; Exner, T.; Ma, D.D.; Joseph, J.E. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. *Thromb. Haemost.* **2010**, *103*, 1044–1052.

30. Ueba, T.; Nomura, S.; Inami, N.; Nishikawa, T.; Kajiwara, M.; Iwata, R.; Yamashita, K. Plasma level of platelet-derived microparticles is associated with coronary heart disease risk score in healthy men. *J. Atheroscler. Thromb.* **2010**, *17*, 342–349. [CrossRef]

31. Badimon, L.; Padró, T.; Vilahur, G. Atherosclerosis, platelets and thrombosis in acute ischaemic heart disease. *Eur. Heart J. Acute Cardiovasc. Care* **2012**, *1*, 60–74. [CrossRef]

32. Merten, M.; Pakala, R.; Thiagarajan, P.; Benedict, C.R. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. *Circulation* **1999**, *99*, 2577–2582. [CrossRef]