Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Antiplatelet and Anticoagulant Activities of *Astragalus sarcocolla* Dymock

Muhammad U. Mazhar[1], Fareeha Anwar[1], Uzma Saleem[1,2], Bashir Ahmad[1], Muhammad U. Mirza[3], Sarfraz Ahmad[4]

[1]Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore, Pakistan, [2]Government College University, Faculty of Pharmacy, Faisalabad, Pakistan, [3]Department of Pharmaceutical and Pharmacological Sciences, Rega Institute for Medical Research, Medicinal Chemistry, University of Leuven, Leuven, Belgium, [4]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

Submitted: 21-Feb-2022 Revised: 27-Mar-2022 Accepted: 27-Sep-2022 Published: 23-Nov-2022

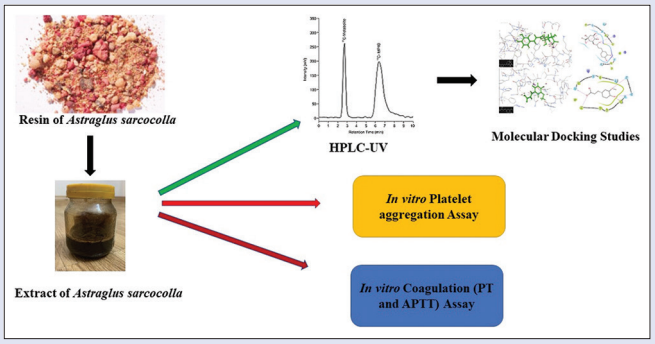
ABSTRACT

Background and Aim of the Study: Antiplatelet drugs available on the market have multiple side effects and pose drug interactions, which actuated the researchers to consider better alternatives by using plant extracts. It is well known that medicinal plants possessing anti-inflammatory action usually have antiplatelet aggregation and anticoagulation potential. Therefore, it was hypothesized that resins of *Astragalus sarcocolla* Dymock possessing anti-inflammatory properties would also manifest antiplatelet aggregation and anticoagulant properties. The objective of the present study was to assess the antiplatelet and anticoagulant effects of four different concentrations of ethanolic extracts (100, 80, 60, and 40%) of *A. sarcocolla* Dymock. **Materials and Methods:** Four different doses of *A. sarcocolla* Dymock extract (2.5, 5, 7.5, and 10 µg) were prepared for assays. *In vitro* antiplatelet and anticoagulant effects using human blood were studied in 42 healthy males aged between 25 and 35 years. Aspirin (0.5 µg) was used as the positive control for the antiplatelet aggregation assay. Furthermore, molecular docking was done to check the interaction between plant constituents and $P2Y_1$, $P2Y_{12}$, and phosphoinositide 3-kinase (PI3K). Chlorogenic acid, ferulic acid, and cinnamic acid were quantified using high-performance liquid chromatography (HPLC)-UV in ethanolic extract. **Results:** An HPLC analysis of the ethanolic extract of *A. sarcocolla* Dymock revealed the presence of chlorogenic acid, ferulic acid, and cinnamic acid. All the extracts of *A. sarcocolla* Dymock significantly inhibited aggregation of platelets against all three agonists' epinephrine (EPI), adenosine 5′ diphosphate (ADP), and collagen (COL) in a concentration-dependent manner. All the extracts significantly affected prothrombin time and activated partial thromboplastin time in a concentration-dependent manner. Molecular docking showed strong interactions of chlorogenic acid and ferulic acid with $P2Y_1$, $P2Y_{12}$, and PI3K. **Conclusion:** The data of the present study revealed the inhibition of platelet aggregation and anticoagulation potential of active compounds of *Astragalus sarcocolla* Dymock, together with their significant interaction profile with the selected therapeutic targets.

Key words: Anticoagulant, antiplatelet, *Astragalus sarcocolla* Dymock, $P2Y_1$, $P2Y_{12}$, PI3K

SUMMARY

- *Astragalus sarcocolla* Dymock extract revealed the presence of chlorogenic acid, ferulic acid, and cinnamic acid upon the HPLC-UV assay. The extract exhibited anticoagulant activity by specifically interfering both intrinsic and extrinsic pathways of blood coagulation. *Astragalus sarcocolla* Dymock inhibited the agonists; COL, ADP, and EPI-induced platelet aggregation, in a dose-dependent manner. Furthermore, it was found that these actions could be due to the strong binding of chlorogenic acid and ferulic acid with PI3K as well as ADP receptors ($P2Y_1$ and $P2Y_{12}$).



Abbreviations used: VWF: Von Willebrand factor; COL: Collagen; GPVI: Glycoprotein VI; ITAM: Immunoreceptor tyrosine-based activation motif; ADP: Adenosine 5′ diphosphate; PI3K: Phosphoinositide 3-kinase; HPLC: High-performance liquid chromatography; PRP: Platelet-rich plasma; PPP: Platelet-poor plasma; PT: Prothrombin time; APTT: Activated partial thromboplastin time; CVD: Cardiovascular disease; CHD: Coronary heart disease; PIP2: Phosphatydilinositol 4,5-bisphosphate; PIP3: Phosphatydilinositol 3,4,5-trisphosphate; ASE100: (*A. sarcocolla* Dymock 100% ethanolic extract); ASE80: *A. sarcocolla* Dymock 80% ethanolic extract; ASE60: *A. sarcocolla* Dymock 60% ethanolic extract; ASE40: *A. sarcocolla* Dymock 40% ethanolic extract.

Correspondence:

Dr. Fareeha Anwar,
Riphah Institute of Pharmaceutical Sciences,
Riphah International University, Lahore, Pakistan.
E-mail: Fareeha.anwar@riphah.edu.pk

DOI: 10.4103/pm.pm_91_22

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Hemostasis is a composite physiological response that results in sealing a break in the vessel and is essential to protect the integrity of the vasculature.[1] Hemostasis comprises two-stage reciprocal processes; the formation of a platelet plug (primary hemostasis) followed by the generation of a fibrin clot (secondary hemostasis).[2] Upon injury to the blood vessel, a subendothelial matrix rich in Von Willebrand factor (VWF), collagen, laminin, fibronectin, fibrinogen, and thrombospondin-1 is exposed, which acts as ligands for platelet surface receptors.[3] Initial platelet adhesion is strongly regulated by the local blood flow conditions. At low shear rates (<1000 s^{-1}) particularly in veins and large arteries,

Cite this article as: Mazhar MU, Anwar F, Saleem U, Ahmad B, Mirza MU, Ahmad S. Antiplatelet and anticoagulant activities of *Astragalus sarcocolla Dymock*. Phcog Mag 2022;18:1066-74.

adhesion is dependent on collagen, fibronectin, and laminin. While at higher shear rates (>1000 s^{-1}), VWF plays a vital role in adhesion, which slows down fast-moving platelets so that a stable adhesion can be formed with the help of additional bonds.[4] Initial platelet binding is interceded by the interaction between the platelet receptor GPIb-IX-V complex and VWF deposited in the subendothelial matrix of the injured vessel wall.[3] Circulating VWF binds with exposed collagen via the collagen-binding domain, particularly A3 resulting in the immobilization of VWF. This immobilization, along with the high shear force of blood, will expose the A1 domain of VWF so that it can interact with the GPIbα subunit of GPIb expressed on the surface of platelets[4] and help in the translocation of platelets along the subendothelial lining.[3] This initial binding of platelets with VWF acts as an anchor to facilitate binding with collagen.[5]

Collagen is the most potent agonist for adhesion and aggregation.[4] Glycoprotein VI (GPVI) is the major signaling receptor for collagen. Within its cytoplasmic domain, FcRγ has an immunoreceptor tyrosine-based activation motif (ITAM). Once collagen binds, a complex is formed as a result of cross-linkage between GPVI/FcRγ that results in the activation of ITAM-dependent triggering of Syk kinase pathways which in turn increases cytoplasmic Ca^{++}.[3]

Furthermore, various phases are involved in the coagulation cascade.[6] (1) Initiation phase is an expression of TF and generation of minor amounts of activated factors VII, FIX, and FX; (2) the amplification phase involves VWF (bearing FVIII) and platelets to facilitate the procoagulant activity, leading to the activation of FX (through activated FVIII and FIX) and prothrombin (via activated FV and FX); (3) thrombin activates FXI via a feedback loop; (4) the propagation phase results in the generation of large quantities of thrombin and fibrin; and (5) extension phase comprises sustained thrombin activity, which guarantees platelet activation, clot remodeling, fibrinolysis, and wound healing. Platelet activation by adenosine 5' diphosphate (ADP) is autocatalytic because once ADP binds with its receptors it leads to the release of more ADP molecules, which will act on the platelets to result in amplification of the reaction.[7] ADP works via two purinergic receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to Gq, while $P2Y_{12}$ is linked with Gi. The two receptors work closely together to ensure the complete activation and aggregation of platelets. The platelet activation and aggregation initiated by $P2Y_1$ are amplified and sustained by coactivation through $P2Y_{12}$ which is vital for full aggregation.[8]

Astragalus sarcocolla Dymock Dymock (Fabaceae) (*A. sarcocolla* Dymock) is an unbranched 6-foot long perennial herb.[9] Saponin is the major chemical constituent in the *Astragalus* genus. The plants of the *Astragalus* genus are also a rich source of flavonoids.[9] Astragalus species have been used in folk medicine as anti-thrombotic,[10-12] anti-inflammatory, immunostimulant, antioxidative, antineoplastic, antidiabetic, cardioprotective, hepatoprotective, and antiviral agents.[9] *Astragalus sarcocolla* Dymock has not so far been studied for its antiplatelet and anticoagulant potential. This present study is going to be a pioneer work on *A. sarcocolla* to identify its antithrombotic effects.

This study was planned to access the *in vitro* antiplatelet and anticoagulation potential of *A. sarcocolla* Dymock. Active compounds of the ethanolic extract were analyzed by HPLC-UV and for further exploration of binary interactions of active compounds with potential therapeutic targets, molecular docking was performed by considering human purinergic P2Y receptor ($P2Y_{12}$), G-protein coupled receptor ($P2Y_1$), and PI3K.

MATERIALS AND METHODS

Reagents

The chemicals and reagents used in this study includes: Absolute ethanol was purchased from Merck (Germany). Acetyl salicylic acid was obtained from Wako Pure Chemical Industries, Ltd. Osaka, Japan. Tri-sodium citrate was purchased from Sigma Aldrich Co, UK. Normal saline (Sterifluid NS) was obtained from Frontier Dextrose Ltd, Pakistan. P/N 384 ADP, P/N 385 Collagen, and P/N 393 Epinephrine were purchased from Chrono-PAR® reagent, Chrono-Log Corp. Havertown, PA, USA. Thromborel® S and Dade Actin® FSL were obtained from Siemen Healthcare Diagnostics Products GmbH Siemens, Germany. All chemicals used were of the highest purity and research grade available on the market.

Collection and identification of plant samples

The *A. sarcocolla* Dymock plant was obtained from the local herbarium market in October 2016, Lahore, Pakistan. The plant sample was identified by Dr Hassan Mushtaq, a taxonomist from Punjab University, with voucher specimen no. 421, for further reference. It was ground to powder in an electric mill to facilitate extraction steps. The powdered sample was stored in dry and clean conditions until use.

Preparation of plant extracts

The powdered resin of the plant was sequentially extracted with four different concentrations of ethanol, that is, 100, 80, 60, and 40%. Pulverized plant material (1 kg) was immersed in the selected solvent (5 L) for 72 h at room temperature with occasional manual stirring. After 72 h, the macerated plant material was filtered through a muslin cloth to separate vegetative debris. For fine filtration, the filtrate was filtered through Whatman filter paper no. 1 by using vacuum filtration. The filtrate was evaporated to dryness under reduced pressure using a rotary evaporator at 40°C and freeze-dried for complete dryness. The dried extract was collected and stored in an airtight container in a refrigerator for further use.[13]

HPLC-UV analysis

The identification of metabolites was carried out by the means of high-performance liquid chromatography (HPLC) system LC-10A (Shimadzu, Nagoya, Japan) using Shim-Pack CLC-ODS C—18 column (25 cm × 4.6 mm, 5 μm).[14] The mobile phase was freshly prepared by filtering through a membrane disc filter (0.45 m) and sonicated before use. Mobile phase contained solvent A (H_2O: Acetic acid—94:6, pH = 2.27) and B (100% acetonitrile). The isocratic elution of fractions was carried out at a flow rate of 0.1 mL/min at 30°C and detected by a ultra-violet (UV)-visible detector at a 280 nm wavelength. The peaks of the samples were characterized by comparing the retention time (t_R) with some standards.

Sample collection

Venous blood (9 mL) was collected from the antecubital vein of healthy subjects who had not taken any medication for the past 14 days by using a 10-mL syringe with a 22-gauge needle. Blood was transferred into the 15 mL conical tube containing anticoagulant (1 mL, 3.8% w/v) trisodium citrate solution at a ratio of 9:1. All these procedures were governed by the guidelines of the British Committee for Standards in Hematology on the laboratory aspects of assays used in hemostasis and thrombosis and the laboratory investigations of blood platelet function.[15]

Sample processing

Platelet count

Platelet count was performed after initial sampling using Sysmex KX-21N™ automated hematology analyzer (Sysmex Corporation Kobe, Japan). A report of the complete blood count was generated, and print was taken. The platelet count was found within the range of 150×10^9 to 490×10^9 cells/L.

Preparation of platelet-rich plasma (PRP)

Citrated blood samples were centrifuged at 1000 rpm for 15 min at 20°C using a Kubota refrigerated centrifuge 8700 (KUBOTA Corporation, Japan) to obtain platelet-rich plasma (PRP). Aggregation studies were carried out at 37°C with PRP having platelet counts of between 2.5×10^9 to 3.0×10^9/L of plasma. All experiments were performed within 3 h of PRP preparation.[16]

Preparation of platelet-poor plasma (PPP)

After carefully removing PRP, blood samples were again centrifuged at 4000 rpm for 10 min using compact centrifuge Z 206 A (HERMLE Labortechnik GmbH Siemens, Germany) to obtain platelet-poor plasma (PPP).[16] PPP was used to dilute PRP if required to adjust the platelet count between the range of 2.5×10^9 and 3.0×10^9/L of plasma.

Sample analysis

Platelets aggregation inhibition assay

The antiplatelet aggregation activity of crude plant extract material on human platelets was assessed by the procedure described by Shah *et al.*[16] Platelet aggregation was measured by using the dual-channel Lumi-aggregometer (Model 700 Chronolog Corporation, Chicago, USA) based on the turbidimetric principle.[17] Citrated PRP was transferred to a cuvette which was placed in a sample receptacle present in a Chronolog aggregometer heated at 37°C. The magnetic bar was added to the cuvette containing PRP, which was stirred at the rate of 1000 times per minute to ensure that platelets encountered one another. The light transmission was adjusted to 0 and 100% with PRP and PPP, respectively. PRP (450 µL) was incubated with 2.5, 5, 7.5, and 10 µg per 50 µL doses of each extract of *A. sarcocolla Dymock* for 1 min at 37°C. Aggregation was induced using three different agonists: ADP, epinephrine (EPI), and COL. ADP and EPI were used in 5 µL (5 µM) concentrations, while COL was used in 2 µg/mL (1 µM) concentration. The inhibition of platelet aggregation was recorded for 5 min after the immediate challenge of the aggregating agent with pre-incubated PRP on the platelet aggregometer. Normal saline was used as a negative control. Aspirin (0.5 µg/50µL) was used as a positive control.[16,18]

Percent inhibition of platelet aggregation was calculated using the formula.

$$\% \text{ Inhibition} = \frac{C - T}{C} \times 100$$

Where, C is percent aggregation of the control and T is percent aggregation of the test solution.

Plasma coagulation assay

Plasma coagulation assays were performed using the Sysmex coagulation analyzer CA-1500 (Sysmex Corporation, Kobe, Japan). The determination of PT and APTT was carried out by the manufacturer's recommended protocols (Siemens).

Preparation of plasma sample

The PPP was freshly prepared as mentioned in the preceding paragraph. Plasma mixtures were prepared by mixing 100 µL of plasma with 50 µL of test sample before performing the PT and APTT assays.[19]

PT

After incubation at 37°C for 180 s, 100 µL of the plasma mixture was blended with 200 µL of Thromborel® S. The reaction was monitored for 120 s.

APTT

After incubation at 37°C for 60 s, 100 µL of the plasma mixture was mixed with 100 µL Dade Actin® FSL and incubated for another 180 s. Then, 100 µL of calcium chloride was added to the reaction mixture and monitored for 190 s. Negative control consisted of normal saline instead of extract.

Molecular docking studies

Molecular docking is an *in-silico* approach used to analyze the binary interactions between small molecules and therapeutic targets.[20] To check the molecular interaction of active compounds from ethanolic and hydroethanolic extracts of *A. sarcocolla* Dymock with respective targets, molecular docking was performed by using the GOLD software of the Cambridge Crystallographic Data Center using default settings and parameters.[21] The X-ray defined structures were downloaded from the Protein Data Bank (PDB) of human purinergic P2Y receptor (P2Y12) (PDB ID: 4NTJ), human G-protein-coupled receptor (P2Y1) (PDB ID: 4XNW), and phosphoinositide 3-kinase (P13K) (PDB ID: 4XEO).[22,23] Before docking, hydrogen atoms and charges were added, all heteroatoms, including water molecules and metal atoms, were removed, and proteins were prepared. Small molecules of benzoic acid, chlorogenic acid, cinnamic acid, and ferulic acid were docked with all these target proteins.[24]

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The study variables in six groups were compared using two-way Analysis of Variance (ANOVA) and differences among groups were determined by Bonferroni's *post hoc* test. All experimental values reflect an average of a minimum of three experiments (mean ± SEM). The *P* values <0.05, <0.01, and <0.001 were considered statistically significant, highly significant, and very highly significant, respectively.

RESULTS

HPLC-UV analysis

The HPLC chromatogram gives 10 compounds peak that are chlorogenic acid (1), catechin (2), taxifolin (3), quercetin-3-O-β-D-glucoside-(1 → 6)-gallic acid (4), Ferulic acid (5), rutin (6), kaempferol-3-O-rutinoside (7), kaempferol-3-O-β-D-glucoside (8), quercetin (9), and Cinnamic acid (10) [Figure 1].

Antiplatelet potential

The platelet aggregation inhibitory effect of various extracts of *A. sarcocolla* Dymock was studied on human platelets using EPI (5 µM), ADP (5 µM), and COL (1 µM) as aggregating agents. Control aggregation induced by these agents was assessed at the beginning of each experiment. Aspirin was used as a positive control.

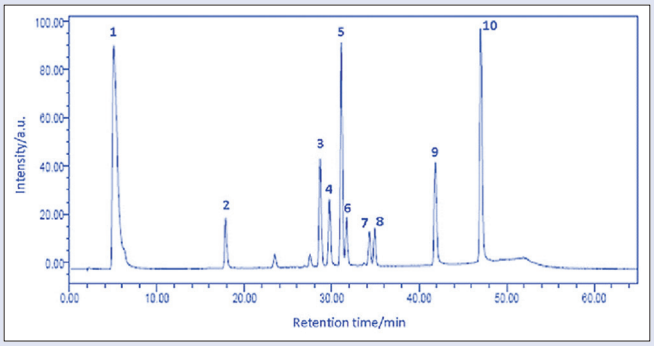


Figure 1: HPLC-UV chromatogram of ethanolic extract of *A. sarcocolla* Dymock

In vitro antiplatelet aggregation activity of *A. sarcocolla* Dymock 40% ethanolic extract (ASE40) against COL, ADP, and EPI induced platelet aggregation

Different doses of ASE100 (2.5–10 µg) were selected. A dose of 2.5 µg reduced the platelet aggregation by 55.35% against COL, 59.98% against ADP, and 64.62% against EPI; the inhibition of platelet aggregation against EPI was significantly greater than that of collagen. The extract at 5 and 7.5 µg caused 100% inhibition of platelet aggregation against EPI and COL. Whereas dose-dependent increase in the antiplatelet aggregation against ADP was observed at these two doses. Against ADP-induced aggregation, the complete inhibition was obtained at 10 µg [Figure 2].

In vitro antiplatelet aggregation activity of *A. sarcocolla* Dymock 80% ethanolic extract (ASE80) against COL, ADP, and EPI-induced platelet aggregation

Different doses of ASE80 (2.5 to 10 µg) were selected. At the lowest dose of 2.5 µg, ASE80 reduced the platelet aggregation by 60.97% against COL, 18.85% against ADP and 70.52% against EPI. The inhibition of platelet aggregation against EPI was significantly greater than that of COL as well as ADP. At the dose of 5 µg, the extract displayed 87.56, 53.02, and 94.86% inhibition of platelet aggregation against COL, ADP, and EPI-induced platelet aggregation, respectively. The percent inhibition of platelet aggregation at 5 µg of ASE80 induced by EPI was more significantly inhibited than that of COL and ADP-induced aggregation. At 7.5 µg, the extract caused 100% inhibition of platelet aggregation against COL while the dose-dependent increase in the antiplatelet aggregation against ADP and EPI was observed. Complete inhibition of platelet aggregation against ADP and EPI-induced aggregation was obtained at 10 µg [Figure 3].

In vitro antiplatelet aggregation activity of *A. sarcocolla* Dymock 60% ethanolic extract (ASE60) against COL, ADP, and EPI-induced platelet aggregation

Different doses of ASE80 (2.5 to 10 µg) were selected. At a dose of 2.5 µg, ASE60 reduced the platelet aggregation by 80.88% against COL, 24.27% against ADP, and 52.31% against EPI. The inhibition of platelet aggregation against COL was significantly greater than that of ADP and EPI. At the dose of 5 µg, the ASE60 displayed 82.37, 62.38, and 73.80% inhibition of platelet aggregation against COL, ADP, and EPI-induced platelet aggregation, respectively. The percent inhibition of platelet aggregation at 5 µg of ASE80 induced by COL was more significantly inhibited than that of ADP and EPI-induced aggregation. At a higher dose of 7.5 µg, the extract showed 88.96% inhibition against COL, 72.72% against ADP and 83.28% against EPI-induced platelet aggregation. 100% inhibition of platelet aggregation was observed at 10 µg [Figure 4].

In vitro antiplatelet aggregation activity of *A. sarcocolla* Dymock 100% ethanolic extract (ASE40) against COL, ADP, and EPI-induced platelet aggregation

Different doses of ASE40 (2.5-10 µg) were selected. At 2.5 µg, ASE40 reduced the platelet aggregation by 75.72% against COL, 31.30% against ADP, and 41.44% against EPI. The inhibition of platelet aggregation against COL was significantly greater than that of ADP and EPI. The extract at 5 µg reduced platelet aggregation by 80.50% against COL, 40.64% against ADP, and 52.74% against EPI revealing that the inhibition was more significant against COL-induced aggregation. At a dose of 7.5 µg, the extract showed 87.27% inhibition against COL, 55.07% against ADP, and 79.27% against EPI-induced platelet aggregation. At the highest dose of 10 µg, 100% inhibition of platelet aggregation was observed only with EPI while the percent inhibition was within the range of 93–96% against ADP and COL [Figure 5].

Anticoagulant potential

The effect of various extracts of *A. sarcocolla* Dymock was studied for anticoagulant potential on human plasma using four doses: 2.5 µg, 5 µg, 7.5 µg, and 10 µg. PT and APTT were measured. Normal saline in PPP was taken as a negative control.

Effect of various extracts of *A. sarcocolla* Dymock on *in vitro* PT

The ethanolic extract of *A. sarcocolla* Dymock (100, 80, 60, and 40%) possessed a dose-dependent response. As the dose was increased, PT was

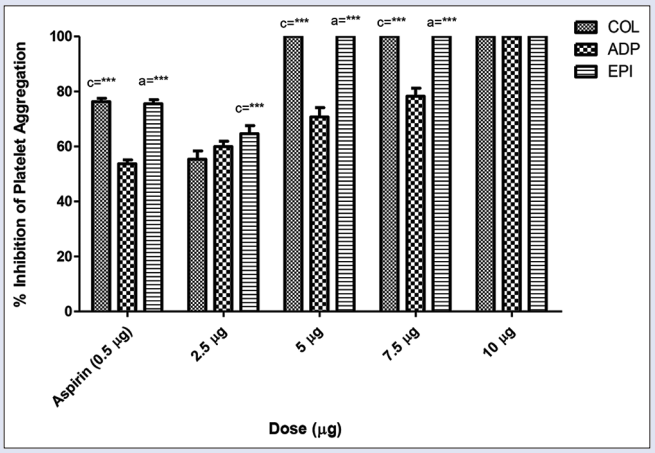


Figure 2: Percent inhibition of platelet aggregation of ASE100 and aspirin (positive control) against COL, ADP, and EPI-induced aggregation using *post hoc* Bonferroni test (a = comparison between ADP and EPI; b = comparison between COL and EPI; c = comparison between COL and ADP) *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM

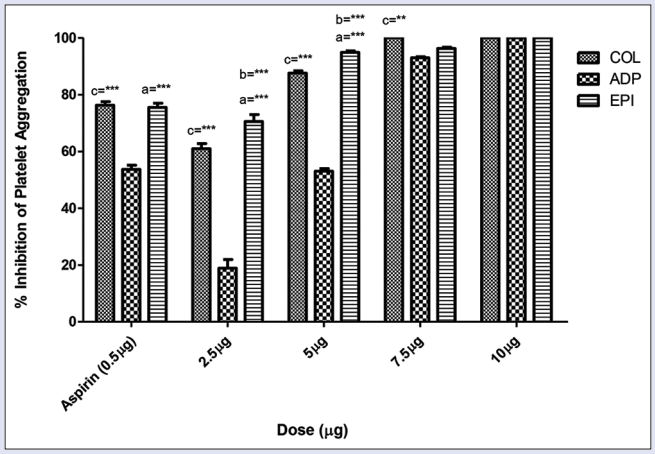


Figure 3: Percent inhibition of platelet aggregation of ASE80 and aspirin (positive control) against COL, ADP, and EPI-induced aggregation using *post hoc* Bonferroni test (a = comparison between ADP and EPI; b = comparison between COL and EPI; c = comparison between COL and ADP) *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM

also increased. All extracts significantly increased the PT in comparison to the control. ASE60 at 10 µg, displayed the most significant results among all (27.15 sec) [Figure 6].

Effect of various extracts of *A. sarcocolla* Dymock on *in vitro* APTT

At a dose of 2.5 µg, no significant increase in APTT was observed with all extracts. At 5 µg ASE100 and ASE80 displayed a significant increase in APTT when compared with control. No increase in APTT was observed with ASE60 and ASE40. At a dose of 7.5 µg, the most significant ($p < 0.001$) increase in APTT was shown by E100 followed by E80, E60, and E40 ($p < 0.01$). At the highest dose of 10 µg, all extracts demonstrated a highly significant ($p < 0.001$) increase in APTT [Figure 7].

Molecular docking analyses

The binding energies were ranked according to the energy function, which consisted of van der Waal's and electrostatic interactions of the protein/ligand complex.[25] All active compounds being docked against selected targets, chlorogenic acid and ferulic acid revealed promising binding affinities with P2Y12 (ΔG = 75.19 and 64.6 kcal/mol), P2Y1 (ΔG = 88.86 and 53.75 kcal/mol) and PI3K (ΔG = 69.4 and 61.15 kcal/mol), while other compounds showed weak binding affinities. Overall, the binding poses of chlorogenic acid and ferulic acid were found deep inside the binding pocket of all respective targets [Figures 8–10] and interacted through several H-bonds (<3 Å) and hydrophobic interactions.

DISCUSSION

Cardiovascular diseases (CVDs) are the leading cause of death in the world. According to a recent survey, it has been pointed out that the subcontinent region of the world is at high risk of morbidity and mortality due to CVD. In 2018, the World Health Organization published a report stating that deaths in Pakistan due to coronary heart diseases (CHDs) have reached 20.28% of the total number of deaths. Pakistan is currently ranked 18 in regards to the number of deaths due to

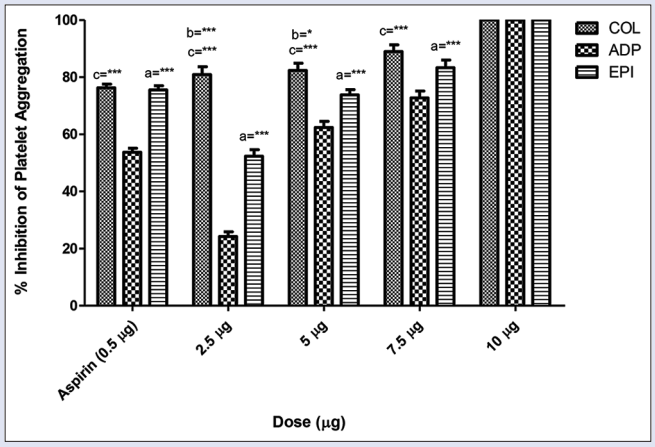


Figure 4: Percent inhibition of platelet aggregation of ASE60 and aspirin (positive control) against COL, ADP, and EPI-induced aggregation using *post hoc* Bonferroni test (a = comparison between ADP and EPI; b = comparison between COL and EPI; c = comparison between COL and ADP) *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM

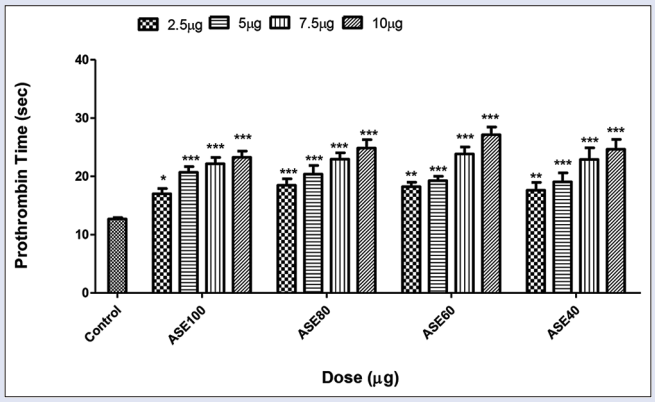


Figure 6: The effect of various extracts of *A. sarcocolla* Dymock in different concentrations on *in vitro* PT against the control using *post hoc* Bonferroni test (compared with control, *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM)

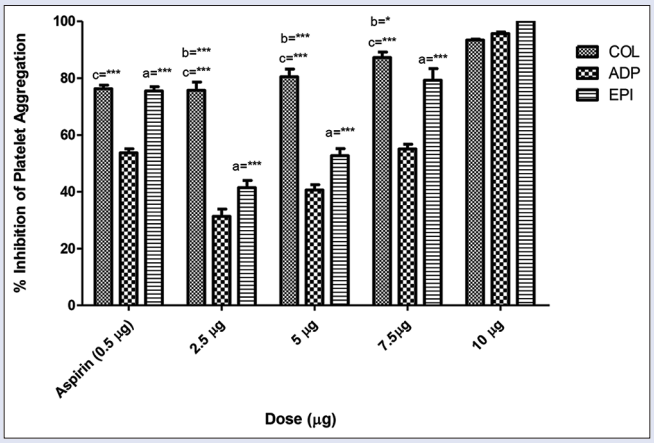


Figure 5: Percent inhibition of platelet aggregation of ASE40 and aspirin (positive control) against COL, ADP, and EPI-induced aggregation using *post hoc* Bonferroni test (a = comparison between ADP and EPI; b = comparison between COL and EPI; c = comparison between COL and ADP) *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM

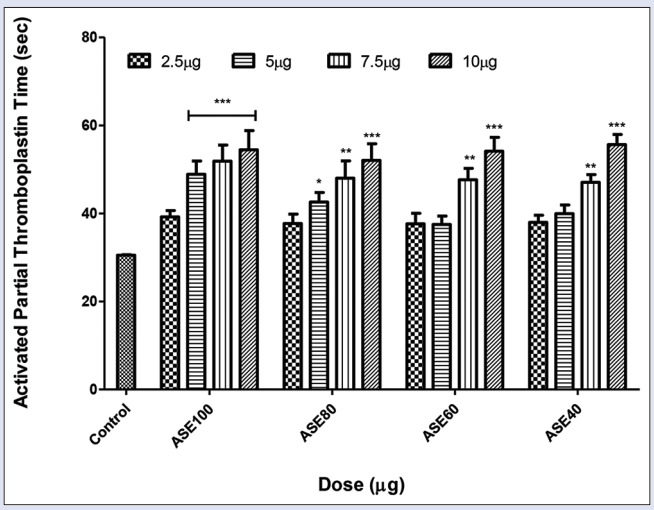


Figure 7: The effect of various extracts of *A. sarcocolla* Dymock in different concentrations on *in vitro* APTT against the control using *post hoc* Bonferroni test (Compared with control, *P < 0.05, **P < 0.01, ***P < 0.001, n = 6, mean ± SEM)

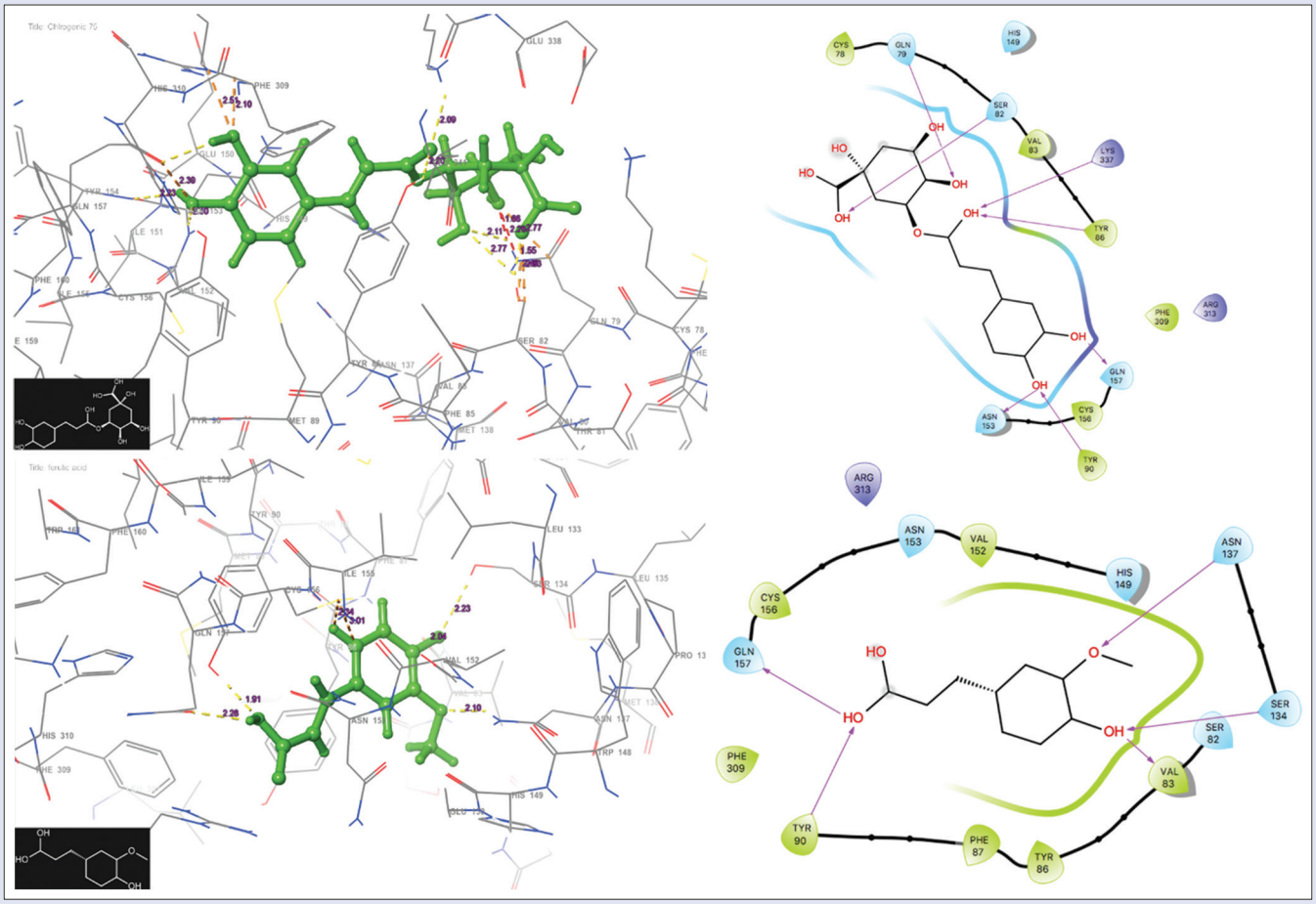


Figure 8: Molecular structure representations of docked poses of chlorogenic acid and ferulic acid in the respective binding pocket of P2Y12

the CHD.[26] Patients with CVDs are usually treated with antithrombotic agents. Antithrombotic agents can be divided into three groups: 1) Drugs which prevent platelet adhesion/aggregation (Antiplatelet drugs); 2) Drugs interfere with fibrin formation (anticoagulants and defibrinating agents); and 3) Drugs which degrade fibrin (thrombolytic drugs).[27] Drug interactions and fatal side effects are the reasons, which researchers throughout the world have been searching for natural compounds with the highest efficacy and fewer side effects.

More than 200 constituents have so far been obtained from the *Astragalus* genus. Though studies were conducted on different species, the chemical composition of the *Astragalus* genus appeared highly uniform, which comprised saponins, flavonoids, and polysaccharides.[9] Preliminary phytochemical screening of Sarcocolla gum resin showed the richness of this plant material with many secondary products like alkaloids, flavonoids, saponins, sterols, terpenoids, and tannins.[28] Flavonoids and polyphenolic compounds are considered to have high antiplatelet properties.[29] Astragalus species are a rich source of a variety of flavonoids, namely, rutin, quercetin, and luteolin.[9] Rutin has displayed concentration-dependent inhibition of human platelet aggregation.[30] Multiple biological activities like antioxidant, antiplatelet, anticoagulant, and antithrombotic have been attributed to polysaccharides in different studies.[9]

The natural anticoagulant/antiplatelet system is suppressed during inflammation, leading to the synthesis and expression of prothrombogenic molecules.[31] Several researchers have pointed out that compounds having anti-inflammatory properties can inhibit platelet aggregation.[32] Major constituents present in the *Astragalus* genus, include flavonoids, saponins, polysaccharides, terpenoids, tannins, alkaloids, and sterols, which have been documented to have anti-inflammatory properties.[9,33] It has been reported that these constituents act by inhibiting Cyclooxygenase (COX), inhibiting inflammatory mediators like cytokines, scavenging free radicals, or via nitric oxide synthase induction. Quercetin (flavonoid) inhibits both cyclooxygenase and lipoxygenase pathways.[33] It has been shown that cyclooxygenase inhibitors suppress platelet aggregation.[32] Quercetin is an important flavonoid that inhibits PI3 kinase.[34]

An HPLC-UV analysis of *A. sarcocolla* Dymock extract revealed the presence of three major components: chlorogenic acid, ferulic acid, and cinnamic acid [Figure 1]. Chlorogenic acid is a potent phenolic antioxidant which has been documented to have antiplatelet action.[35] Ferulic acid is believed to have antioxidant, anti-inflammatory, antiobesity actions as along with antithrombotic potentiality.[36] Cinnamic acid has been studied to produce anticoagulation effects by causing clotting factor deficiency of intrinsic pathway.[37] The results of present study has validated the *in vitro* antithrombotic potential of *A. sarcocolla* Dymock.

The reason for selecting three agonists (EPI, COL, and ADP) was to evaluate the possible mechanism of the plant extract which could inhibit platelet aggregation against the agonists and induced the platelet aggregation by the pathways and mechanisms which were already known.

At the lowest dose of the extracts (2.5 µg), the percent inhibition demonstrated by ASE80 (70.52%) was comparable to the percent

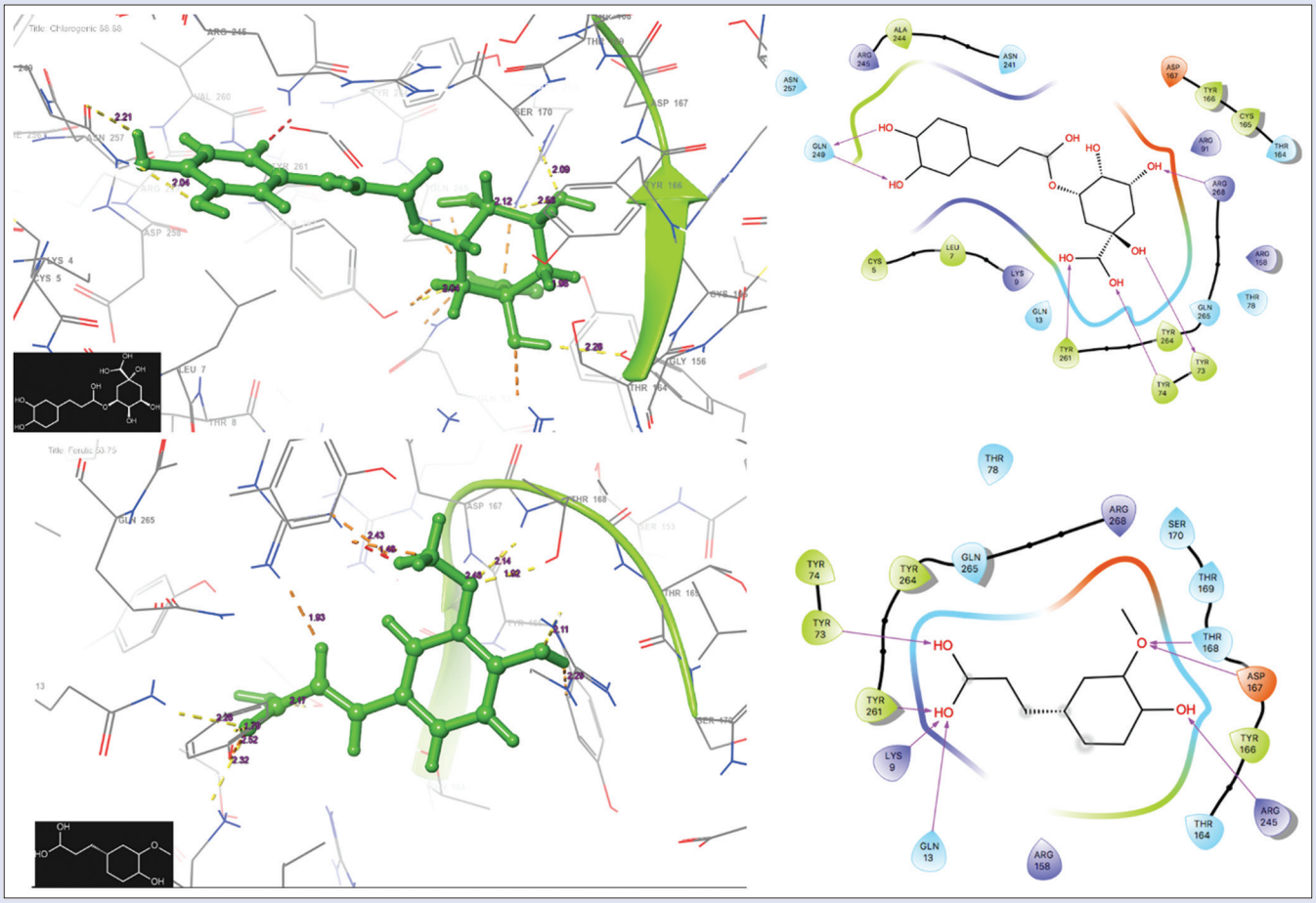


Figure 9: Molecular structure representation of docked poses of chlorogenic acid and ferulic acid in the respective binding pocket of P2Y1

inhibition shown by aspirin (75.51%). Strangely, ASE100, ASE60, and ASE40 showed less percent inhibition than the control. Strikingly, an increase in the dose of extracts from 2.5 to 5 µg drastically changed the degree of platelet inhibition with ASE100, ASE80, and ASE60. The maximum percent inhibition was 100% which was displayed by ASE100, followed by ASE80 (94.86%) [Figures 2–5]. This indicates that at a lower dose of the extract, the number of constituents responsible for antiplatelet activity was less.

Among hydroethanolic extracts, the most significant inhibition of platelet aggregability was shown by ASE80, which could be due to the synergistic effect of constituents extracted via ethanol and distilled water. It was also observed that by increasing the water content and decreasing the percentage of ethanol in a hydroethanolic extract, there was a significant decrease in antiplatelet aggregation activity. It suggests that water-soluble components are interfering with the activity of ethanolic components, but this interference was limited to the dose of 7.5 µg. Surprisingly, 100% inhibition was seen at the dose of 10 µg.

With all extracts, dose-dependent inhibition of platelet aggregation induced by ADP was seen [Figures 2-5]. The activity shown by ASE100 was different for ADP as compared to EPI. Except for the effects at the lowest dose (2.5 µg) at which ASE100 displayed a comparable effect to aspirin, as dose was increased the antiplatelet aggregation effect of ASE100 was more significant than aspirin. It appears that at a higher dose of 7.5 µg 100% inhibition of platelet aggregation was seen, unlike EPI in which 100% inhibition was achieved at 5 µg [Figure 2]. This may be due to the reason that ADP works via two receptors, unlike EPI which works via a single receptor. May be at low doses, ASE100 targets only one receptor of ADP but with the increase in the dose, both the receptors got completely blocked. Against COL-induced aggregation, ASE100 displayed similar results as seen with EPI [Figure 2]. Among hydroethanolic extracts, the activities of ASE60 and ASE40 were comparable [Figures 4 and 5]. At the dose of 2.5 µg, the percent inhibition of ASE60 and ASE40 was higher than ASE80 which indicated that at this dose, the components isolated through ethanol and water were working synergistically to induce inhibition against COL. The synergistic effect between constituents of ethanol and water is dependent on an optimal concentration, which in the case of COL-induced aggregation was 2.5 µg.

Findings from the *in vitro* anticoagulation assay suggest that *A. sarcocolla* Dymock has constituents that affect the blood coagulation pathway. To ensure which pathway of the coagulation cascade was targeted by the *A. sarcocolla* Dymock, PT and APTT were estimated. PT is used to evaluate the integrity of the extrinsic pathway of blood coagulation. All ethanolic and hydroethanolic extracts displayed a significant increase in PT than control, while they showed comparable activities among themselves at all doses [Figure 6]. APTT is used to assess the integrity of the intrinsic pathway of blood coagulation as it monitors factors XII, XI, IX, VIII, V, II, I, prekallikrein, and high molecular weight kininogen. A significant dose-dependent increase in APTT was observed with ASE100 at 5, 7.5, and 10 µg. Among hydroethanolic extracts, only ASE80 displayed a significant increase in APTT at 5 µg. The activities of all hydroethanolic extracts were comparable at 7.5 and 10 µg [Figure 7].

Several studies have demonstrated that PI3K inhibition attenuates

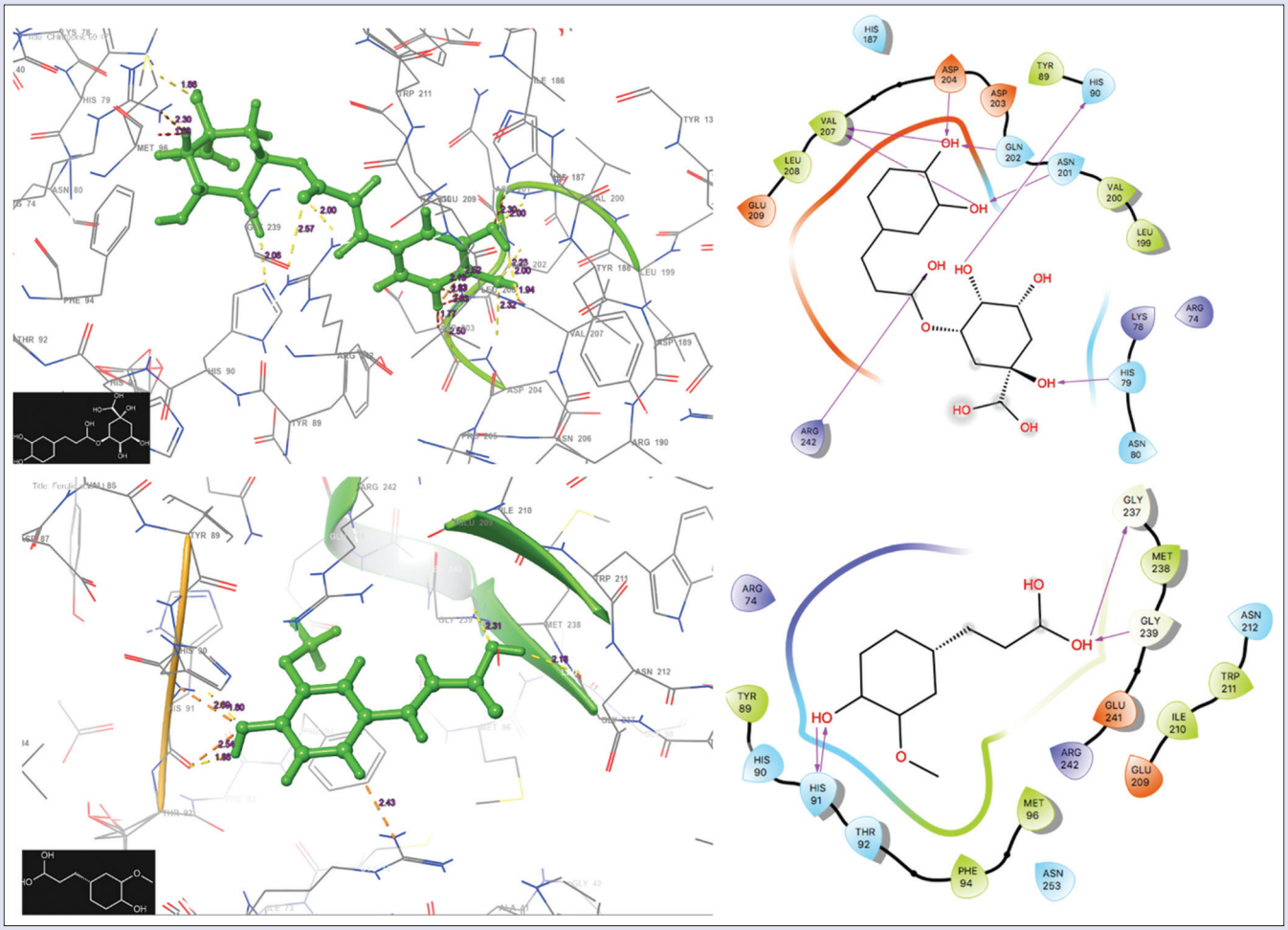


Figure 10: Molecular structure representation of docked poses of chlorogenic acid and ferulic acid in the respective binding pocket of PI3K

platelet activation and thrombus formation.[38] It has been documented that four major types of receptors support platelet activation during hemostasis and thrombosis: GPIb-IX-V (VWF receptor), ITAM-bearing receptors (GPVI), G-protein-coupled receptors (able to bind soluble agonists such as thrombin, EPI, and ADP), and integrins (mainly integrin a2b1 for collagen and αIIbβ3 for fibrinogen).[39] Important coagulation factors like tissue factor and fibrinogen have also been linked with the activation of the PI3K cascade. Studies have revealed that by inhibiting PI3K, the expression of TF and binding of fibrinogen binding with αIIbβ3 are reduced.[38] They promote activation of class I PI3Ks and conversion of phosphatydilinositol 4,5-bisphosphate (PIP2) to phosphatydilinositol 3,4,5-trisphosphate (PIP3). PIP3 triggers the recruitment, phosphorylation, and activation of the Ser/Thr kinase Akt (Protein kinase B), which ultimately results in integrin activation, platelet aggregation, and thrombus formation.[38] Because of the central role of the PI3K pathway in platelet activation and expression of coagulation factors it can be inferred that this pathway may be a therapeutic target to reduce coagulopathies in various CVDs. Since the last decade, $P2Y_{12}$ receptor presented on the platelet membrane has been a target for antithrombotic strategies. Inhibition of this receptor is a major part of the treatment strategy for the patients with various CVD. In order to understand the mechanism of strong inhibition of platelet aggregation and a significant increase in PT and APTT by various extracts of *A. sarcocolla Dymock* molecular docking was carried out by choosing three targets; PI3K and both ADP receptors ($P2Y_1$ and $P2Y_{12}$). The results revealed that only chlorogenic acid and ferulic acid showed promising binding affinities with all three targets [Figures 8–10]. By binding PI3K *A. sarcocolla* Dymock is likely to prevent the stimulation of PI3K-mediated platelet aggregation and coagulation. Similarly, both ADP receptors were also found to have strong binding affinities with chlorogenic acid and ferulic acid, thus ADP failed to stimulate these receptors, which leads to the inhibition of platelet aggregation.

CONCLUSION

The study validates the potential of *Astragalus sarcocolla Dymock* for its antiplatelet aggregation and anticoagulant properties in human blood. Four different extracts of *A. sarcocolla* Dymock remarkably inhibited the aggregation of platelets against three agonists: EPI, ADP, and COL in a concentration-dependent manner. These extracts significantly affected PT and APTT in a concentration-dependent manner. The correlation of these activities along with the bonafide anti-inflammatory potential of this plant can open a new window of opportunity toward its therapeutic realm.

Acknowledgements

This research was a collaborative study by the Riphah Institute of Pharmaceutical Sciences (RIPS), Lahore Campus, Armed Forces Institute

of Pathology (AFIP), Rawalpindi, Pakistan and Malaya University, Malaysia, for the resources (Grant no.: IIRG003A-2019).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Monroe DM. Basic principles underlying coagulation. In: Practical Hemostasis and Thrombosis. John Wiley and Sons, Ltd.; Hoboken, New Jersey. 2017. p. 1-11.
2. Heemskerk JW, Cosemans JM, van der Meijden PE. Platelets and coagulation. In: Platelets in Thrombotic and Non-Thrombotic disorders. Cambridge, UK: Springer; 2017. p. 447-62.
3. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, *et al.* Platelets are versatile cells: new discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci 2016;53:409-30.
4. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. Blood Rev 2011;25:155-67.
5. Fareed J, Iqbal O. Physiology of hemostasis. In: Anticoagulation and Hemostasis in Neurosurgery. Toronto: Springer; 2016. p. 3-16.
6. Mackman N. Triggers, targets and treatments for thrombosis. Nature 2008;451:914-8.
7. Imran I, Hussain L, Ahmed S, Rasool N, Rasool S, Abbas G, *et al.* Antiplatelet activity of methanolic extract of Acacia leucophloea bark. J Med Plants Res 2012;6:4185-8.
8. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A 1998;95:8070-4.
9. Li X, Qu L, Dong Y, Han L, Liu E, Fang S, Zhang Y, Wang T. A review of recent research progress on the astragalus genus. Molecules 2014;19:18850-80.
10. Dharmananda S. Astragalus Practical Aspects of Administering the Herb. 2006. Available from: http://www.itmonline.org/arts/astragalus.htm. [Last accessed on 2016 May 10].
11. Wang Y, Wang J, Guo L, Gao X. Antiplatelet effects of Qishen Yiqi dropping pill in platelets aggregation in hyperlipidemic rabbits. Evid Based Complement Alternat Med 2012;2012:205451. doi: 10.1155/2012/205451.
12. Rui-kai Z, Jian L. Effects of Xinfeng capsules on expression of platelet granule membrane protein 140 and platelet cluster of differentiation 40 ligand in peripheral blood of adjuvant arthritis rats. Int J Rheumatol 2012;2012:139696. doi: 10.1155/2012/139696.
13. Siddiqui WA, Mazhar MU, Malik JA, Talat A, Zaffar S, Rashid H, Chattha IR. The spasmolytic effect of astragalus sarcocolla on the intestinal smooth muscles of rabbit *in vitro*: Potassium channel opening. Cureus 2020;12:e9066. doi: 10.7759/cureus. 9066.
14. Abdel-Hameed E-SS, Bazaid SA, Shohayeb MM. RP-HPLC-UV-ESI-MS phytochemical analysis of fruits of *Conocarpus erectus* L. Chem Pap 2014;68:1358-67.
15. Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laffan M, British Committee for Standards in Haematology. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol 2013;35:1-13.
16. Shah BH, Nawaz Z, Pertani SA, Roomi A, Mahmood H, Saeed SA, Gilani AH. Inhibitory effect of curcumin, a food spice from turmeric, on platelet-activating factor- and arachidonic acid-mediated platelet aggregation through inhibition of thromboxane formation and Ca2+signaling. Biochem Pharmacol 1999;58:1167-72.
17. Born GV, Cross MJ. The aggregation of blood platelets. J Physiol 1963;168:178-95.
18. Hussain J, Jamila N, Gilani SA, Abbas G, Ahmed S. Platelet aggregation, antiglycation, cytotoxic, phytotoxic and antimicrobial activities of extracts of *Nepeta juncea*. Afr J Biotechnol 2009;8:935-40.
19. Gao B, Huang L, Liu H, Wu H, Zhang E, Yang L, *et al.* Platelet P2Y12 receptors are involved in the haemostatic effect of notoginsenoside Ft1, a saponin isolated from Panax notoginseng. Br J Pharmacol 2014;171:214-23.
20. Ikram N, Mirza MU, Vanmeert M, Froeyen M, Salo-Ahen OMH, Tahir M, *et al.* Inhibition of oncogenic kinases: An *in vitro* validated computational approach identified potential multi-target anticancer compounds. Biomolecules 2019;9:124. doi: 10.3390/biom9040124.
21. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. Improved protein–ligand docking using gold. Proteins 2003;52:609-23.
22. Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, *et al.* Structure of the human P2Y 12 receptor in complex with an antithrombotic drug. Nature 2014;509:115-8.
23. Zhang D, Gao ZG, Zhang K, Kiselev E, Crane S, Wang J, *et al.* Two disparate ligand-binding sites in the human P2Y 1 receptor. Nature 2015;520:317-21.
24. Kumar RB, Priya BS, Suresh MX. In silico analysis of potential inhibitors of Ca (2+) activated K(+) channel blocker, Charybdotoxin-C from Leiurus quinquestriatus hebraeus through molecular docking and dynamics studies. Indian J Pharmacol 2015;47:280-4.
25. Kumar RB, Suresh MX. A computational perspective of molecular interactions through virtual screening, pharmacokinetic and dynamic prediction on ribosome toxin A chain and inhibitors of Ricinus communis. Pharmacognosy Res 2012;4:2-10.
26. Coronary heart disease in Pakistan [Internet]. World Life Expectancy. Available from: https://www.worldlifeexpectancy.com/pakistan-coronary-heart-disease. [cited 2022 Nov 08].
27. Jain S, Dangi C, Kaur M, Singh H, Peter J, Kosta S. Plant as anticogulant/antithrombotic agent. World J Pharm Res 2014;3:4573-83.
28. Ababutain IM. Antimicrobial activity and phytochemical screening of sarcocolla gum resin. Pak J Biol Sci 2017;20:571-6.
29. Olas B, Wachowicz B. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. Platelets 2005;16:251-60.
30. Widowati W, Ratnawati H, Rusdi UD, Winarno W, Immanuel V. Phytochemical assay and antiplatelet activity of fractions of velvet bean seeds (*Mucuna pruriens* L.). Hayati 2010;17:85-90.
31. Mosa RA. *In vitro* Anti-Platelet Aggregation Activity of the Extracts of *Protorhus Longifolia*. University of Zululand; South Africa 2011.
32. Bukhari IA, Khan RA, Gilani AH, Ahmed S, Saeed SA. Analgesic, anti-inflammatory and anti-platelet activities of the methanolic extract of Acacia modesta leaves. Inflammopharmacology 2010;18:187-96.
33. Mohammed MS, Osman WJA, Garelnabi EAE, Osman Z, Osman B, Khalid HS, *et al.* Secondary metabolites as anti-inflammatory agents. J Phytopharmacol 2014;3:275-85.
34. Saeed SA, Connor JD, Imran, Quadri J, Tasneem S, Ahmed S, *et al.* Inhibitors of phosphatidylinositide 3-kinase: Effects on reactive oxygen species and platelet aggregation. Pharmacol Rep 2007;59:238-43.
35. Fuentes E, Caballero J, Alarcon M, Rojas A, Palomo I. Chlorogenic acid inhibits human platelet activation and thrombus formation. PLoS One 2014;9:e90699.
36. Choi JH, Park JK, Kim KM, Lee HJ, Kim S. *In vitro* and *in vivo* antithrombotic and cytotoxicity effects of ferulic acid. J Biochem Mol Toxicol 2018;32:e22004.
37. Khoo LT, Abdullah JO, Abas F, Tohit ERM, Hamid M. Bioassay-guided fractionation of melastoma malabathricum Linn. Leaf solid phase extraction fraction and its anticoagulant activity. Molecules 2015;20:3697-715
38. Guidetti GF, Canobbio I, Torti M. PI3K/Akt in platelet integrin signaling and implications in thrombosis. Adv Biol Regul 2015;59:36-52.