REVIEW ARTICLE

Anthocyanin Supplementation Alleviates Antithrombotic Risk by Inhibiting Platelet Activity in Humans

Almottesembellah Gaiz, PhD; Avinash Reddy Kundur, PhD; Elham Nikbakht, PhD; Lada Vugic, PhD; Natalie Colson, PhD; Sapha Shibeeb, PhD; Indu Singh, PhD

ABSTRACT

Background • Platelet hyperactivity has a crucial role in initiating vascular thrombosis and subsequent cardiovascular disease (CVD).

Objective • This study aimed to assess the effect of anthocyanins (AC) on platelet aggregation and activation and lipid profile.

Study Design • A total of 26 healthy participants consumed 320 mg of AC/day in the form of Medox® capsules for 28 days.

Setting • This study was conducted in the laboratories of the School of Medical Sciences, Griffith University, Gold Coast, Australia.

Participants • A total of 26 randomly recruited healthy 25- to 75-year-old participants completed this study.

Primary Outcome Measures • Fasting blood samples were collected pre- and post-the intervention period to perform platelet activation studies by measuring platelet surface marker expression of CD41a and P-selectin, and platelet-monocyte aggregates in adenosine diphosphate (ADP) stimulated platelets. Platelet aggregation studies were performed by stimulating platelets with various agonists such as ADP, collagen and arachidonic acid. Full blood examination, coagulation and biochemistry profile analyses were also performed pre- and post-intervention. Flow cytometric analysis showed a significant effect of AC on the expression of P-selectin as measured by the platelet surface expression of CD62p.

Results • There was a significant reduction of ADP-stimulated platelet aggregation. Hematologic analysis showed a significant reduction of mean platelet volume, mean cell hemoglobin, and mean cell hemoglobin concentration. Coagulation analysis demonstrated significant attenuation of fibrinogen level in the blood.

Conclusion • This study showed inhibition of platelet activity, platelet aggregation and mean platelet volume (MPV). These results suggest that AC has a positive impact on attenuating platelet activity, which might minimize thrombotic risk. (*Altern Ther Health Med.* [E-pub ahead of print.])

Almottesembellah Gaiz, PhD, School of Medical Science, Griffith University, Southport, Australia; National Centre of Hematology, Almustansiriyah University, Baghdad, Iraq. **Avinash Reddy Kundur, PhD; Elham Nikbakht, PhD; Lada Vugic, PhD; Natalie Colson, PhD; Indu Singh, PhD;** School of Medical Science, Griffith University, Southport, Australia. **Sapha Shibeeb, PhD**, School of Medical Science, Griffith University, Southport, Australia; Monash University, Melbourne, Australia.

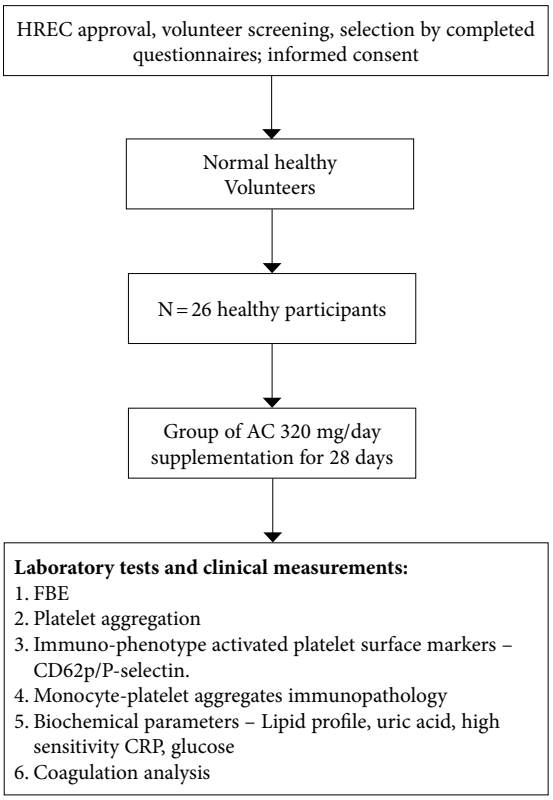
Corresponding author: Almottesembellah Gaiz, PhD
E-mail: almotesem78@gmail.com

INTRODUCTION

Increased platelet activation and aggregation play a central role in the development of intravascular thrombosis and the progression of cardiovascular disease (CVD).[1-3] Intravascular platelet activation is triggered in the event of endothelial vessel wall damage, which exposes the collagen component of the subendothelial matrix. The adhesion of platelets to the sub-endothelial matrix is crucial to thrombus formation. Current antiplatelet therapies specifically target different pathways of platelet activation, thus reducing the risk of thrombosis. However, recent studies conducted on the efficacy of antiplatelet drugs have reported increased resistance, loss of efficiency in reducing platelet activation and development of adverse events in some patients.[4,5]

Polyphenols such as anthocyanins are phytochemicals that are naturally present in colored fruits and berries such as apples, blueberries, strawberries, bilberries and blackcurrants.

Figure 1. An illustration of AC study design



Abbreviations: AC, anthocyanin; CRP, C-reactive protein; FBE, full blood examination; HREC, Human Research Ethics Committee.

Previous studies have shown that consumption of an AC-rich diet can significantly lower the risk of developing CVD.[2,6-8] Furthermore, several *in vitro* and *in vivo* trials have demonstrated that AC supplementation improves lipid profile, endothelial function and flow-mediated vasodilation, while reducing inflammation, hyperglycaemia, lipid peroxidation and endothelial dysfunction.[9-11] The potential of AC to target multiple pathways of platelet activation is of great interest and further research is warranted to fully explore AC's potential in reducing the risk for arterial thrombosis. Accordingly, the aim of this study is to investigate the ability of pure AC extract from bilberries and blackcurrants (Medox®) on various risk markers of thrombosis such as platelet function, coagulation and biochemical profile following a 28-day supplementation period. Medox capsules were sourced from Medpalett AS, Sandnes, Norway.

METHODS

Participant Recruitment and Study Design

This study was approved by Griffith University Human Research Ethics Committee, Griffith University, Queensland, Australia (GU Ref No: MSC/07/14/HREC) and is registered with Australia and New Zealand Clinical Trails Registry (ACTRN12615000293561). For the study, 26 healthy individuals were recruited from the general population and signed an informed consent form prior to the commencement of the study. All the participants were carefully screened using health questionnaires and interviews to ensure that they were healthy, non-smokers and without any history of CVD, bleeding disorders or liver disease. Furthermore, participants taking anti-inflammatory, antiplatelet or anticoagulant agents were not included in the study.

Prior to the commencement of the study, baseline fasting blood samples were collected to determine the presence of any underlying health condition using the results from full blood examination, platelet function assays, coagulation and biochemistry profiles. Participants who met the inclusion criteria were asked to consume 4 capsules of AC extract (Medox capsules, Medpalett AS, Sandnes, Norway) for 28 days with each capsule containing 80 mg of AC for a total of 320 mg/day. Each capsule contained AC concentrate (equivalent to 80 mg) from wild Scandinavian bilberries and blackcurrants, maltodextrin and acidity regulator citric acid. Fasting blood samples were collected before and after the 28-day supplementation period. Adherence and compliance with AC capsule intake were monitored by checking the capsule strips returned by the participant after the supplementation and also by personally interviewing them.

Blood Sample Collection and Full Blood Examination

Fasting blood samples pre- and post-AC supplementation were collected from the median cubital vein by a trained phlebotomist. The blood was then carefully aliquoted into 1 ethylenediamine tetraacetic acid (EDTA; 1.8 mg/ml) tube for full blood examination (FBE) analysis, 3 tri-sodium citrate (28.12 g/L) tubes for platelet function and coagulation studies and 1 serum separation tube (SST) for biochemical analysis. Beckman Coulter ACT™ 5Diff CP hematology analyser (Coulter Corporation, Miami, Florida, USA) was used to perform FBE analysis.

Platelet Aggregation Assay

Platelet-rich plasma obtained (PRP) from whole blood collected in tri-sodium citrate anticoagulant tubes was used to perfrom platelet aggregation studies. PRP was extracted by spinning citrated whole blood at 180 × g for 10 minutes, followed by which platelet-poor plasma (PPP) was obtained by spinning the same tube at 2000 × g for 10 minutes. Platelet aggregation was stimulated by adding platelet agonists ADP (5 μM), collagen (2 μg/mL) and arachidonic acid (AA) (200 μg/mL) and recording the percentage aggregation for 6 minutes at a constant temperature of 37° C. Platelet aggregation studies were performed using Helena AggRam Platelet Aggregometer (Helena Laboratory, Beaumont, Texas, USA). Platelet aggregation testing was completed within 2 hours of the blood collection.

Evaluation of Platelet Activation and Monocyte Platelet Aggregates

Tri-sodium citrate anticoagulated whole blood was used to evaulate platelet activation. Monocloncal antibodies

conjugated with specific flurophores were used to identify and evaluate platelet activation, degranulation and formation of monocyte-platetelet aggregates. CD 41a conjugated with peridinin-chlorophyll-protein complex CY5.5 (PerCP-CY5.5) was used to identifying platelets, while CD62P conjugated with allophycocyanin (APC) was used to quantify platelet degranulation. CD14 conjugated with fluorescein isothiocyanate (FITC) was used to identify monocytes. CD 41a- PerCP-CY5.5/CD14-FITC expression was used to determine the formation of monocte-platelet aggregates, which were defined as CD14+ monocytes that were simultaneously positive for the CD41a marker. For analysis, citrated whole blood was diluted in 1:5 ratio with modified Tyrod`s Buffer (MTB). A mixture of monoclonal antibodies was added to the diluted blood and incubated for 15 minutes at room temperature in the dark. ADP (5 μM) was added as an agonist to stimulate platelet activation, and the samples were incubated for a further 10 minutes. The samples were then fixed by adding 800 μl of 10% RBC lysing solution (BD Biosciences, San Diego, California, USA) and later analysed on BD LSRFortessa™ flowcytometer (BD Biosciences, San Diego, California, USA).

Coagulation Profile

Platelet-poor plasma (PPP) was used to perform coagulation assays. The prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration testing were performed on the Stago STAR-Evolution® Coagulation Analyser (Stago, Asnieres sur Seine, France) per the manufacturer's instructions.

Biochemistry Profile

Blood collected in serum separation tubes (SST) was centrifuged for 10 minutes at 2000xg at RT to extract serum for biochemical analysis. Serum levels of glucose, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), and uric acid (UA) were determined using Integra Cobas 400 Biochemistry Analyser (Roche Diagnostics, Basel, Switzerland). Quality controls and calibrators were run prior to testing to ensure the accuracy of the analyzer.

Statistical Analysis

Statistical analysis was performed using an Graph Pad Prism® version 6 for Windows. Paired *t* test was used to analyze the data. The values are expressed as mean ± standard error of the mean (SEM). $P < .05$ was considered statistically significant.

RESULTS

Full Blood Count

The baseline FBE parameters for all participants were within the reference range. However, AC supplementation significantly reduced MPV (see Table 1).

Platelet Activation and Aggregation

AC supplementation for 28 days significantly lowered ADP-induced platelet aggregation (Figure 2; $P < .05$).

Table 1. Descriptive Values of Full Blood Examination (FBE) Parameters in 26 Participants Pre- and Post-AC Supplementation

Hematologic Indices	Pre-AC Mean ± SEM	Post-AC Mean ± SEM	*P* Value
WBC	4.97 ± 0.31	5.38 ± 0.23	.1906
RBC	4.65 ± 0.30	4.72 ± 0.14	.7457
HGB	131.16 ± 8.47	136.64 ± 2.39	.5237
HCT	0.38 ± 0.02	0.39 ± 0.01	.6973
MCV	83.70 ± 1.31	83.84 ± 1.23	.6647
MCH	29.63 ± 0.59	29.13 ± 0.49	.0122[a]
MCHC	353.16 ± 3.74	347.12 ± 1.61	.0249[a]
RDW	10.83 ± 0.16	10.87 ± 0.11	.6586
PLT	224.66 ± 13	239.28 ± 11	.6586
MPV	8.14 ± 0.15	7.96 ± 0.15	.0319[a]
NE%	48.51 ± 4.12	54.48 ± 1.67	.2147
LY%	28.24 ± 2.5	33.34 ± 1.46	.0709
MO%	7.10 ± 0.66	8.49 ± 0.41	.0599
EO%	2.88 ± 0.36	3.13 ± 0.35	.0987
BA%	1.15 ± 0.38	0.50 ± 0.03	.3510

[a]represents a statistically significant value ($P < .05$).

Note: A statistically significant reduction in MPV ($P = .01$), MCH ($P = .02$) and MCHC ($P = .03$) values was observed pre- and post-AC supplementation. Values are represented as mean ± SEM.

Abbreviations: AC, anthocyanin; BA, basophil; EO, eosinophil; HCT, hematocrit; HGB, hemoglobin; LY, lymphocyte; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean cell volume; MO, monocyte; MPV, mean platelet volume; NE, neutrophil; PLT, platelet; RBC, red blood cell; RDW, red cell distribution width; WBC, white blood cell.

AC intervention also significantly reduced the expression of activation-dependent platelet surface maker P-selection (CD62p) on platelets that were stimulated with ADP (Figure 3; $P < .05$). No difference in the expressions of monocyte-platelet aggregates was observed post-AC supplementation.

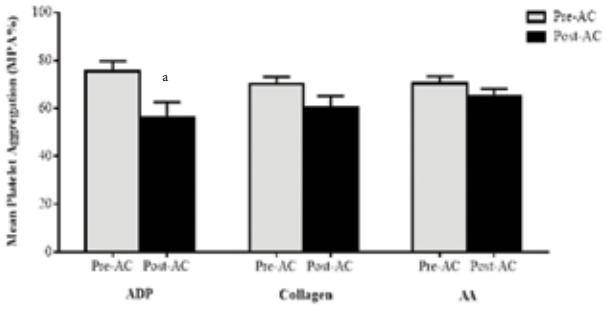
Coagulation Analysis

A statistically significant reduction in plasma fibrinogen concentration was observed post-AC supplementation (Figure 4; $P < .05$). However, AC supplementation did not influence clotting times for PT and aPTT coagulation assays.

Biochemical Analysis

No significant differences in the lipid profile, fasting blood glucose or uric acid concentrations were observed pre- or post AC-supplementation.

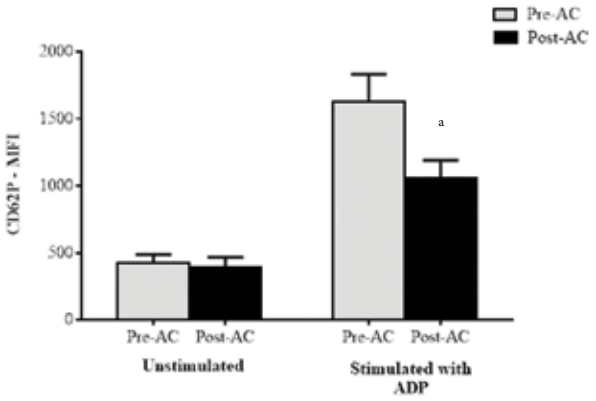
Figure 2. A statistically significant reduction in ADP-induced platelet aggregation was observed post-anthocyanin supplementation (P = .03). No significant reduction in collagen and AA-induced platelet aggregation was observed post-supplementation. Data represented as mean ± SEM.



[a]represents a statistically significant value (P < .05).

Abbreviations: AA, arachidonic acid; AC, anthocyanin; ADP, adenosine diphosphate acid.

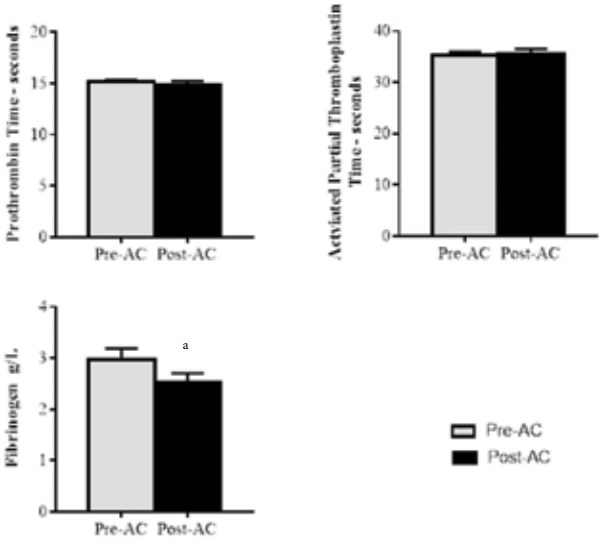
Figure 3. A significant reduction in P-selectin expression on ADP-stimulated platelets was observed post-anthocyanin supplementation (P = .03). Data represented as mean ± SEM



[a]represents a statistically significant value (P < .05).

Abbreviations: AC, anthocyanin; MFI, mean fluorescence intensity; SEM, standard error of the mean.

Figure 4. 4-week anthocyanin supplementation has been shown to reduce fibrinogen concentration in healthy adults (P = .03). No significant difference in the clotting times of PT and aPTT were observed post-AC supplementation. Data represented as mean ± SEM.



[a]represents a statistically significant value (P < .05).

Abbreviations: AC, anthocyanin; PT, prothrombin time; aPTT, activated partial thromboplastin time; SEM, standard error of the mean.

Table 2. General Biochemistry Profile Results Pre- and Post-AC Supplementation

Biochemical Assay	Pre-AC Mean ± SEM	Post-AC Mean ± SEM	*P* Value
TC	4.57 ± 0.42	3.57 ± 0.41	.0982
HDL	1.14 ± 0.13	1.03 ± 0.10	.8380
LDL	3.13 ± 0.35	2.45 ± 0.33	.0831
TG	1.53 ± 0.32	0.96 ± 0.13	.3649
FBG	4.59 ± 0.29	3.87 ± 0.27	.1866
UA	277 ± 20.8	252 ± 19.7	.3430
Hs-CRP	1.37 ± 0.26	1.53 ± 0.31	.6242

Note: Values are represented as mean ± SEM.

Abbreviations: AC, anthocyanin; FBG, fasting blood glucose; HDL, high-density lipoprotein; Hs-CRP, high sensitivity C-reactive protein; SEM, standard error of the mean; TC, total cholesterol; TG, triglycerides; UA, uric acid.

DISCUSSION

The aim of this study was to investigate the antithrombotic effects of AC supplementation in healthy adults. The data demonstrate a significant inhibitory effect of AC on ADP-induced platelet aggregation and the expression of P-selectin in ADP-stimulated platelets, which indicates reduced platelet activation and degranulation, suggesting that AC may reduce platelet activation, degranulation and aggregation.[12] Furthermore, AC supplementation has also been shown to reduce circulating fibrinogen concentration and mean platelet volume in healthy adults.

Different exogenous agonists (ADP, collagen and AA) were used to stimulate platelet aggregation *ex vivo.* These agonists induce platelet aggreagtion via different pathways. Our data demonstrates that AC supplementation for 28 days significanlty inhibited ADP-induced platelet aggregation, suggesting that AC extract from bilberries and blackcurrants may exert its antiplatelet effect by acting as an P_2Y_1 and P_2Y_{12} receptor antagonist. The observed inhibitory effect of AC supplementation is in aggreement with the findings of several other studies that showed that an AC-rich diet can inhibit ADP-induced platelet aggregation. In a recent study by Thomson, et al, 28-day AC supplementation reduced ADP-induced platelet aggregation by 29% in a sedentary population.[13] Although in this study AC supplementation did not show a statistically significant inhibition of collagen and AA-induced platelet aggregation, several other *in vivo* studies have demonstrated that AC from other sources such as strawberries and Queen Garnet plums do show the potentail to inhibit collagen and AA-induced platelet aggregation.[14,15]

The inhibition of P-selectin expression on the surface of ADP-stimulated platelets by AC suggests the potential of AC to inhibit platelet activation, degranulation and subsequent α granule release, thereby reducing the risk for thombosis. Andreas, et al reported an inhibitory effect of *in vitro* AC on the expression of CD62P in resting and activated platelets.[16] The data is consistent with the findings of others demonstrating the effect of AC on the expression of CD62P on platelet surfaces.[2,17] A few other studies have also investigated the effect of AC in reducing P-selectin expression on platelets; however, the source of AC and its concentration, the sample population, or the agonist used for platelet activation were different. Song, et al reported an inhibitory effect of AC on P-selection in patients with hypercholesteremia.[18] Yao, et al consistently demonstrated a significant inhibitory effect of cyanidin-3-glucoside (a member of the anthocyanin family) on the expression of CD62P.[8] Yang, et al showed a sigificant reduction of P-selection in dyslipidemic rats that were supplemented with AC extract from black rice.[2,17,19,20] It is believed that the desensitization of platelet activation-dependent superficial receptors by AC interferes with signal transduction, thus reducing P-selectin release of α-granule contents following platelet activation.[21] Flavonoids, including AC, may reduce platelet production of superoxide anion and increase platelet nitric oxide production,[22] which in turn inhibit platelet adhesion and activation. The inhibitory effect of AC on the expression of P-selectin in activated platelets can reduce platelet hyperactivity in response to various stressors such as oxidative stress and shear stress that lead to thrombotic events and CVD.[17,23]

A reduction in fibrinogen concentration was observed post-AC supplementation. Fibrinogen is a plasma protein that is highly susceptible to oxidative modification and previous studies have shown that antioxidants can affect circulating fibrinogen concentration and function. It is believed that antioxidants such as AC may inhibit fibrin synthesis by blunting the enzymatic activity of thrombin and protecting fibrin from oxidative modification in the presence of free radicals. Several studies have also reported a reduction in circulating fibrinogen concentration following AC supplementation from various sources such as Queen Garnet plum juice.[2,17] However, the data from the current study for post-intervention fibrinogen levels lie within the normal reference range, indicating no clinical risk of bleeding due to AC. In this study, AC extract from bilberries and blackcurrants did not influence the clotting time of PT and aPTT in healthy individuals. However, Santhakumar, et al have demonstrated that supplementing healthy individuals with AC-rich plum juice prolongs aPTT clotting time, suggesting that AC may have inhibitory effects on coagulation factors associated with the intrinsic pathway of the coagulation cascade.[2,17]

There was a significant suppressive effect of AC on MPV. MPV is a commonly used measure of platelet size and it has been suggested as a potential marker of platelet reactivity.[24] Larger platelets are metabolically and enzymatically more active[25] and have greater prothrombotic potential.[26] Elevated MPV is associated with other mediators of platelet activity, including increased platelet aggregation, increased thromboxane synthesis and β-thromboglobulin release and increased expression of adhesion molecules.[27] Furthermore, higher MPV is observed in patients with diabetes mellitus,[28] hypertension,[29] hypercholesterolemia,[30] smoking[31] and obesity, suggesting a common mechanism by which these factors may increase the risk for CVD.[24]

AC supplementation did not influence the lipid profile, fasting blood glucose or inflammation in the participants. The inhibitory effect of antioxidants on these biochemical parameters was shown in other studies.[32,33] It has been hypothesised that AC may improve the lipid profile by lowering β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase gene activation, thus reducing the synthesis of cholesterol; by inhibiting cholesteryl ester transfer protein (CEPT), which reduces circulating concentrations of LDL,[34] and by lowering apolipoprotein B and apolipoprotein C-III-lipoprotein levels in blood.[35] In addition, AC facilitates excretion of cholesterol through feces.[36] The link between dyslipidemia and inflammation may be attributed to the fact that elevated serum cholesterol is associated with a higher level of proinflammatory cytokines and hence the protective effect of AC could also be dual.[37]

CONCLUSION

This study demonstrates a significant inhibitory effect of AC on ADP-induced platelet aggregation and alpha granule exocytosis as measured by P-selectin expression. Furthermore, AC supplementation significantly reduced plasma fibrinogen levels and MPV. In addition, reduced blood levels of cholesterol, glucose, triglycerides and uric acid were reported. Together, the data suggest that long-term AC supplementation may potentially exert cardioprotective effects. However, future studies are warranted to fully elucidate the effect of chronic AC supplementation in patient populations such as patients with existing CVD and diabetes, in which the risk for thrombosis is increased.

CONFLICT OF INTEREST

There is no conflict of interest declared by any investigator, and they are all responsible for all contents and writing of this article.

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