# Chapter 3

# Evaluation of the antiplatelet and antithrombotic activity of Diindolylmethane

## 3 Introduction

In previous findings (objective 2), we found that oral administration of I3C generates high amounts DIM and oral administration of I3C protected the brain from cerebral ischemia. These findings accordance with previous earlier findings, we conclude that DIM may be involved in mediating the pharmacological activities of I3C (Bradlow & Zeligs, 2010). However, the influence of DIM on I3C-induced antiplatelet and antithrombotic activity is not explicit. Therefore, the present study investigated the antiplatelet and antithrombotic activities of DIM.

Thrombotic diseases are gradually escalating with high mortality and morbidity (Del Brutto et al., 2019; Powers et al., 2018; Virani et al., 2020). Platelets contribute to thrombus formation in several coagulative disorders, including ischemic stroke, myocardial infarction, pulmonary thromboembolism, and atherosclerosis (Yeung, Li, & Holinstat, 2018). To lessen this burden, antiplatelet and antithrombotic therapy are widely used to manage clotting disorders (Hackam & Spence, 2019). Collagen in the subendothelial matrix interacted with glycoprotein receptor VI (GPVI) on platelets, leading to platelet adhesion (Hosseini, Hojjati, & Ghasemzadeh, 2020). At the same time, collagen forms a variety of chemical bonds with amino acid residues present in the GPVI receptors(Horii, Kahn, & Herr, 2006) and promotes platelet adhesion, and activation causes the release of platelet agonists, including ADP and thromboxane A2 (TXA2) (Shin, Kwon, Irfan, Rhee, & Lee, 2021). Further, ADP binds to amino acid residues in the $P_2Y_{12}$ receptors and activates these receptors (K. Zhang et al., 2014), leading to conformational changes or activation of GP IIa/IIIb receptors. The conformational changes lead the GP IIb/IIIa receptors to bind fibrinogen between adjacent platelets (Coller et al., 1991). Moreover, collagen, TXA2, and thrombin stimulate the dense granules to release ADP, thereby activating the $P_2Y_{12}$ receptors (Dorsam & Kunapuli, 2004).

Therefore, altering collagen binding to GPVI receptors and ADP binding to $P_2Y_{12}$ receptors could be a promising approach to mitigate platelet aggregation and thrombus generation.

The ferric chloride ($FeCl_3$) induced arterial thrombosis model is widely used to screen antithrombotic drugs (W. Li, Nieman, & Gupta, 2016; Tanaka, Sato, & Kurimoto, 2000). The application of FeCl3 to the artery produces a significant amount of reactive oxygen species (ROS), which thereby causes endothelial damage and generates the thrombus (W. Li, McIntyre, & Silverstein, 2013; Prakash et al., 2014). Moreover, $FeCl_3$ produces significant, reproducible, and low variation in platelet aggregation which is relevant to thrombosis pathology (Eckly et al., 2011; W. Li et al., 2013). Therefore, inhibition of platelet adhesion in early events provides a novel and safe therapeutic approach to treating coagulative diseases (Kleinschnitz et al., 2007). I3C inhibited platelet aggregation mediated by collagen and ADP (P. Paliwal, Chauhan, Gaurav, Gautam, Deepa, Dash, Debabrata, Patne, Shashikant CU, Krishnamurthy, Sairam, 2018; Park, 2008). Earlier reports suggested that half of the I3C is transformed into DIM, and most of the I3C pharmacological properties are driven by DIM (Bradlow & Zeligs, 2010). We hypothesized that DIM plays a vital role in the antiplatelet and antithrombotic activities of I3C. However, the antiplatelet and antithrombotic activities of DIM were not explored. Therefore, we have analysed the interaction of DIM with GPVI and $P_2Y_{12}$ receptors using docking studies. Further, to validate the *in silico* results, we proceeded with the DIM for *in vitro* antiplatelet aggregation and antithrombotic studies. The present study further ascertained DIM's antiplatelet and antithrombotic activities in $FeCl_3$ induced carotid artery thrombosis using *in vivo* studies. The proposed hypothesis is depicted in figure 3.1.

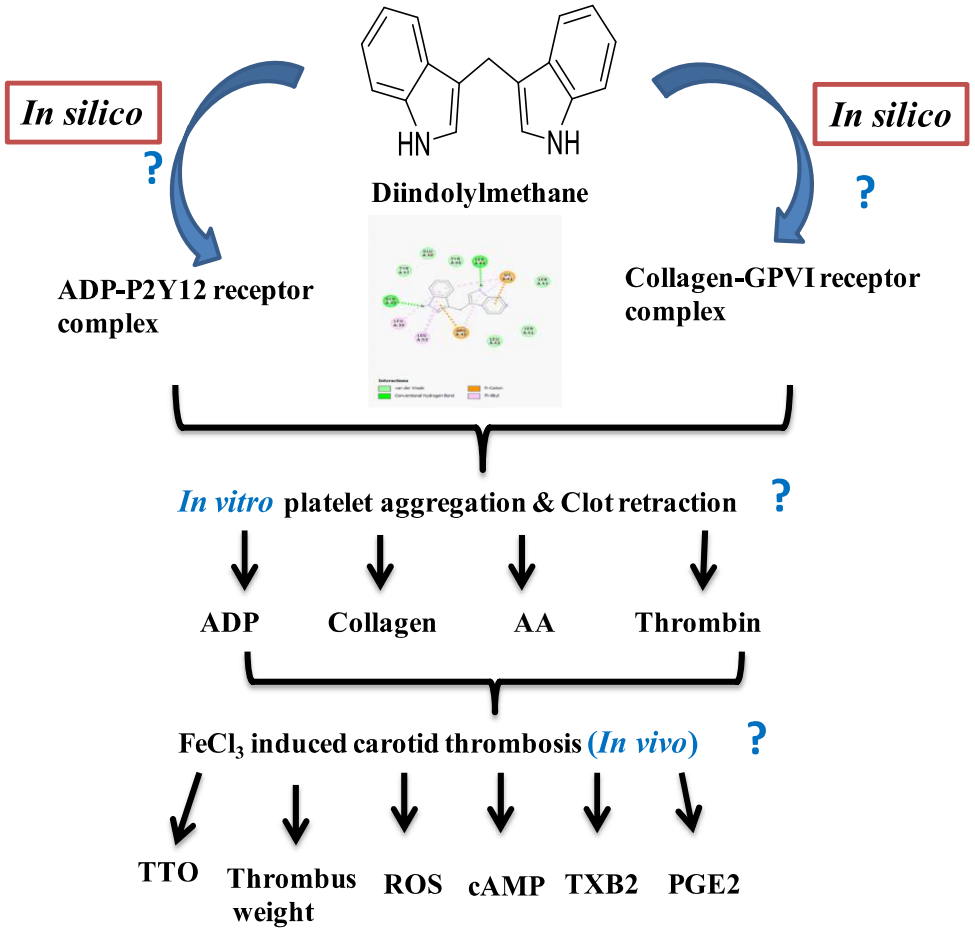


*Figure 3.1. The proposed hypothesis.*

## 3.1 Materials and Methods

### 3.1.1 Chemicals

Diindolylmethane (CAS no:1968-05-4, TCI Chemicals, Japan), Indole-3-carbinol (CAS no: 700-06-1, Sigma Aldrich, USA), Clopidogrel (CLOP) (Dr. Reddys, India), Aspirin (ASP) (Alta laboratories, India), Losartan (Karnataka antibiotics and pharmaceutical limited, India), Dabigatran etixelate (Disto Pharma, India), Streptokinase (Cadila pharmaceuticals, India), Adenosine diphosphate (ADP) (CAS no:16178-48-6, Sigma Aldrich, USA), Thrombin (CAS no: 9002044, Sigma Aldrich, USA), Collagen (Sigma Aldrich, USA), Arachidonic acid (Sigma Aldrich, USA), Dichlorodihydrofluorescein diacetate(CAS no: 4091-99-0, DCFH-

DA) (Sigma Aldrich, USA), and Thiopentone sodium (Neon Labs, India) were purchased. Thromboxane B2 ($TXB_2$) ELISA kit (501020, Cayman Chemical, USA), cyclic adenosine monophosphate (cAMP) ELISA kit (K11-0299, KinesisDx, USA), cycloxygenase 1 (COX-1) ELISA kit (K11-0909, KinesisDx, USA), nicotine adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) (KLR2344, Krishgen Biosystems, India), and prostaglandin E2 (PGE2) (K11-0505, KinesisDx, USA) ELISA kits were obtained. Amplex Red kit for the estimation of hydrogen peroxide ($H_2O_2$) was purchased from Thermofisher, USA (A22188). All other reagents and chemicals used in the present study were obtained from local suppliers.

### 3.1.2 Animals

Male Wistar rats (240-260 gm) were acquired from the central animal house, the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Rats were homecaged under the standard laboratory conditions, like free access to food, water, constant light, and dark cycle (12/12hr). Before experiments, a two-week acclimatization period was given to experimental animals. Approval of the Institutional Animal Ethics Committee (Protocol no. Dean/2021/IAEC/2548) was granted before commencing the experiment. All the experimental procedures were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH, Publication-2011).

### 3.1.3 Experimental Design

The present study had three sets of experiments. Set one experiment is evaluating *in vitro* platelet aggregation, clot retraction assay, and thrombolytic activity (n=6). Blood (5 – 6 ml) was collected from each animal via cardiac puncture, and platelet-rich plasma (PRP) was used to measure the *in vitro* platelet aggregation and clot retraction assays. 0.5 ml of blood was used to measure the thrombolytic activity. The second set of experiments includes evaluating the antithrombotic potential of DIM using ferric chloride ($FeCl_3$) induced carotid

artery thrombosis (n = 6). Time to occlusion (TTO), thrombus weight, platelet reactive oxygen species (ROS), hydrogen peroxide ($H_2O_2$), NOX2, and cAMP were evaluated as per the manufacturer's guidelines. Serum was obtained by centrifuging the blood at 4000 rpm for 10 min at 4°C (M. Kumar, Kasala, Eshvendar Reddy, Bodduluru, Lakshmi Narendra, Dahiya, Vicky, Lahkar, Mangala, 2016). Further, serum COX-1, $TXB_2$, and prostaglandin E2 ($PGE_2$) were estimated as per previous reports using ELISA kits (Lourenço et al., 2015; Xu et al., 2021). The third set of experiments investigated the effect of DIM on bleeding and clotting time (n = 6). The complete study design is depicted in figure 3.2.

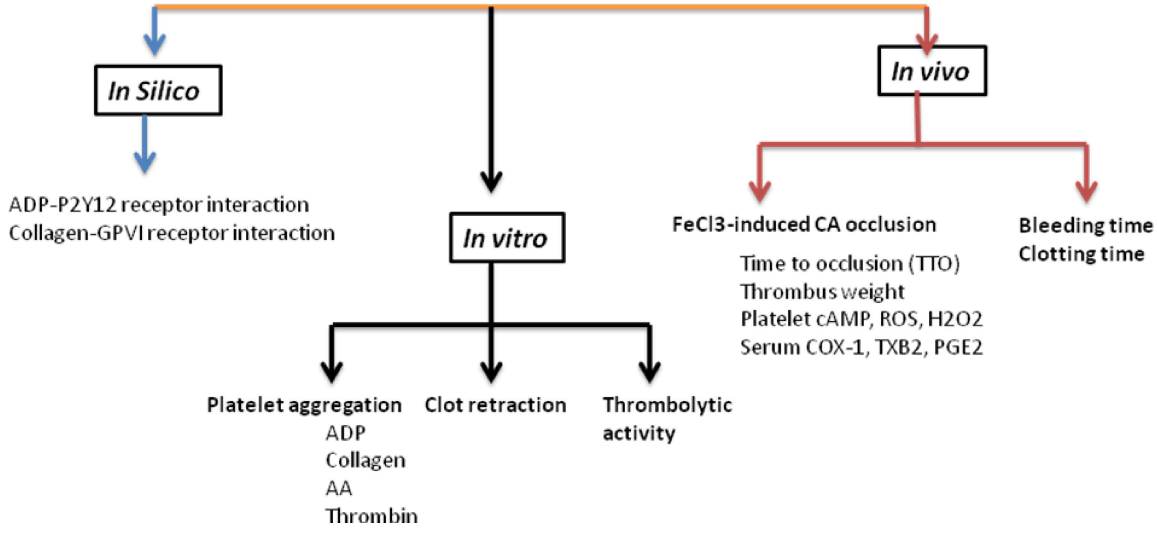


*Figure 3.2. Study design.*

### 3.1.4 *In silico* studies

The computational investigation was performed by using the ADT tools 1.5.6, Discovery Studio 2016, Chem 3D, and Pubchem. Glycoprotein VI crystal structures (PDB ID: 2GI7) and $P_2Y_{12}$ receptor crystal structures (PDB ID: 4NTJ) were obtained from the protein data bank (https://www.rcsb.org/). A chain of proteins from GPVI and the entire $P_2Y_{12}$ receptor were selected for docking. Protein was prepared by removing the nonessential residues using

Discovery Studio Visualiser and atom types, charges, and protonation states of amino acids were added through the PDB-PQR server using the Amber force field at pH 7.4 (Dolinsky et al., 2007). The obtained refined protein was converted into pdbqt format by using Autodock tools. 1.5.6. Losartan is known to inhibit the GPVI receptors (Onselaer et al., 2020) and $P_2Y_{12}$ receptor antagonist clopidogrel (CLOP) (Michelson, 2008) was chosen in the present study to compare the potential of I3C and DIM. Further, I3C, DIM, losartan, and CLOP were downloaded from Pubchem, and energy was minimised using Perkin Elmer Chem 3D with Generalized Amber Force Field (GAFF). Afterward, the obtained ligand in pdb format was converted into a pdbqt format by utilizing Autodock tools 1.5.6. The size of the grid was such that it covered the complete spike protein with grid dimension points 92, 56, 124 for GPVI and 66, 66, 68 for the $P_2Y_{12}$ receptor, along with grid spacing of 0.392 Å for GPVI and 0.481 Å for $P_2Y_{12}$ receptor. The grid centre was placed at coordinates 21.538, 30.285, 35.357 (x, y, z respectively for GPVI) and 20.798, 95.505, 41.25 (x, y, z respectively for the $P_2Y_{12}$ receptor). Furthermore, docking was performed with AutoDock 4.0 using the Lamarckian Genetic Algorithm (LGA) (Morris et al., 1998). It applied a semiempirical free energy force field to evaluate conformers generated by docking simulations. We have computed the free binding energy of the ligand-receptor complex for scoring several conformations. I3C, DIM, and losartan were docked against the target enzyme GPVI. The floor of the binding groove is formed by different types of residue, including Lys41, Lys59, Arg60, Arg66, Leu53, Phe54, Pro56, Leu62, Tyr66, Ser43, Ser44 (S), Glu48, Glu50, Ser61, Val34, Leu3 present in the domain of the GPVI receptor majorly involved in collagen binding (Horii et al., 2006). Discovery Studio 2016 was used for the visualisation of the docked structures and binding interactions were obtained (Morris et al., 1998).

### 3.1.5 Platelet and PRP isolation

Rat blood was withdrawn from the cardiac puncture with a sodium citrate anticoagulated syringe (3.8%, 1:9, citrate:blood). Platelet-rich plasma was obtained after centrifugation of blood at 1000 rpm for 10 min at 37 °C. Further, residual blood was centrifuged again at 3000 rpm for 10 min at 37 °C to isolate the platelet-poor plasma (PPP) (Xue Li et al., 2020a). Platelets were isolated from PRP by centrifugation at 350g for 10 min and the platelet number was adjusted to $4X10^8$ cells/ml using Tyrodes buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, and 0.3 mM $NaHPO_4$, pH 7.4) (Irfan, Kwon, Kwon, & Rhee, 2020).

### 3.1.6 *In vitro* platelet aggregation

Platelet number was adjusted to $4X10^8$ cells/ml using Tyrodes buffer (Irfan et al., 2020). Further, the *in vitro* antiplatelet aggregation properties of DIM, I3C, CLOP, and ASP were evaluated using the 96-well plate aggregometry method (Bednar, Condra, Gould, & Connolly, 1995; T.-F. Cheng et al., 2020). I3C, CLOP, ASP, losartan, and dabigatran doses were selected from previous studies (Q.-f. Chen et al., 2016; P. Li, Ferrario, & Brosnihan, 1998; P. Paliwal, Chauhan, Gaurav, Gautam, Deepa, Dash, Debabrata, Patne, Shashikant CU, Krishnamurthy, Sairam, 2018; Wu et al., 2020). *In vitro* platelet aggregation was performed after incubating the PRP (200 µl) with DIM (12.5, 25, and 50 µM), I3C (25 µM), clopidogrel (3 µM), aspirin (3 µM), dabigatran etexilate (10 µM), losartan (10 µM), and DMSO (0.5% v/v). Before stimulation with platelet aggregating agents, PRP absorbance was measured at 570 nm on a microplate reader (BioTek, USA). Then, 10 µM ADP, 100 µM AA, 5µg/ ml of collagen, and thrombin (0.05U/ml) were added to initiate the platelet aggregation (E. S. Kim, Lee, & Lee, 2020; Shin et al., 2021), then continuously incubated at 37 °C for 5 min with shaking. The change in absorbance values was recorded at 570 nm. Inhibitory concentrations ($IC_{50}$) of DIM against ADP, collagen, thrombin, and AA-induced platelet aggregation were

calculated using nonlinear regression analysis using GraphPad Prism software. The PPP absorbance was counted as a blank and platelet aggregation (%) was calculated as follows:

$$Platelet\ aggregation\ rate\ (\%) = PRP\ abs\ (before\ stimulation) - PRP\ abs\ (after\ stimulation) \div PRP\ abs\ (before\ stimulation) - PPP\ abs) * 100$$

### 3.1.7 Clot retraction

PRP (500 µL) was preincubated with DIM, I3C, ASP, and CLOP in polyethylene tubes for 15 min at 37°C. Then, clot retraction was initiated by the addition of thrombin (0.05 U/mL). Further, polyethylene tubes were allowed for 2 hours at 37°C. After that, photographs were taken, and then clot retraction weight was measured (Shin et al., 2021). Further, the clot area was measured using Image J (v1.46, National Institutes of Health, USA).

### 3.1.8 $FeCl_3$ induced carotid artery thrombosis

DIM, I3C, and CLOP were dissolved in 0.5% DMSO and administered orally for one week. I3C, ASP, and CLOP (15 mg/kg) were selected from previous studies (N. Ma et al., 2016; P. Paliwal, Chauhan, Gaurav, Gautam, Deepa, Dash, Debabrata, Patne, Shashikant CU, Krishnamurthy, Sairam, 2018). After 30 min of the final dose, rats were anesthetized with thiopentone sodium (40 mg/kg) (Ramakrishna et al., 2021). The right common carotid artery (CCA) was carefully isolated, and presoaked Whatman filter paper (2x1mm) in ferric chloride (10%) was applied to the carotid artery for 5 minutes. The Whatman filter paper was removed after 5 min, and the CCA was washed with normal saline to prevent the $FeCl_3$ effect on surrounding tissues. Then, blood flow was observed using a laser Doppler instrument (Omegazone OZ-2 STD, Japan). Time to occlude (TTO) CCA was measured. If the CCA was not occluded, the TTO was counted as 60 min (Misra et al., 2018). After that, the CCA was dissected, and the thrombus weight was measured (E. Choi, Oh, & Sung, 2020; Misra et al., 2018). Platelet ROS level was measured using DCFH-DA fluorescence dye. DCFH-DA (10

M) was added to platelets and incubated for 30 min at 37°C in the dark. Platelet fluorescence was measured at 485 nm and 520 nm, respectively (C. Liu et al., 2019). Platelet $H_2O_2$ was measured by using an Amplex Red colorimetric kit. Platelet cyclic adenosine monophosphate (cAMP) and serum levels of $TXB_2$, COX-1, and $PGE_2$ were estimated by ELISA kits.

### 3.1.9 Bleeding and clotting time

Bleeding time and clotting time were measured after 30 min of drug administration. Briefly, rats were anaesthetized with thiopentone sodium (40 mg/kg) after 30 min of administration of DIM (12.5, 25, and 50 mg/kg), I3C (50 mg/kg), CLOP (15 mg/kg) (P. Paliwal, Chauhan, Gaurav, Gautam, Deepa, Dash, Debabrata, Patne, Shashikant CU, Krishnamurthy, Sairam, 2018), and ASP (20 mg/kg) (N. Ma et al., 2016). According to the pharmacokinetic profile, 50 % of I3C is converted to DIM (Bradlow & Zeligs, 2010). Therefore, we have chosen DIM 25 mg of (I3C dose is 50 mg/kg) as the median dose. Further, DIM lower (12.5 mg/kg) and higher (50 mg/kg) doses were fixed in geometric progression. Blood (0.5 ml) was collected to measure the clotting time. Clotting was confirmed when fibrin mesh was picked up by a dissecting needle (J. Huang, Fan, Yin, & Huang, 2019). Further, bleeding time was evaluated by transecting rats distal tails (2 mm) and submerging them in saline (40 ml). Bleeding time was noticed when bleeding ceased continuously for 3 minutes, and the cut-off time was 60 minutes (L. Chen et al., 2018).

### 3.1.10 *In vitro* thrombolytic activity

Rat blood (0.5 ml) was transferred into a preweighed sterile centrifuge (W1) and incubated for 45 min at 37°C. After that, the serum was decanted from the centrifuge tube and weighed (W2). Then, the clotted blood was calculated using the following formula:

*Weight of clotted blood = (Weight of clot containing tube, W2) – (pre-weighed tube without clot, W1).* Further, 100 µL of 12.5, 25, and 50 mg/ml of DIM, I3C (50 mg/ml), and

streptokinase (100 µL, 1500000 IU) were added to clots. DMSO (100 µL) was added to the blank tubes. Furthermore, all the centrifuge tubes were incubated for 90 min at 37°C. After that, the weight of the centrifuge tubes was taken to calculate the amount of clot lysed (thrombolysis) (Zaman, Parvez, Jakaria, Sayeed, & Islam, 2015). The clot lysis (%) was calculated as:

Clot lysis (%) = [1-(Weight of clot after lysis/Weight of clot before lysis)] X 100.

### 3.1.11 Statistical analysis

Data are expressed as Mean ± S.D. GraphPad Prism 5 was used to analyse the statistical data. One-way ANOVA was used for the multiple comparisons. If ANOVA shows a significant difference, then post hoc analysis was performed with the Newman-Keuls test. $IC_{50}$ values of DIM against platelet aggregating agents, ADP, collagen, thrombin, and AA were calculated by linear regression analysis using GraphPad Prism5. A P-value of less than 0.05 was considered statistically significant.

## 3.2 Results

### 3.2.1 DIM interacted with platelet GPVI receptors

The interaction between I3C, DIM, and losartan with the GPVI receptor is depicted in Figure 3.3. Figure 3.3B shows that the hydroxyl group (oxygen and hydrogen) of I3C forms two conventional hydrogen bonds (Ser44 and Arg46) with GPVI receptors, while indole forms -alkyl bonds (Lys41 and Arg46). The interaction of DIM and GPVI receptors is depicted in Figure 3.3C. Indole hydrogens interact via conventional hydrogen bonding at Ser44 and Gln48. Indoles of DIM form π–carbon bonding (Arg46 and Lys41) and π –alkyl bonding (Leu39, Leu53, Arg46 and Lys41) van der Waals (Leu62, Ser61, Tyr66, Gln60, Tyr47, and Ser63). Figure 3.3D displays the interaction between losartan and GPVI; the nitrogens of tetrazole in losartan interacted through the conventional hydrogen bonding at Arg65 and

Ser43 and observed the presence of unfavorable donor-donor at Arg65 and Lys41, hydrogen of imidazolemethanol formed the traditional bonding of hydrogen at Ala63, imidazole interacted with Ser61 (π–sigma bond), biphenyl group forms π–π-t shaped bond at Tyr169, and π-alkyl bond at Pro168 and Ala63.

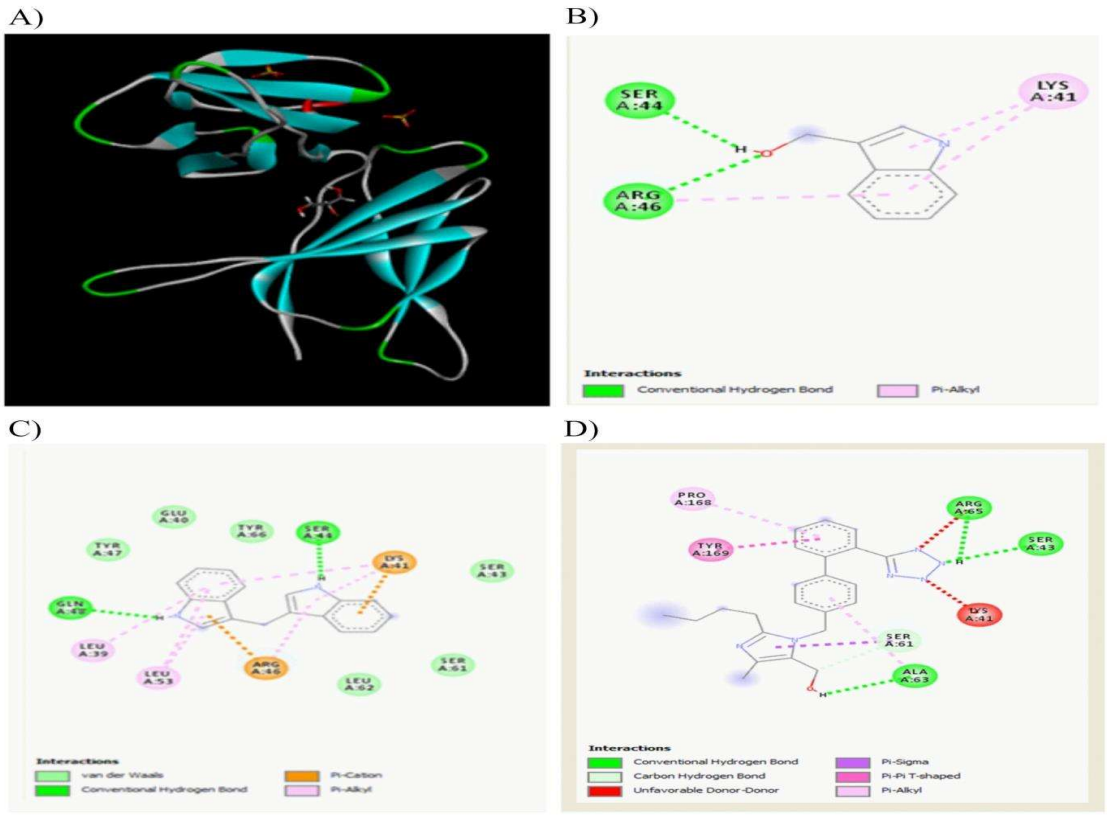


*Figure 3.3: Interaction of I3C, DIM, and Losartan with GPVI (2GI7). Figure 3.3A depicts the crystal structure of GPVI, Figure 3.3B depicts the interaction of I3C with GPVI, Figure 3.3C depicts the interaction of DIM with GPVI, and Figure 3.3D depicts the interaction of losartan with GPVI.*

### 3.2.2 DIM interacted with platelet $P_2Y_{12}$ receptors

The interaction between I3C, DIM, and CLOP is illustrated in figure 3.4. Figure 3.4B represents the interaction of I3C with the $P_2Y_{12}$ receptor, hydrogen of indole group of DIM

forms conventional hydrogen bonding with Phe252, indole forms amide-π stacked at Val279 and Ala255, and π-alkyl bonding at Lys280 and Arg256. Figure 3.4C illustrated the interaction between DIM and $P_2Y_{12}$ receptor, hydrogens of indoles interacted through the conventional hydrogen bonding at Arg256 and Phe252, indoles form amide-π stacked at Phe252 and Ala255, π-π stacked at Phe252 and Ala255, and π-alkyl bonding with Ala255, Lys280, Val279, and Arg256, and π-carbon bonding at Arg256. Figure 3.4D represents the interaction of CLOP with $P_2Y_{12}$ receptor, ester group of carbonyl oxygen makes conventional hydrogen bonding with Arg256, phenyl group forms π-sigma bond with Leu276 and alkyl and π-alkyl bonds with Lys280, benzyl carbon makes alkyl and π-alkyl bonds at Leu276 and Tyr259, dihydropyridine makes alkyl and π-alkyl bonds with Arg256, Lys280, and Phe252, and thiophene makes interaction with Ala255 (π-sigma bonds) and alkyl and π-alkyl bonds with Lys280, Val279, and Arg256.

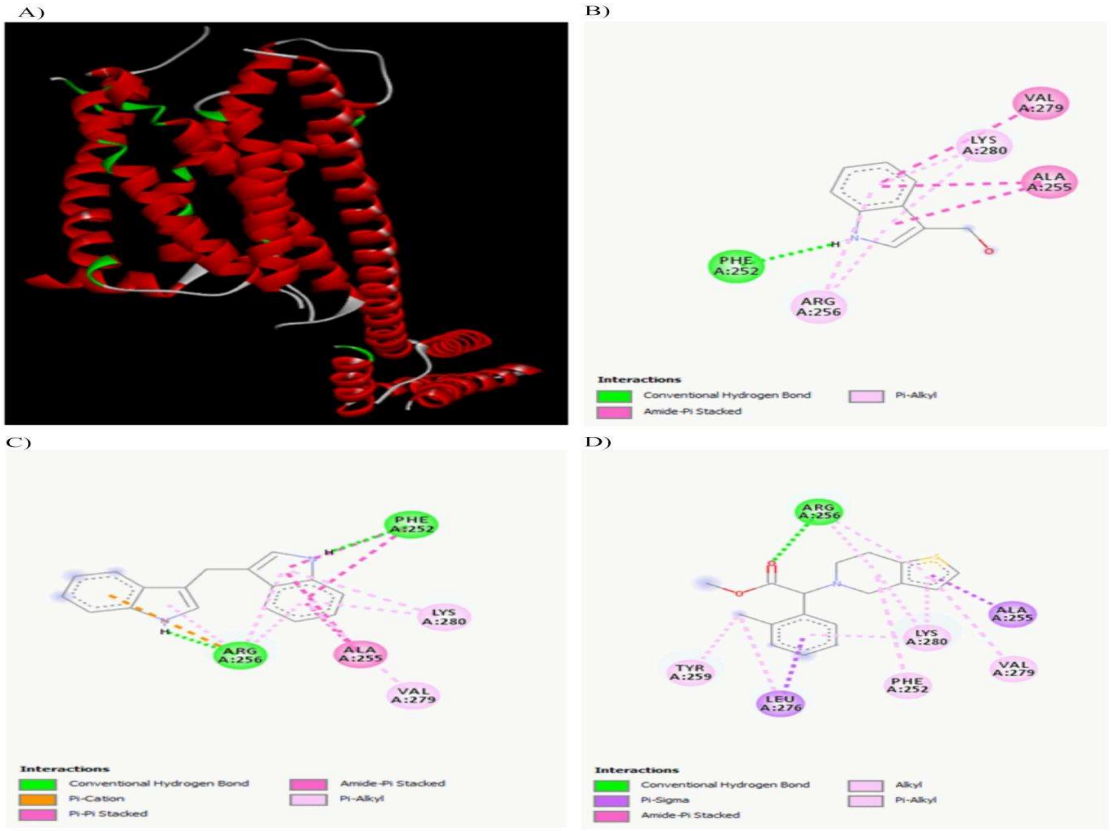

*Figure 3.4: Interaction of DIM with the $P_2Y_{12}$ receptor. Figure 3.4A indicates the crystal structure of $P_2Y_{12}$ receptors. Figure 3.4B represents the interaction between I3C and $P_2Y_{12}$ receptors. Figure 3.4C shows the interaction between DIM and $P_2Y_{12}$ receptors, and Figure 3.4D represents the interaction between CLOP and $P_2Y_{12}$ receptors.*

### 3.2.3 DIM exhibited low binding energy to GPVI and $P_2Y_{12}$ receptors

The binding affinities of DIM with GPVI and $P_2Y_{12}$ receptors are depicted in table 3.1. DIM (-6.32) exhibited a higher binding affinity to GPVI receptors when compared to I3C (-4.72). Losartan (-7.5) showed a higher binding affinity than DIM and I3C. DIM (-7.8) displayed a higher binding affinity to $P_2Y_{12}$ receptors than I3C (-5.49) and CLOP (-6.5). I3C exhibited a lower binding affinity to $P_2Y_{12}$ receptors than CLOP. DIM interacted with more amino acid residues (12) in GPVI receptors, whereas I3C and losartan interacted with 3 and 7 amino acid residues, respectively. DIM and I3C interacted with five amino acid residues in $P_2Y_{12}$ receptors, whereas CLOP interacted with seven amino acid residues.

| S.No. | Compound ID | Compound | Binding energy (kcal/mol) | | Total amino acids interacted | |
|---|---|---|---|---|---|---|
| | | | $P_2Y_{12}$ receptors | GPVI receptors | $P_2Y_{12}$ receptors | GPVI receptors |
| 1 | 3712 | I3C | - 5.49 | -4.72 | 5 | 3 |
| 2 | 3071 | DIM | -7.8 | -6.32 | 5 | 12 |
| 3 | 60606 | CLOP | -6.5 | - | 7 | - |
| 4 | 3961 | Losartan | - | -7.5 | - | 7 |

*Table 3.1: Binding energy of I3C, DIM, and CLOP with $P_2Y_{12}$ receptors and GPVI receptors and number of amino acids involved in interaction with respective receptors.*

### *3.2.4* DIM decreased *in vitro* platelet aggregation

$IC_{50}$ concentrations of DIM against platelet aggregation are depicted in figure 3.5 and *in vitro* platelet aggregation inhibitory properties of DIM are depicted in figure 3.6. One-way analysis identified the significant changes in platelet aggregation among groups: ADP [F (5, 35) = 122.6; P < 0.0001], collagen [F (5, 35) = 201.9; P < 0.0001], thrombin [F (5, 35) = 352.8; P < 0.0001], and arachidonic acid [F (5, 35) = 340.9; P < 0.0001]. $IC_{50}$ values of DIM were identified as 10.72, 11.33, 11.82, and 10.62µM against ADP, collagen, thrombin, and AA-induced platelet aggregation, respectively. We found that 95% confidence interval (CI) values of DIM ranged from 10.01 to 11.48, 10.07 to 12.76, 11.20 to 12.8, and 10.62 to 13.44, respectively, against ADP, collagen, thrombin, and AA-induced platelet aggregation. Post hoc analysis revealed that DIM treatment reduced the percentage of platelet aggregation in a dose-dependent manner. DIM 25µM significantly decreased the platelet aggregation compared to I3C 25$\mu M$. ASP and clopidogrel significantly ameliorated the platelet aggregation than I3C 25$\mu M$. However, a significant difference was not observed between losartan, dabigatran, ASP, clopidogrel, and DIM 25$\mu M$ treated groups. These findings indicate that DIM was a more potent antiplatelet agent than I3C.

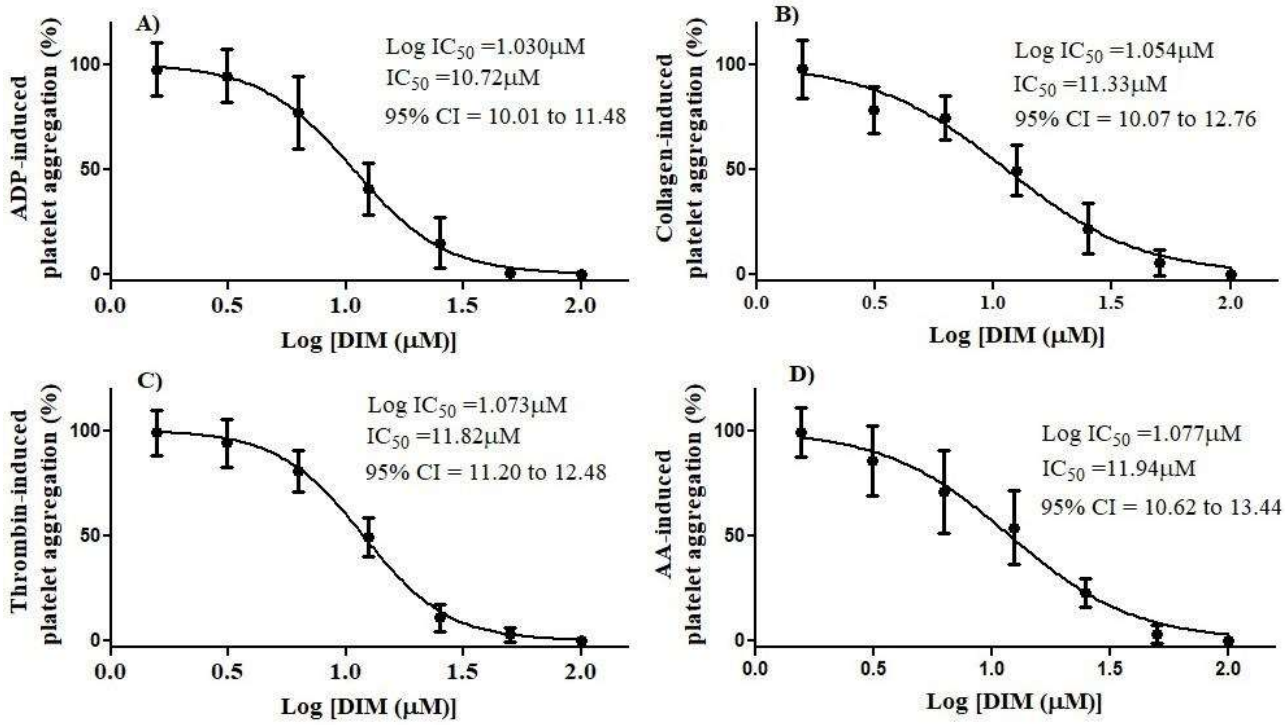


Figure 3.5. *$IC_{50}$ of DIM. Figures 3.5A, 3.5B, 3.5C, and 3.5D indicate the $IC_{50}$ of DIM against ADP, collagen, thrombin, and AA-induced platelet aggregation, respectively.*

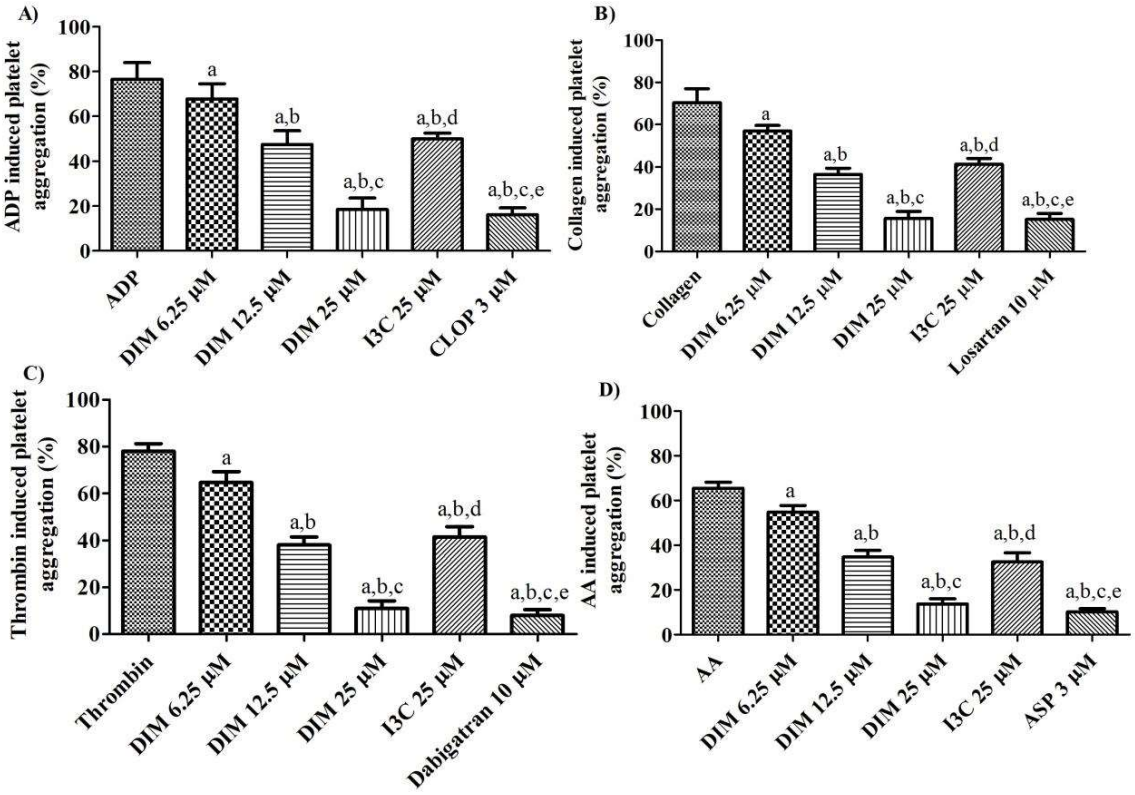


*Figure 3.6. Effect of DIM treatment on in vitro platelet aggregation. Figures 3.6A, 3.6B, 3.6C, and 3.6D indicate the alterations in ADP (**10 µM**), collagen (**5µg/ ml**), thrombin (**0.05U/ml**), and AA (**100 µM**)-induced platelet aggregation, respectively. [a]p<0.001 vs. ADP, collagen, thrombin, and AA in their respective groups, [b]p<0.001 vs. DIM 6.25µM, [c]p<0.001 vs. DIM 12.5µM, and [d]p<0.001 vs. DIM 25µM, [e]p<0.001 vs. I3C 25µM in their respective groups. All the results are Mean ± SD (n=6). One-way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.5 DIM reduces clot retraction

The effect of DIM on thrombin-induced clot retraction is illustrated in figure 3.7. One way analysis revealed that there was significant difference among the groups: clot retraction (%) [F (7, 47) = 478.3; P < 0.0001] and clot retraction weight [F (7, 47) = 904.2; P < 0.0001]. Further, post hoc analysis retrieved that DIM dose-dependently alleviated the clot retraction

and increased the clot retraction weight. I3C (25 *μM)* showed a significant difference compared to DIM 12.5 and 25*μM* treated rats. However, significant differences were observed between DIM 25*μM* and I3C 25*μM* treated groups. Moreover, treatment with DIM 25*μM*, CLOP, and ASP did not show any significant difference as seen in post hoc analysis. These results demonstrated that DIM inhibited the clot retraction than I3C.

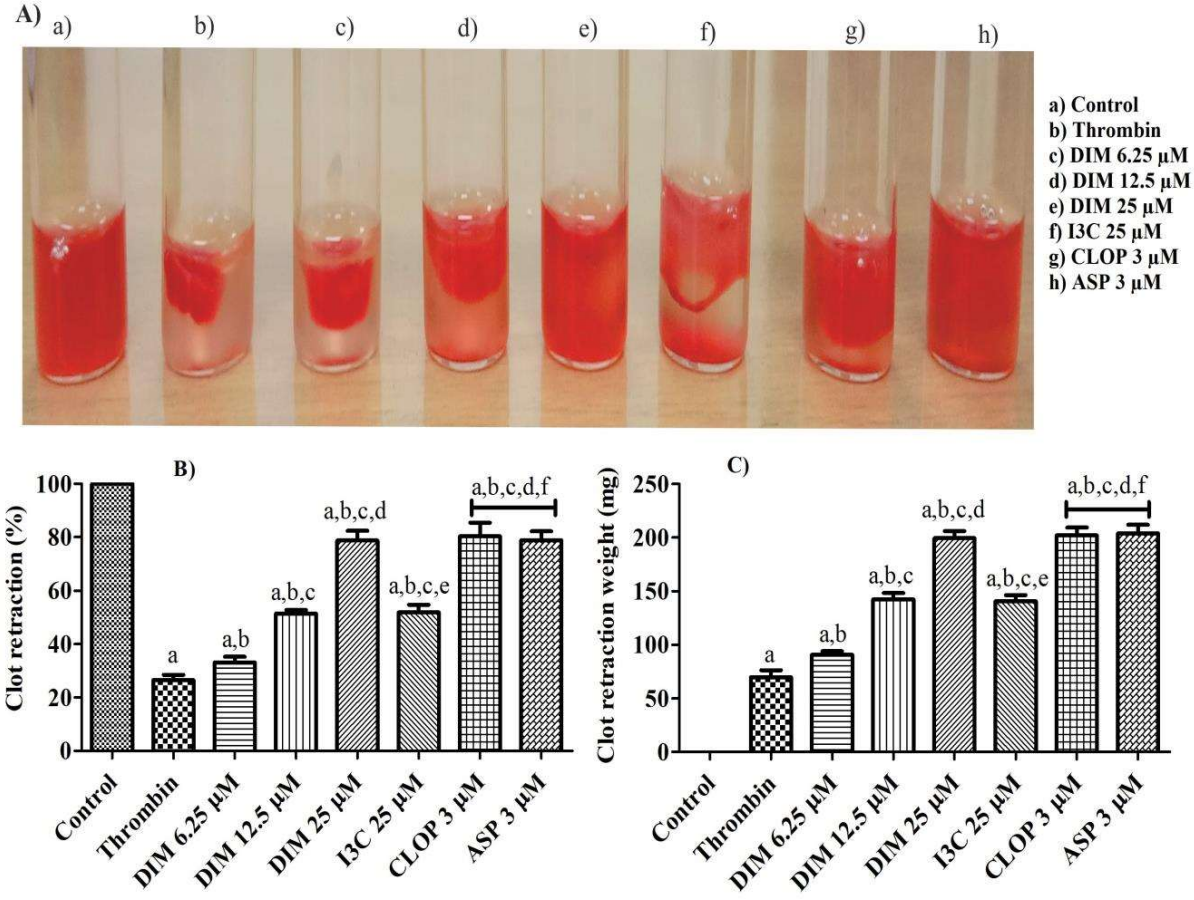


*Figure 3.7. Effect of DIM treatment on thrombin-induced clot retraction. Figures 3.7A, 3.7B, and 3.7C represent the images of clot retraction, clot retraction (%), and clot retraction weight (mg).* $^{a}p<0.001$ *vs. control,* $^{b}p<0.001$ *vs. thrombin,* $^{c}p<0.001$ *vs. DIM 6.25μM,* $^{d}p<0.001$ *vs. DIM 12.5 μM, and* $^{e}p<0.001$ *vs. DIM 25μM,* $^{f}p<0.001$ *vs. I3C 25μM. All the results are Mean ± SD (n=6). One-way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.6 DIM ameliorated the $FeCl_3$ induced TTO and thrombus weight

Changes in TTO and thrombus weight after DIM treatment are depicted in figure 3.8. One-way analysis revealed that there were significant differences among groups: TTO [F (7, 47) = 140.0; P < 0.0001] and thrombus weight [F (7, 47) = 148.7; P < 0.0001]. Post hoc analysis revealed that $FeCl_3$ rats significantly decreased the TTO and increased the thrombus weight. DIM treatment dose-dependently increased the TTO and decreased the thrombus weight.

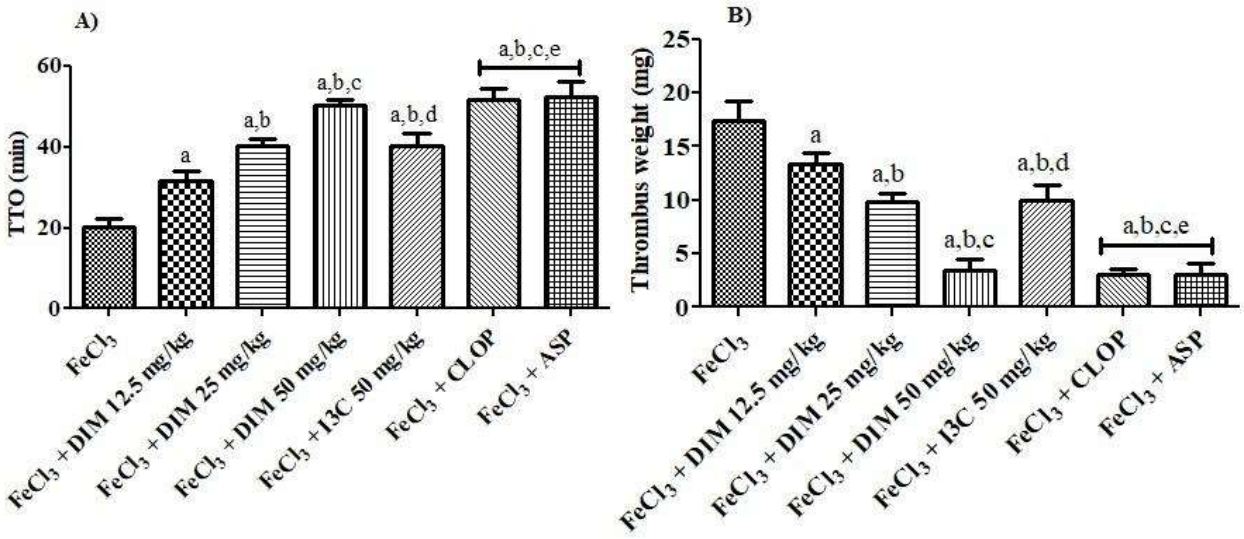


*Figure 3.8. Effect of DIM treatment on TTO and thrombus weight. Figures 3.8A and 3.8B indicates the changes in TTO and Thrombus weight, respectively.* $^{a}p<0.001$ *vs.* $FeCl_3$, $^{b}p<0.001$ *vs.* $FeCl_3$*+ DIM 12.5mg/kg, and* $^{c}p<0.001$ *vs.* $FeCl_3$ *+ 25mg/kg,* $^{d}p<0.001$ *vs.* $FeCl_3$ *+ DIM 50 mg/kg, and* $^{e}p<0.001$ *vs.* $FeCl_3$ *+ I3C 50 mg/kg. All the results are expressed as Mean ± SD (n=6). One-way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.7 DIM improved cAMP and alleviated the COX-1, $TXB_2$, and $PGE_2$ in $FeCl_3$ induced thrombosis

Alterations in ROS, cAMP, $TXB_2$, and $PGE_2$ levels after DIM treatment are illustrated in figure 3.9. One-way analysis identified the significant differences among groups: cAMP [F (6,41) = 223.1; P < 0.0001], $TXB_2$ [F (6,41) = 122.9; P < 0.0001], COX-1 [F (6,41) = 318.7;

$P < 0.0001$], and $PGE_2$ [$F(6,41) = 224.8$; $P < 0.0001$]. Post hoc analysis revealed that DIM treatment significantly increased the cAMP levels and decreased the $TXB_2$, COX-1, and PGE2 levels. The DIM 50 mg/kg significantly elevated the cAMP levels and decreased the $TXB_2$, COX-1, and $PGE_2$ levels compared to the DIM 12.5, 25 mg/kg, and I3C 50 mg/kg treated groups. I3C 50 mg/kg dose significantly increased the cAMP and decreased the $TXB_2$, COX-1, and PGE2 levels compared to FeCl3 and DIM 12.5 mg/kg groups. DIM 12.5 mg/kg significantly alleviated the $TXB_2$, COX-1, and $PGE_2$ levels and improving the cAMP levels as compared to the $FeCl_3$ group. When compared to the DIM 12.5 mg/kg treated group, the DIM 25mg/kg treated group significantly increased cAMP while decreasing $TXB_2$, COX-1, and $PGE_2$. No significant differences were observed between I3C 50 mg/kg and DIM 25 mg/kg as well as between ASP, CLOP, and DIM 50 mg/kg treated rats.

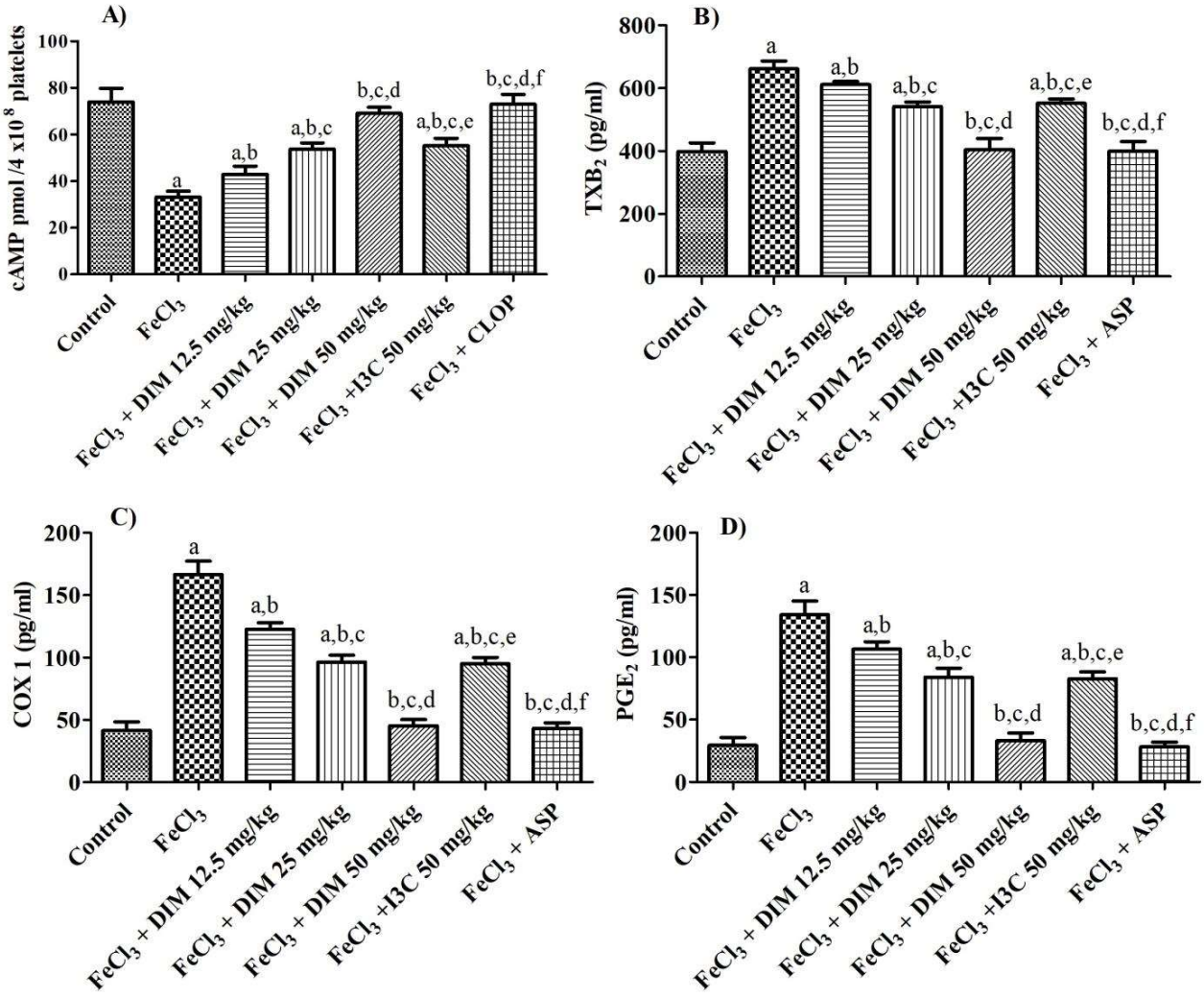


*Figure 3.9. Effect of DIM treatment on cAMP, $TXB_2$, COX-1, and $PGE_2$ levels. Figures 3.9A, 3.9B, 3.9C, and 3.9D indicate the changes in cAMP, COX-1, $TXB_2$, and $PGE_2$ levels, respectively. $^{a}p<0.001$ vs. control, $^{b}p<0.001$ vs. $FeCl_3$, and $^{c}p<0.001$ vs. $FeCl_3$ + DIM 12.5mg/kg, $^{d}p<0.001$ vs. $FeCl_3$ + 25mg/kg, and, $^{e}p<0.001$ vs. $FeCl_3$ + I3C50 mg/kg, and $^{f}p<0.001$ vs. $FeCl_3$ + I3C 50 mg/kg. All the results are expressed as Mean ± SD (n=6). One way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.8 DIM alleviated the platelet ROS and $H_2O_2$

Alterations in platelet ROS, $H_2O_2$, and NOX2 levels after DIM treatment are illustrated in figure 3.10. One-way analysis identified the significant differences among groups: ROS [F (7, 47) = 223.1; P < 0.0001], $H_2O_2$ [F (7, 47) = 468.9, P < 0.0001], and NOX2 [F (7, 47) = 52.63, P < 0.0001], respectively. Post hoc analysis revealed that DIM treatment significantly reduced platelet ROS and $H_2O_2$ levels in a dose-dependent manner. Platelet ROS and $H_2O_2$ levels were significantly elevated in $FeCl_3$ treated groups compared to control animals. When compared to the I3C and CLOP treated groups, DIM and ASP significantly reduced platelet ROS and $H_2O_2$ levels. Moreover, DIM and I3C treatment did not alter the platelet NOX2 levels. These results indicate that DIM acts as a free radical scavenger, thereby reducing platelet aggregation.

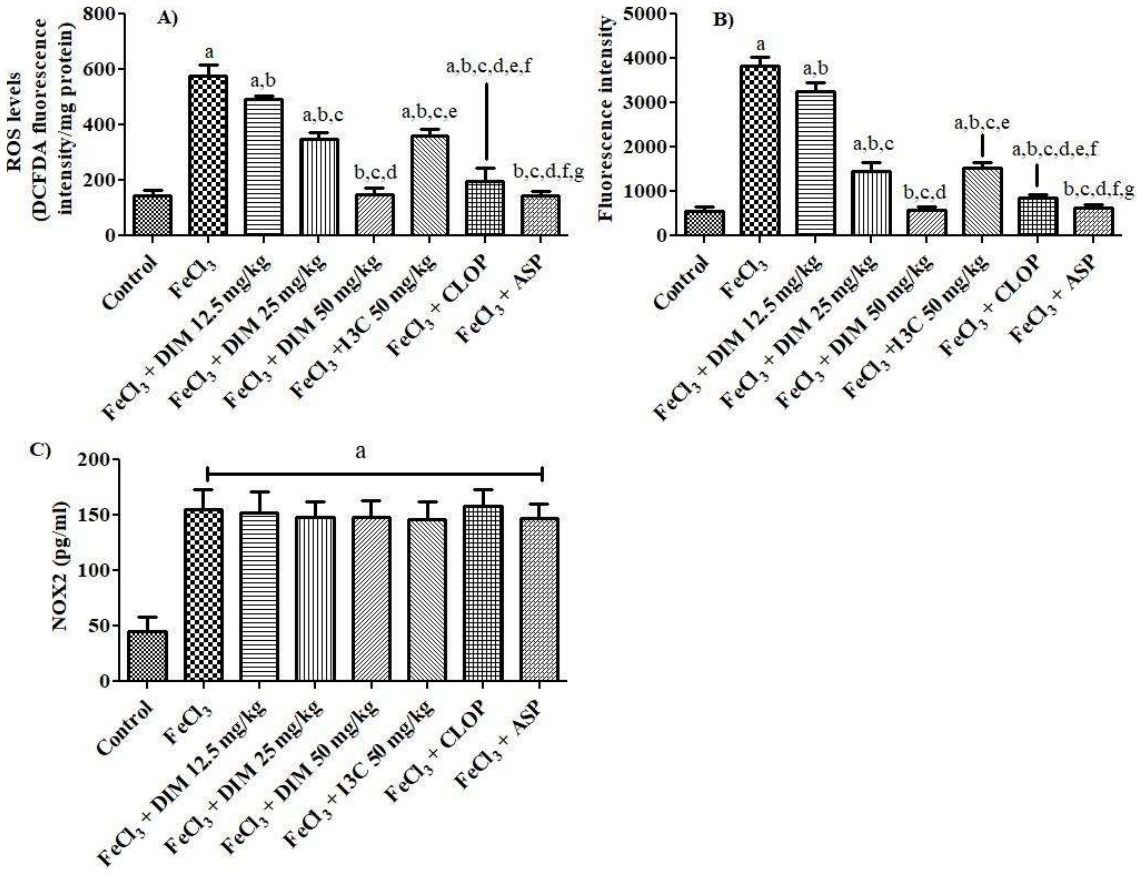

*Figure 3.10. Effect of DIM treatment on platelet ROS, $H_2O_2$, and NOX2 levels. Figure 3.10A, 3.10B, and 3.10C indicate the platelet ROS, $H_2O_2$, and NOX2, respectively. [a]p<0.001 vs. control, [b]p<0.001 vs. $FeCl_3$, and [c]p<0.001 vs. $FeCl_3$+ DIM 12.5mg/kg, [d]p<0.001 vs. $FeCl_3$ + 25mg/kg, and, [e]p<0.001 vs. $FeCl_3$ + I3C50 mg/kg, and [f]p<0.001 vs. $FeCl_3$ + I3C 50 mg/kg. All the results are expressed as Mean ± SD (n=6). One way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.9 DIM improved the bleeding and clotting time

Figure 3.11 represents the effect of DIM treatment on bleeding and clotting time in rats. One way analysis revealed that significant differences were observed among the groups: bleeding time [F (6, 47) = 123.8; P < 0.0001] and clotting time [F (6, 47) = 233.6; P < 0.0001]. Post hoc analysis revealed that DIM treatment dose-dependently increased the BT and CT. The DIM 50 mg/kg treatment significantly prolonged the BT and CT times more than the I3C 50 mg/kg treatment. Moreover, treatment with DIM 50 mg/kg, CLOP, and ASP did not significantly differ. These results indicate that DIM potentially prolongs the BT and CT more than I3C.

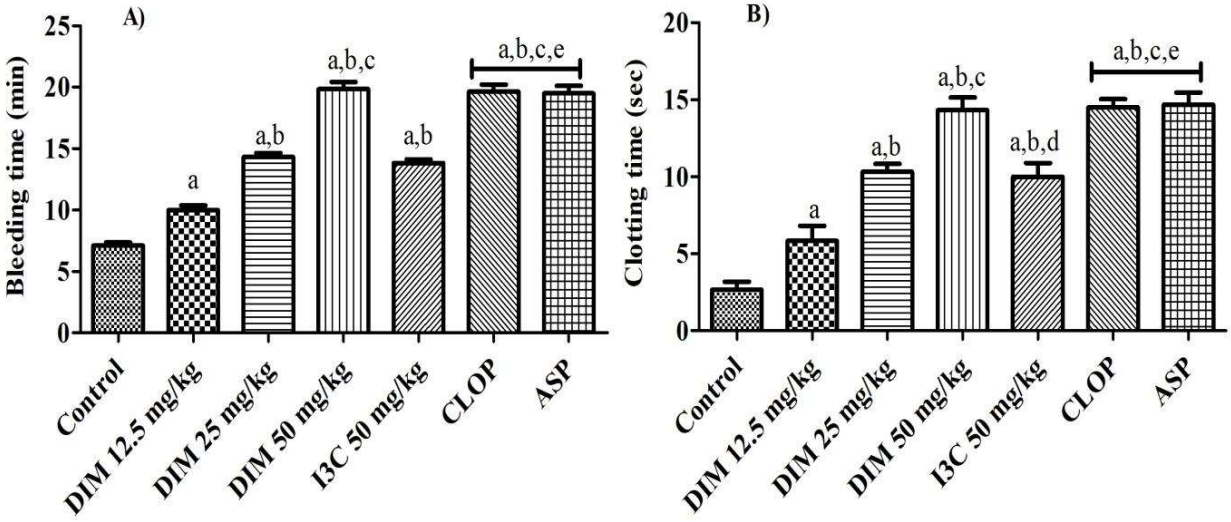

*Figure 3.11. Effect of DIM treatment clotting and bleeding time. Figure 3.11A represents the bleeding time and figure 3.11B indicates the clotting time.* $^{a}p<0.001$ *vs. control,* $^{b}p<0.001$ *vs.* $FeCl_3$ *+ DIM 12.5mg/kg,* $^{c}p<0.001$ *vs.* $FeCl_3$ *+ DIM 25 mg/kg,* $^{d}p<0.001$ *vs.* $FeCl_3$ *+ DIM 50 mg/kg, and* $^{e}p<0.001$ *vs.* $FeCl_3$ *+ I3C 50 mg/kg. All the results are Mean ± SD (n=6). One-way ANOVA followed by Newman-Keuls post hoc test.*

### 3.2.10 DIM did not cause thrombolysis

The thrombolytic potential of DIM is depicted in figure 3.12. One-way analysis revealed that there was a significant difference among the groups in thrombolytic activity [F (5, 35) = 177.5; P < 0.0001]. Post hoc studies revealed that there were no significant differences between DIM, I3C treated, and control groups. However, SK-treated rats show substantial differences compared to DIM and I3C treated groups. DIM and I3C treated rats did not show significant differences when compared to control rats.

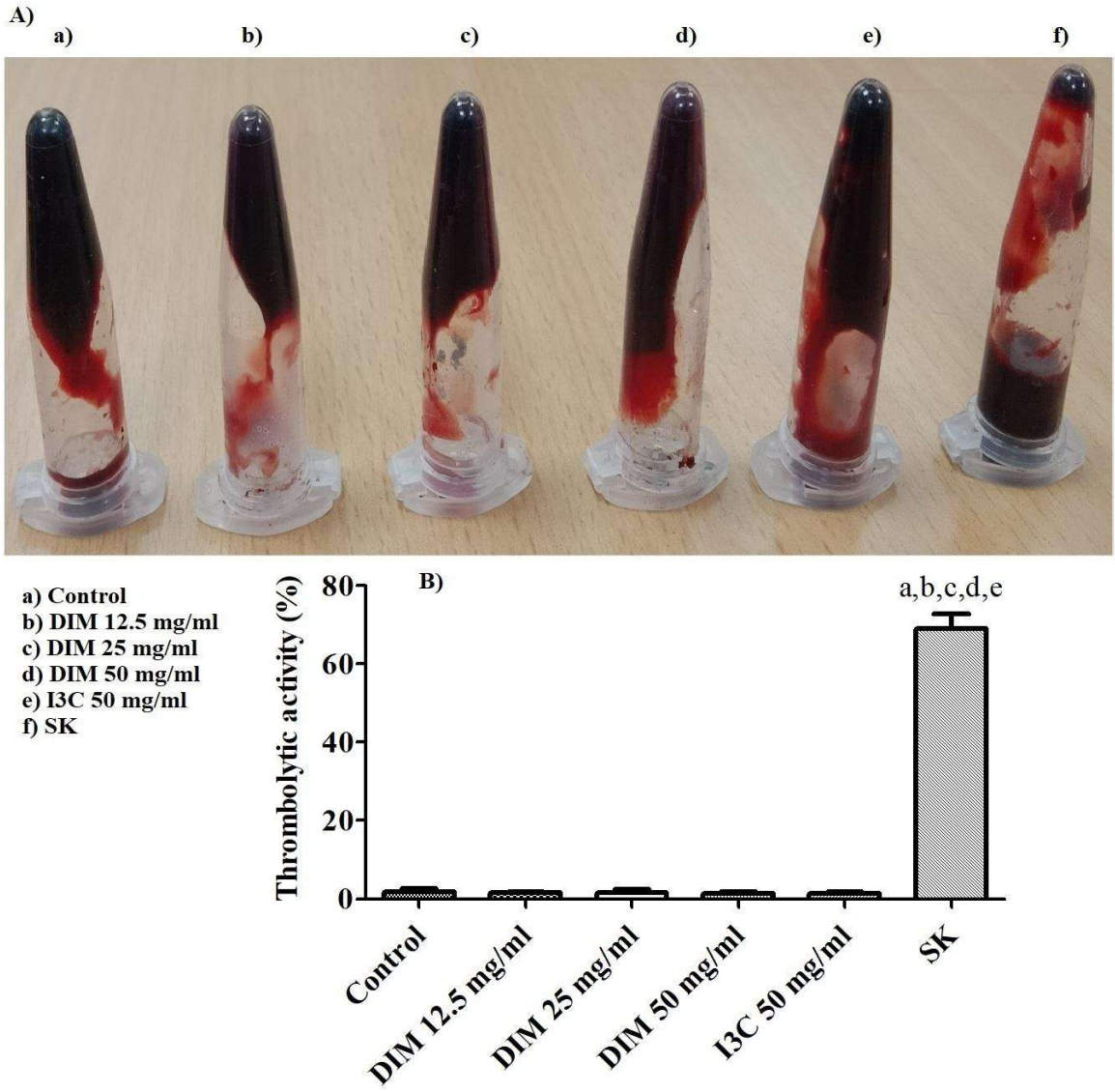


*Figure 3.12. The thrombolytic activity of DIM. Figure 3.12A illustrates the thrombolytic images of DIM treatment and Figure 3.12B indicates the thrombolytic activity (%), respectively. SK-streptokinase.* $^{a}p<0.001$ *vs. control,* $^{b}p<0.001$ *vs. DIM 12.5mg/kg,* $^{c}p<0.001$ *vs. DIM 25 mg/kg,* $^{d}p<0.001$ *vs. DIM 50 mg/kg, and* $^{e}p<0.001$ *vs. I3C 50 mg/kg. All the results are Mean ± SD (n=6). One-way ANOVA followed by Newman-Keuls post hoc test.*

### 3.3 Discussion

The present study showed that DIM exhibited antiplatelet and antithrombotic activity. DIM interacted with platelet GPVI and $P_2Y_{12}$ receptors. Further, DIM ameliorated platelet

aggregation, clot retraction, and prolonged clotting and bleeding time. DIM prolonged the TTO, mitigated the thrombus weight, oxidative stress, COX-1, and improved the cAMP levels. These effects were more significant than the parent compound, I3C.

Collagen binds and facilitates the platelets to adhere to the injured endothelium through the GPVI receptors and promotes platelet aggregation (Jung et al., 2017). *In silico* results from this study indicated that DIM modulated the collagen with GPVI receptors than its parent compound I3C, indicating that DIM is a potent modulator of GPVI receptors. Correspondingly, DIMs low binding energy to GPVI receptors than I3C demonstrates that DIM is strongly bonded to GPVI receptors. Therefore, these findings indicate that DIM bonded and hindered the interaction between collagen and GPVI receptors. Further, DIM modulated the ADP-$P_2Y_{12}$ receptor complex by bonding to Arg256. Similar to other reports, these findings indicate that modulation of Arg256 of the $P_2Y_{12}$ receptor could impede the ADP-$P_2Y_{12}$ receptor complex activity by DIM (Hoffmann, Sixel, Di Pasquale, & von Kügelgen, 2008). *In silco,* findings suggested that DIM, when compared to its parent compound, I3C, may modulate the interaction of collagen with the GPVI receptor and ADP with the P2Y12 receptors.

In support of these findings, *in vitro* results indicate that DIM ameliorated the ADP and collagen-induced platelet aggregation, which indicates that DIM may be altering the GPVI and $P_2Y_{12}$. Moreover, GPVI ectodomain shedding contributes to the elevation of soluble GPVI (sGPVI), which is an indication of platelet activation (Gardiner et al., 2007). Therefore, sGPVI estimation would validate the role of the downstream pathway of GPVI in the mechanism of DIM. However, the effect of DIM on ectodomain shedding or GPVI downstream pathways has to be further evaluated. This is a limitation of the study. Further, DIM also attenuated the AA-induced platelet aggregation, indicating that DIM was involved

in ameliorating AA-mediated platelet aggregation. Thrombin causes platelet activation and platelet aggregation by converting fibrinogen to fibrin, which enables clot formation and activates the GP IIb/IIIa receptor by stimulating other platelet aggregating agents such as ADP and collagen, which further potentiates the clot retraction (Jiansong Huang et al., 2019; Seiffert et al., 2002; Shin et al., 2021). We found in amelioration of thrombin-induced platelet aggregation with the treatment of DIM. Further, *in vitro* clot retraction studies indicated that DIM also mitigated the thrombin-induced clot retraction and increased the clot retraction weight. These findings concluded that DIM acts as an antithrombotic agent. Furthermore, DIM mitigated the platelet aggregation and clot retraction more effectively than I3C, implying that DIM is more effective than I3C.

$FeCl_3$-induced arterial thrombosis model is widely used to evaluate the antithrombotic activities of chemicals. $FeCl_3$ produces consistent platelet aggregation, as commonly observed in thrombosis pathology (Honda, Kamisato, & Morishima, 2016; Kaku et al., 2013). We found that $FeCl_3$ application to the carotid artery was confirmed by measuring the carotid artery occlusion time (TTO) and thrombus weight. DIM therapy increased TTO and increased thrombus weight, implying that DIM is an antithrombotic drug. Moreover, these effects of DIM are as much as similar to FDA-approved antiplatelet drugs such as CLOP and ASP. Like $FeCl_3$, platelets release a greater amount of ROS in thrombotic diseases during the platelet activation phase, further enhancing platelet aggregation and activation (Qiao et al., 2018). Theoretically, antioxidants are reported to have antiplatelet aggregation and anticoagulant activity (Jang et al., 2014). DIM has been reported to mitigate oxidative stress (Lee et al., 2020). DIM therapy substantially reduced platelet ROS and $H_2O_2$ levels while having no effect on platelet NOX2. NOX2 is found on platelets and contributes to reactive oxygen species (ROS), such as $H_2O_2$ (Vara, Campanella, & Pula, 2013). Conversely, the

NOX2 enzyme is not responsible for platelet activation or aggregation (Sonkar et al., 2019; Vara et al., 2020). These findings from our results suggest that DIM ameliorates platelet ROS and $H_2O_2$ in NOX2 independent mechanisms. Thus, DIM-mediated antiplatelet activity could be due to its antioxidant activity. Furthermore, *in vivo* pre-treatment with DIM and I3C substantially raised platelet cAMP, showing that the $P_2Y_{12}$ receptor was inhibited. As a result, our findings revealed that DIM reduced platelet aggregation via inhibiting ADP-induced activation of the P2Y12 receptor.

The COX-1 enzyme synthesizes prostaglandin E2 (PGE2) and thromboxane A2 (TXA2) from arachidonic acid, which are important mediators of platelet activation and aggregation (Sakata et al., 2013). *In vitro* data suggested that DIM mitigated the AA-induced platelet aggregation. In support of these findings, *in vivo* data suggest that DIM mitigated the COX-1 and its downstream platelet aggregating agents, such as $TXB_2$ and $PGE_2$. Therefore, I3C and DIM act as COX-1 inhibitors to inhibit platelet aggregation. Moreover arachidonic acid and its downstream pathway enzymes (COX-1, $TXB_2$, and $PGE_2$) are activated due to phospholipase A2 enzyme activity (PLA2) (Sakata et al., 2013). However, it needs to be determined whether I3C and DIM suppress PLA2 activity is considered one of the limitations of the present study. Moreover, prolongation of bleeding and clotting time by DIM than I3C treatment indicated that DIM is a more potent antiplatelet and antithrombotic agent. Ischemic strokes are often treated with thrombolytics to remove the clots (Sharobeam, Jones, Walton-Sonda, & Lueck, 2020). DIM treatment did not cause thrombolysis. Therefore, DIM cannot be a substitute for rTPA therapy to treat primary strokes. Hence, DIM can be used as an antiplatelet and antithrombotic agent to treat primary and secondary strokes.

Previous reports suggested that I3C inhibited ADP and collagen-induced platelet aggregation (P. Paliwal, Chauhan, Gaurav, Gautam, Deepa, Dash, Debabrata, Patne, Shashikant CU, Krishnamurthy, Sairam, 2018; Park, 2008). Furthermore, it is thought that DIM mediates I3C's pharmacological activities (Bradlow & Zeligs, 2010). Our in *silico*, *in vitro*, and *in vivo* results indicated that DIM significantly ameliorated the platelet aggregation, thrombus formation, ROS, $H_2O_2$, $TXB_2$, COX-1, $PGE_2$, thrombus weight, improved the cAMP, and prolonged the TTO. DIM and I3C inhibited collagen, AA, thrombin-induced platelet aggregation, and the interaction of ADP and $P_2Y_{12}$ receptors. Therefore, DIM and I3C act in multiple ways to inhibit platelet aggregation and thrombus generation. Corroborating, *in silico, in vitro,* and *in vivo* results indicate that DIM, a metabolite of I3C, is a more potent antiplatelet and antithrombotic agent than its parent compound. Therefore, DIM could be a potential antiplatelet and antithrombotic compound to treat thrombotic diseases. However, *in vivo* studies need to be investigated for the beneficial effects of DIM in treating coagulative diseases. The pharmacological effects of DIM against platelet aggregation and thrombus generation are illustrated in figure 3.13.

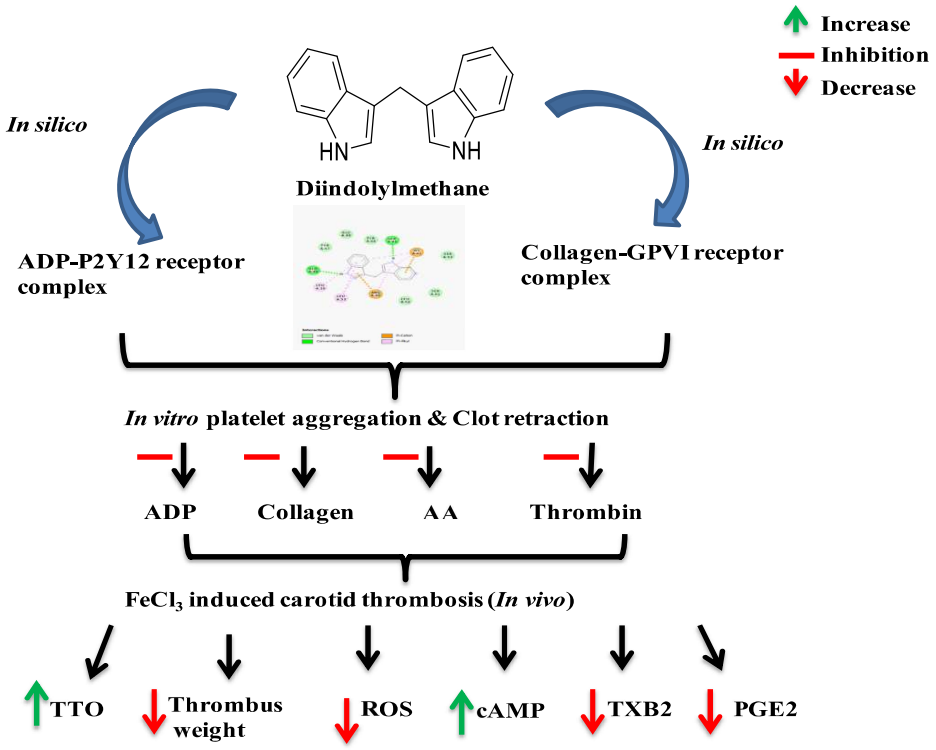


Figure 3.13. The summary of pharmacological activities of DIM against platelet aggregation and thrombosis.

### 3.4 Summary

- *In silico* studies predicted that the indole group of DIM and the hydroxyl group of I3C are responsible for the modulation of platelet interaction with the GPVI and $P_2Y_{12}$ receptors.
- DIM has a stronger interaction with GPVI and $P_2Y_{12}$ receptors compared to its parent compound, I3C.
- *In vitro* studies indicate that DIM more potently inhibited platelet aggregation and thrombin-induced clot retraction than its parent compound, I3C.
- *In vivo* results from $FeCl_3$ induced carotid artery thrombosis showed that DIM inhibited the oxidative stress and biosynthesis of $TXB_2$, $PGE_2$, and increased the cAMP and TTO.

- DIM prolonged the bleeding and clotting time than I3C.
- These findings conclude that DIM mediates the antiplatelet and antithrombotic activities of I3C and DIM is more potent inhibitor of platelet aggregation and thrombosis.