CHAPTER II

Crocin, A Dietary Additive Protects Platelets from Oxidative Stress-Induced Apoptosis and Inhibits Platelet Aggregation
Introduction

With a lifespan of just about a week, platelets are very sensitive to various external and internal stimuli and are a vulnerable target for the ROS generated in the endothelium during oxidative stress. It has been reported that oxidative stress is a major contributing factor for the pathophysiology of several diseases including CVDs. Therefore, it seems plausible to inter-relate oxidative stress, altered platelet functions and CVDs, going by the fact that accelerated platelet activation and aggregation eventually lead to the development of ischemia/reperfusion, atherothrombosis and MI. Consequently, many of the current treatment strategies for CVDs include antiplatelet drugs which target the various events of platelet activation and aggregation. However, recent studies claim that high amount of circulating MPs are also involved in the progression of CVDs. MPs are PS-positive membrane fractions released from apoptotic platelets. Despite their anuclear nature, platelets undergo apoptosis, the process of programmed cell death, similar to nucleated cells, but sans the nuclear events (Leytin, 2012). The apoptotic events include the increased production and release of ROS, $\text{H}_2\text{O}_2$, depolarization of $\Delta\Psi m$, release of apoptotic factors and PS externalization (Gyulkhandanyan et al., 2012). Eventually they release MPs possessing pro-inflammatory and pro-coagulatory properties, thereby affecting vascular function and leading to the pathogenesis of CVDs (Morel et al., 2011; Mallat et al., 2000; VanWijk et al., 2003).

In the present medical scenario, the available antiplatelet therapies are accompanied by several harmful side effects including severe internal bleeding owing to reduced platelet count or thrombocytopenia. Hence, people nowadays are turning towards plant-based therapeutics. Besides lacking harmful side effects, many of them
are potent antioxidants, and hence have become the choice of treatment of late for many of the oxidative stress-induced ailments including CVDs. But, their effect on oxidative stress-induced alterations in platelet functions has not been copiously assessed. Thereby in the current study, an attempt has been made to evaluate the effect of crocin on oxidative stress-induced platelet apoptosis and aggregation. Crocin (MW. 976.96) is a natural dietary carotenoid found in the stigma of flowers of *Crocus sativus* L. (saffron) and fruits of *Gardenia jasminoides*. Chemically, it is a diester of disaccharide gentiobiose and the dicarboxylic acid crocetin (Fig. 2.1). It is the major active principle and colouring pigment of the widely used spice saffron. Saffron apart from its culinary usage has been used in many traditional systems of medicine including Indian, Chinese and Iranian to treat various disorders of the digestive system, muscle spasms, spleen and liver enlargement, problems associated with urinary tract and menstruation, CVDs and depression (Wani et al., 2011).

**Fig. 2.1**

![Structure of Crocin](image)

**Fig. 2.1 Structure of Crocin**
Studies emphasize that it is an excellent antioxidant with immense therapeutic potentials including tumoricidal, anticarcinogenic, antihyperlipidemic, and antidepressant properties (Chen et al., 2008; Abdullaev, 2002; Lee et al., 2005; Wang et al., 2010c). The current piece of work aims to project crocin as a novel antiplatelet phytochemical, which protects the platelets from oxidative stress-induced apoptosis and inhibits aggregation and thereby assert its role as a cardioprotective compound.

**Materials and Methods**

**Chemicals/ Reagents**

Rotenone, crocin, collagen (Type-I), benzamidine hydrochloride, N-acetyl-Leu-Glu-His-Asp trifluoromethylcoumarin (AC-LEHD-FMC), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), glutaraldehyde, sodium orthovanadate (Na$_3$VO$_4$), fluorescein isothiocyanate (FITC)-labeled annexin V, 5-(and-6)-chloromethyl-2’,7´-dichlorodihydrofluorescein diacetate acetyester (CM-H$_2$DCFDA), 5,5´-6,6´-tetrachloro-1,1´,3,3´-tetraethyl benzimidazolylcarbocyanine iodide (JC-1), leupeptin hydrochloride, N-(2-Hydroxyethyl)piperazine-N´-ethanesulfonic acid (HEPES), fura-2/AM, dithiothreitol (DTT), enhanced chemiluminescence (ECL) detection reagents and hyperfilm were from Sigma Chemicals, St. Luois (USA). Homovanillic acid (HVA) was from Sisco Research laboratories Pvt Ltd., Mumbai (India). Horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody and anti-cytochrome c antibody were purchased from Epitomics, Inc., Burlingame (USA). Collagen was from Vitrogen 100; Cohesion, Palo Alto, CA (USA). All other reagents were of analytical grade.
Preparation of Washed Platelets

Venous blood was drawn from healthy, drug-free human volunteers (non-smokers) with informed consent as per the guidelines of Institutional Human Ethical Committee (IHEC–UOM No. 72/Ph.D/2012-13), University of Mysore, Mysore. It was immediately mixed with acid citrate dextrose (ACD) anticoagulant (85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose) in the ratio 6:1 (blood : ACD v/v). The anti-coagulated whole blood was then centrifuged at 90×g for 15 min and the supernatant obtained was the platelet-rich plasma (PRP). The PRP was centrifuged at 1,700×g for 15 min at 37 °C. The platelet pellet thus obtained was suspended and incubated for 10 min. in Tyrode’s albumin buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na2HPO4, 1 mM MgCl2, 6 mM glucose, and 0.3 % bovine serum albumin) pH 6.5 and washed thereafter at 1,700×g for 15 min at 37 °C. The previous washing step was repeated one more time. Finally, the washed platelets were suspended in the Tyrode’s albumin buffer, pH 7.4. The cell count was determined in both PRP and washed platelet suspension using a Neubauer chamber and adjusted to required number of platelets in the final suspension using Tyrode’s albumin buffer (pH 7.4) (Kumar et al., 2011).

Determination of Endogenously Generated ROS

Endogenous ROS production in platelets was determined according to the method of Lopez et al. (2007), with slight modifications using CMH2DCFDA, a ROS-sensitive fluorescent probe. PRP as well as washed platelet suspension were taken separately in polystyrene 96-well microtiter plates and treated with collagen/A23187 (10 µg/mL), final volume was made up to 200 µL with HEPES-buffered saline (HBS), pH 7.45, containing 145 mM NaCl, 10 mM HEPES, 10 mM
D-glucose, 5 mM KCl, 1 mM MgSO₄ and supplemented with 0.1% Bovine Serum Albumin (BSA) incubated at 37 °C for 1 h. For inhibition studies, platelets were pre-incubated with different doses of crocin (dissolved in HBS) for 10 min. at 37 °C prior to collagen treatment. The control (untreated) and treated platelets were then incubated with 10 μM CMH2DCFDA for 30 min at 37 °C, fluorescence was recorded using a Varioskan multimode plate reader (Thermo Scientifics, USA.) by exciting the samples at 488 nm and measuring the resulting fluorescence at 530 nm.

**Determination of Endogenously Generated H₂O₂**

HVA, a specific H₂O₂-sensitive fluorescent probe was used to detect the endogenously generated H₂O₂. With slight modifications to the method of Barja (2002), briefly, pre-treated (as described in the previous section) and control PRP and washed platelets were incubated with 100 μM HVA for 30 min at 37 °C, centrifuged and the pellets were suspended in HBS, fluorescence was recorded using a multimode plate reader by exciting the samples at 312 nm and measuring the resulting fluorescence at 420 nm.

**Estimation of Intracellular Calcium**

Intracellular Ca²⁺ concentration was measured in PRP and washed platelets as described previously (Asai et al., 2008). Both PRP and washed platelets, taken in 96-well polystyrene microtiter plates were treated with H₂O₂ (2 mM) and the final volume made up to 200 μL with modified Tyrode’s solution (pH 7.4) containing NaCl (150 mM), KCl (2.7 mM), KH₂PO₄ (1.2 mM), MgSO₄ (1.2 mM), CaCl₂ (1.0 mM), and HEPES (10 mM) with 1% BSA and incubated for 1 h at 37 °C to induce the release of Ca²⁺ from the intracellular Ca²⁺ stores. Inhibition studies with crocin were done as described in the previous section. The platelets were then incubated for 45
min at room temperature with 2 μM fura-2/AM, a fluorescence Ca\(^{2+}\) indicator. The cells were subsequently washed twice with the modified Tyrode’s solution to remove the dye from the extracellular fluid and finally the platelet pellet suspended in modified Tyrode’s solution. The fura-2/AM absorption was determined by exciting the cells at 340 and 380 nm and the resulting fluorescence was measured at 500 nm. Data were presented as absorption ratios (340/380 nm).

**Determination of Changes in Mitochondrial Membrane Potential (ΔΨ\(_m\))**

The cationic dye JC-1 was used to detect changes in the ΔΨ\(_m\) according to the method of Salvioli et al. (1997). JC-1 accumulates in mitochondria as red fluorescent aggregates at high membrane potentials, whereas, it exists as a green fluorescent monomeric form at low membrane potential. Treated (as described in the previous section) and control PRP/washed platelets were loaded with 10μg/mL JC-1 at 37 °C for 10 min. The cells were then excited at 488 nm and emission was detected at 585 nm for JC-1 aggregates and 516 nm for JC-1 monomers using multimode plate reader. Data were presented as emission ratios (585/516). Agonist-induced changes in ΔΨ\(_m\) were quantified as the integral of the decrease in JC-1 fluorescence ratio.

**Preparation of Platelet Lysate**

Platelet lysate was prepared by immediately adding an equal volume of 2X Triton buffer (2% TritonX-100, 2mM EGTA, 100 mM Tris/HCl - pH 7.2, 100 μg/mL leupeptin, 2 mM PMSF, 10 mM benzamidine, 2 mM Na\(_3\)VO\(_4\)) to the treated and control platelets from PRP and washed platelet suspension and allowed to undergo lysis for 30 min at 4 °C. The lysate was centrifuged at 16000×g for 5min. The pellet thus obtained is the cytoskeleton-rich (triton-insoluble) fraction, which was subjected to caspase activity and western blotting (Rosado et al., 2000).
Detection of Cytochrome c Release

Cytochrome c release was detected by western blotting samples from cytosolic fractions of washed platelets (Lopez et al., 2006). Platelets were pre-treated with different concentrations of crocin (0-100 µg/mL) for 10 min and then stimulated with H$_2$O$_2$ (2 mM) for 1 h at 37 °C. Cytosolic proteins were separated by 10% SDS-PAGE and electrophoretically transferred on to a nitrocellulose membrane for 1 h at 50 V using a wet blotter. Blots were then incubated overnight with 10% BSA in tris-buffered saline with 0.1% tween 20 (TBST) to block residual protein binding sites. Membranes were incubated with anticytochrome c antibody (1:1000) in TBST for 2 h. Blots were incubated with horseradish-peroxidase (HRP)-conjugated anti IgG antibody (1:10,000) in TBST and exposed to enhanced chemiluminescence for 3 min. Finally the blots were exposed to photographic films.

Assay of Caspase Activity

Caspase activity was determined by incubating cell lysate in a microtiter plate with substrate solution (20mM HEPES, pH-7.4, 2mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 µM caspase substrate (AC-DEVD-AMC for caspase 3 and AC-LEHD-AFC for caspase 9) for 2 h at 37 °C (Amor et al., 2006). Substrate cleavage was measured with a multimode plate reader (excitation wavelength 360 nm and emission at 460 nm).

Determination of Phosphatidylserine Externalization

Samples of treated and control PRP/washed platelets were transferred to equal volume of ice-cold 1% (w/v) glutaraldehyde in HBS for 10 min, and then incubated for 10 min with annexin V fluorescein isothiocyanate (0.6 µg/mL) in HBS. The cells
were collected by centrifugation for 60 s at 3000×g and resuspended in HBS. Cell staining was measured in a multimode plate reader by exciting the samples at 496 nm and emission was recorded at 560 nm (Rosado et al., 2006).

**Platelet Aggregation**

Platelet aggregation was determined by turbidimetric method (Kumar et al., 2011) with a dual channel Chrono-log model 700-2 aggregometer (Havertown, USA). Briefly, 240 μL of PRP was incubated at 37 °C in a siliconized glass cuvette and pre-incubated with different concentrations of crocin (0-200 µg/mL) in 5 μL HBS for 3 min, and the aggregation was initiated by the addition of collagen (10 µg/mL)/ ADP (10 µM)/ epinephrine (5 µM). The aggregation was then followed with constant stirring at 900 rpm for 6 min. Aggregation induced by collagen/ ADP/ epinephrine alone was considered as 100% aggregation.

**Platelet Adhesion Assay**

Platelet adhesion assay was carried out according to the method of Bellavite et al. (1994). Briefly, collagen was immobilized on to 96-well polystyrene microtiter plates by adding 20 µg of collagen type I in 200 µl phosphate buffered saline (PBS) to each of the wells and left overnight at 4 °C. Following which, 200 µl of 1% (w/v) BSA in PBS was added to block the wells and incubated at 37 °C for 1 h. The wells were then washed three times with PBS. In the first set of experiments, crocin (10-200 µg/mL) was directly added to the collagen-coated wells, pre-incubated for 10 min and then PRP was added. In the second set of experiments, PRP pre-treated with crocin (10-200 µg/mL) for 10 min was added to the collagen-coated wells. Total reaction volume was made up to 200 µL. The reaction mixture was incubated at 37 °C for 90 min, and then washed three times with PBS. The adherent platelets were then lysed
with 150 μl lysis buffer (0.1 M citrate buffer pH 5.4 containing 5 mM p-nitrophenyl phosphate and 0.1% Triton X-100) by incubating at 37 °C for 90 min. The reaction was terminated by inactivating the platelet membrane acid phosphatase activity with the addition of 100 μL stopping reagent (2 N NaOH). The colour developed was measured at 405 nm. Platelet adhesion was expressed as percent adhesion, considering PBS-treated platelet suspension as 100%.

Statistical Analysis

Results were expressed as mean ± SEM of five independent experiments. Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means. Here, data were shown as mean ± SEM (n = 5), p<0.05 (*).

Results

Effect of Crocin on ROS and H₂O₂

Crocin was firstly evaluated for its efficacy on collagen/A23187-induced endogenous generation of ROS, H₂O₂ in platelets. Treatment of PRP with collagen induced 2.25 fold and 1.61 fold increase in the generation of ROS and H₂O₂ respectively as compared to the control. Treatment of washed platelets with A23187 induced 1.6 fold and 2.36 fold increase in the generation of ROS and H₂O₂ respectively as compared to the control. Inhibition studies with crocin at different concentration ratios of 1:0.5, 1:1, 1:1.5, 1:2 (Collagen: Crocin; w/w), significantly reduced both collagen-evoked ROS as well H₂O₂ generation in a concentration-dependent manner in PRP. Similarly, crocin at different concentration ratios of 1:0.25, 1:0.5, 1:0.75 1:1, 1:2 (A23187: Crocin; w/w), significantly reduced both A23187-
evoked ROS as well as $H_2O_2$ generation in a concentration-dependent manner in washed platelets. At the ratio 1:1 there was complete inhibition of ROS and at the ratio 1:2 there was 98% inhibition of $H_2O_2$ generation in both PRP as well as washed platelets (Fig. 2.2 A and B).

**Effect of Crocin on $H_2O_2$-Induced Generation of Intracellular Calcium**

Treatment with 10 µg/mL A23187 and 2 mM $H_2O_2$ respectively resulted in 1.5 and 1.4 folds increase in intracellular $Ca^{2+}$ in PRP, whereas in washed platelets it resulted in 1.5 and 2.26 folds increase respectively. Inhibition studies with crocin in the concentration range 0-50 µg/mL resulted in the dose-dependent amelioration in $Ca^{2+}$ generation. The $Ca^{2+}$ concentration was restored to basal level in A23187- and $H_2O_2$-treated PRP at crocin concentration 25 and 50 µg/mL respectively. In the case of washed platelets inhibition studies were carried out at concentration range 0-150 µg/mL of crocin. The $Ca^{2+}$ concentration was restored to 80 and 87% of the basal level in A23187- and $H_2O_2$-treated PRP at crocin concentration 100 and 150 µg/mL respectively (Fig. 2.3 A and B)

**Effect of Crocin on $H_2O_2$-Induced Activation of Caspases in Platelets**

Caspase activation was analyzed using two specific fluorescent substrates AC-DEVD-AMC and AC-LEHD-AFC for caspase 3 and 9 respectively. Treatment of platelets with 2 mM $H_2O_2$ induced the activation of caspase 3 by 1.3 and 1.6 folds in PRP and washed platelets respectively. Activation of caspase 9 by $H_2O_2$ was to a greater extent than that of caspase 3, reaching a maximal activity after 2 h of stimulation with 1.8 and 2.0 folds increase respectively in PRP and washed platelets. Pre-treatment of platelets for with crocin in the concentration range 25-100 µg/mL significantly reduced $H_2O_2$-evoked activation of caspase 3 and 9 in a concentration-
dependent manner. At the concentration 100 µg/mL, there was almost complete neutralization of both caspase 9 and 3 (Fig. 2.4 A and B).

**Crocin Diminishes the Effect of H$_2$O$_2$-Induced Mitochondrial Membrane Potential ($\Delta\Psi_m$) Depolarization and PS Scrambling**

Mitochondrial membrane depolarization was detected by the decrease in JC-1 fluorescence ratio (585/516 nm). Treatment with 10 µM rotenone induced maximal decrease in JC-1 fluorescence ratio and was considered as 100% $\Delta\Psi_m$ dissipation. Treatment with 2 mM H$_2$O$_2$ resulted in 87 and 62% $\Delta\Psi_m$ dissipation in PRP and washed platelets as compared to that of rotenone. Pre-treatment with crocin at 0-100 µg/mL induced a dose-dependent restoration of membrane potential. At 100 µg/mL concentration, crocin was able to restore H$_2$O$_2$-induced changes in $\Delta\Psi_m$ to 96% in PRP as compared to control. In the case of washed platelets, 50 µg/mL crocin was able to restore the $\Delta\Psi_m$ up to 9% (Fig. 2.5).

As shown in Fig. 2.6, treatment of platelets with 2 mM H$_2$O$_2$ induced a 1.6 and 2.15 fold increases in PS exposure in PRP and washed platelets respectively when compared to control. Pre-treatment of platelets with crocin at concentrations between 0-200 µg/mL significantly reduced H$_2$O$_2$-evoked PS externalization in a concentration-dependent manner and at the concentration 100 and 200 µg/mL, crocin completely abolished the PS externalization in PRP and washed platelets respectively.

**Effect of Crocin on H$_2$O$_2$-Induced Cytochrome c Release**

Treatment with 2 mM H$_2$O$_2$ for 1 h at 37 °C induces cytochrome c release into the cytosol that was detected by Western blotting technique. However, pre-treatment with crocin at concentrations of 50 and 100 µg/mL inhibited the cytochrome c release.
by the platelets in a concentration-dependent fashion. At the 100 µg/mL crocin, there was almost complete inhibition of cytochrome c expression (Fig. 2.7).

**Effect of Crocin on Collagen-Induced Platelet Aggregation and Adhesion**

Pre-treatment of PRP with crocin at different concentrations prior to collagen treatment resulted in a dose-dependent inhibition of platelet aggregation. Crocin at a concentration 25 µg/mL was able to inhibit aggregation by 97%, and at higher concentrations there was 100% inhibition (Fig. 2.8 A and B). However, it was not able to inhibit the aggregation induced by ADP and epinephrine (data not shown). On the other hand crocin was able to prevent the binding of platelets to collagen in a dose-dependent manner. In the case of crocin pre-treated collagen coated well, the platelet adhesion was brought down to 45 ± 5% and in the case of crocin pre-treated PRP, the adhesion was brought down to 65% as against 100% adhesion with PBS-treated PRP (Fig. 2.8 C).

**Discussion**

In view of the current trend of people choosing plant-based therapeutics for treatment of various ailments including CVDs, the present study investigated the influence of crocin on human platelets. Crocin, being a natural antioxidant, has been claimed to have remarkable curative properties. It is the pigment responsible for the coloration of saffron, which by itself is used in many traditional systems of medicine including Chinese and Iranian. In the traditional Ayurveda system of medicine, it is used as a medication to improve blood circulation and libido and also used as a supplement in the diet of pregnant women. Therefore, an attempt has been made to probe the effect of crocin on oxidative stress-induced platelet apoptosis as well as, platelet aggregation. The study was based on the fact that one of the causes for
platelet apoptosis is oxidative stress induced by free radicals and ROS such as $\text{H}_2\text{O}_2$ as evidenced by recent studies and that crocin being a potential antioxidant, a fact validated by several independent studies, may possibly attenuate the free radical-induced platelet apoptosis. Since platelets are anuclear, the effects of crocin on the cytoplasmic events of programmed cell death, which are the parameters for platelet apoptosis, were evaluated.

In the first set of experiments, collagen/A23187 was used to induce the endogenous generation of ROS in platelets. When the platelets were pre-treated with crocin prior to collagen/ A23187 treatment; there was impairment in the ROS generation in a dose-dependent manner. According to the study by Lopez et al. (2007), $\text{H}_2\text{O}_2$ in particular triggers the events of apoptosis in platelets through the intrinsic or mitochondrial pathway by altering $\Delta\Psi_m$. Therefore the endogenous generation of $\text{H}_2\text{O}_2$ was also measured, where in crocin-pre-treated platelets upon collagen/A23187 treatment exhibited a dose-dependent decrease in $\text{H}_2\text{O}_2$ generation. Carotenoids including crocin have long been investigated for their antioxidant functions, which have been shown to involve radical scavenging, quenching, and enzyme-inhibiting actions. Thereby, the results of the present work are in tune with the fact that crocin is an excellent antioxidant and further validate this fact. In addition, several existing studies suggest that the endothelial cell dysfunction is associated with the pathogenesis of atherosclerosis. Vascular endothelial cells are highly sensitive to ROS, because they are in close contact with the constituents of blood (e.g., leucocytes) and activated leucocytes release ROS including $\text{H}_2\text{O}_2$ for biophylaxis, which damage endothelial cells. This in turn facilitates adhesion of platelets and other blood cells to the vascular wall, which is considered as an initial cause of atherogenesis. In a recent study, it was found that crocin could prevent
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**Crocin Inhibits Platelet Apoptosis and Aggregation**

endothelial cells from apoptosis induced by H$_2$O$_2$, which suggested that treatment of atherosclerosis by crocin might correlate with its antiapoptotic effect (Xu et al., 2006). For further experiments H$_2$O$_2$ was used as the agonist. Rotenone was used as standard agonist to induce changes in $\Delta \Psi m$. Crocin could effectively prevent H$_2$O$_2$-induced changes in $\Delta \Psi m$ and restore the membrane potential up to 79% of the original membrane potential in the case of PRP. While in washed platelets, it could almost completely restore the $\Delta \Psi m$. Mitochondrial membrane depolarization is the initial step of the intrinsic/mitochondrial pathway of apoptosis and hence is the most crucial one (Leytin et al., 2009). From the result it can be stated that crocin has the capacity to inhibit H$_2$O$_2$-induced intrinsic pathway of platelet apoptosis. To further ascertain the efficacy of crocin in protecting $\Delta \Psi m$, its effect on intracellular Ca$^{2+}$ was investigated. The presence of high concentration of Ca$^{2+}$ in the cytosol is one of the factors responsible for the changes in $\Delta \Psi m$ and formation of MPTP. Yet again crocin could inhibit the increase in intracellular Ca$^{2+}$. In support, further experiments were carried out, which included the protein expression levels of cytosolic cytochrome c. Leakage of mitochondrial membrane cytochrome c following the membrane depolarization is the next event of intrinsic apoptotic pathway. Crocin was able to dose-dependently hinder cytochrome c expression. Release of apoptogenic cytochrome c from mitochondrial intermembrane space to the cytosol due to formation of a channel MPTP, in the outer mitochondrial membrane, serves a regulatory function as it precedes morphological changes associated with apoptosis. Once cytochrome c is released it binds with apoptotic protease activating factor-1 (Apaf-1) and ATP, which then bind to procaspase 9 to create a protein complex known as apoptosome. The apoptosome cleaves the procaspase to its active form.
caspase 9, which in turn activates the effector caspase 3. Hence cytochrome c release can be regarded as a pivotal event in the intrinsic pathway of apoptosis.

Caspases are a group of cysteine-dependent aspartate-directed proteases that cleave proteins at aspartic acid residues and play a vital role in apoptosis, inflammation and cell necrosis. Generally caspases are expressed in an inactive proenzyme form known as procaspases and once activated, can activate other procaspases, allowing initiation of a protease cascade. The proteolytic cascade amplifies the apoptotic signalling pathway and thus leads to rapid cell death. There are ten major caspases, which are classified into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5). Crocin was able to impair the H$_2$O$_2$-induced activation of both the caspases 9 and 3 in a concentration-dependent manner. These findings further confirm the antiapoptotic effects of crocin in platelets. Consistent with this, it was shown that H$_2$O$_2$ was able to induce PS exposure, another biochemical feature of apoptosis (Schoenwaelder et al., 2009). The expression of cell surface markers results in the early phagocytic recognition of apoptotic cells, permitting quick phagocytosis. This event is achieved by the movement of the normal inward-facing PS of the cell’s lipid bilayer to expression on the outer layers of the plasma membrane. H$_2$O$_2$-induced PS externalization was clearly abrogated by crocin treatment. All these results firmly highlight the antiapoptotic effects of crocin on human platelets.

Platelets undergoing apoptosis expose negatively charged PS on their surface and also release PS-positive membrane fractions called microparticles, both of which cause fibrin deposition by providing competent surfaces for the assembly of coagulation factors and thrombus formation. They have a potent pro-inflammatory
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effect, promote coagulation and affect vascular function. These processes are all involved in the development of CVDs. Circulating MP numbers are also altered in many CVDs. Hence, MPs play a major role in the pathogenesis of CVDs including transient ischemia and MI, and post surgery complications in cardiopulmonary bypass patients. There are numerous studies, which signify the protective role of crocin against CVDs such as the anti-atherosclerotic effects of crocin through decreasing the Ox-LDL levels, the cardioprotective role of crocin due to its ability to maintain redox potential and thus prevent atherosclerosis, inflammation, cardiotoxicity, and its hypotensive, anti-diabetic effects (He et al., 2005; Nam et al., 2010; Goyal et al., 2010; Imenshahidi et al., 2010; Mousavi et al., 2010). Also, a recent study claims it to be a potential therapeutic candidate in the treatment of cerebral ischemia (Zheng et al., 2007). The current study suggests another possible mechanism through which this compound can be considered as a therapeutic constituent in the treatment of CVDs, i.e., crocin through its antiapoptotic effect on platelets can possibly reduce the extent of generation of MPs and thus prevent CVDs. Moreover, recent study by Hemshekhar et al. (2012a) demonstrated that crocin by reducing oxidative stress and inflammation in arthritic rats, acts as a potential anti-arthritic molecule. Arthritis is accompanied with reduction in platelet count and a concurrent increase in the PMPs in the synovial joints resulting in the aggravation of inflammation. Therefore it can be articulated that crocin by blocking platelet apoptosis controls the production of MPs and consequently reduces inflammation at the joints.

If platelet apoptosis exceeds beyond the threshold level the platelet count may drop rapidly leading to a condition called thrombocytopenia. This condition is presumed to be serious if the platelet count is 50,000/μL as against the normal count of 150,000 to 450,000 platelets/μL of blood. The adverse effects of thrombocytopenia
include spontaneous bleeding from organs and delay in the normal process of clotting. In this context it is worth mentioning the beneficial impact of crocin in reversing the apoptosis-induced drop in platelet count. Though there are several studies on platelet apoptosis and its causes, there is only one report of a phytochemical exhibiting antiapoptotic effects in human platelets i.e., cinnamomannin B-1 from bay wood extract (Bouaziz et al., 2007). To further highlight crocin’s role as a cardio-protective compound, its influence on platelet aggregation was scrutinized. Platelets are deemed to play a critical role in the development of atherothrombotic disease. During vascular injury, they initiate the formation of hemostasis plug and mediate pathophysiological thrombosis, which in turn promulgates a multiplicity of CVDs. Platelet adherence and aggregation are involved in the instigation of intraluminal thrombosis and thus hasten MI, peripheral vascular occlusions and stroke. Therefore, platelet aggregation plays a critical role in the propagation of CVDs (Ueno et al., 2011). The results obtained firmly suggest that it has the ability to mitigate collagen-induced platelet aggregation. The inhibitory effect of crocin on aggregation was probably due to its interaction with collagen, which was verified with the platelet adhesion assay. In the case of crocin pre-treated collagen-coated wells, the inhibition of platelet adhesion was better compared to crocin pre-treated PRP directly added to collagen-coated wells. This was probably due to the existence of an interaction between crocin and collagen. According to a study by Hadley et al. (1998), glycation of collagen alters the charge distribution and influences the quaternary structure as well as the interaction of the glycated collagen with other proteins. Thus, it has been hypothesized that the disaccharide gentiobiose present in crocin may cause glycation of collagen and thereby prevents its interaction with collagen receptors present on the platelets.
Conclusion

In conclusion it can be articulated that crocin is a promising molecule, which can be implemented in the treatment strategy for CVDs as it has been clearly demonstrated in the current study. It exerts excellent antiapoptotic effects on oxidative stress-induced platelets, besides inhibiting platelet aggregation. It has the ability to retard the various events of the intrinsic pathway probably mediated through its antioxidant activity (Fig. 2.9). Although crocin has been shown to have proapoptotic effects on tumour cells and many reports claim crocin to be a promising cancer therapeutic agent, the present study signifies its antiapoptotic effects in normal cells (Mousavi et al., 2011). This study has further scope because crocin’s effects on the extrinsic pathway of apoptosis in platelets. Thus crocin, a component of saffron used in various traditional system of medicine, appears to be a potential molecule and nature’s answer to combat a host of modern life style- and stress- associated ailments such as CVDs.
Fig. 2.2 Effect of crocin on endogenous generation of ROS and H₂O₂: (A) Crocin inhibits collagen (10 μg/mL)-induced endogenous generation of ROS and H₂O₂ in PRP. (B) Crocin inhibits A23187 (10 μg/mL)-induced endogenous generation of ROS and H₂O₂ in washed platelets. Values are presented as means ± SEM (n = 5), expressed as fold increase in DCF fluorescence (for ROS) and HVA fluorescence (for H₂O₂) relative to control. a*: significant compared to control; b*: significant compared to collagen/A23187-treated (p<0.05).
Fig. 2.3 Effect of crocin on intracellular calcium release: crocin dose-dependently ameliorates H$_2$O$_2$ (2mM)- and A23187 (10 μg/mL)-induced increase in intracellular Ca$^{2+}$ in PRP (A) and in washed platelets (B). Values are presented as means ± SEM (n = 5), expressed as percentage increase in fura-2/AM fluorescence relative to control. a*: significant compared to control; b*: significant compared to H$_2$O$_2$/A23187-treated (p<0.05).
Fig. 2.4 Effect of crocin on caspase activity: crocin concentration-dependently abrogates H$_2$O$_2$ (2 mM)-induced caspases 9 and 3 activation in PRP (A) and in washed platelets (B). Values are presented as means ± SEM (n = 5), expressed as fold increase caspase activity relative to control. a*: significant compared to control; b*: significant compared to H$_2$O$_2$-treated (p<0.05).
Fig. 2.5: Effect of crocin on mitochondrial membrane potential: Crocin dose-dependently restores of H₂O₂ (2 mM)-induced changes in ΔΨₘ in PRP (A) and in washed platelets (B). Values are presented as means ± SEM (n = 5), expressed as percentage increase (JC-1) fluorescence relative to ΔΨₘ depolarization induced by 10 µM rotenone (considered as 100%). a*: significant compared to control; b*: significant compared to H₂O₂-treated (p<0.05).
Fig. 2.6 Effect of crocin on phosphatidylserine externalization: Crocin concentration-dependently inhibits H$_2$O$_2$ (2 mM)-induced PS externalization in PRP (dashed line) and in washed platelets (solid line). Values are presented as means ± SEM ($n=5$), expressed as percentage increase in annexin V- FITC fluorescence. a*: significant compared to control; b*: significant compared to H$_2$O$_2$-treated ($p<0.05$).
Fig. 2.7

**Fig. 2.7 Immunoblot of the effect of crocin on the expression of cytosolic cytochrome c:** Platelets were pre-treated with different concentrations of crocin (0–100 µg/mL) for 10 min and then stimulated with H$_2$O$_2$ (2 mM) for 1 h at 37 °C. The cytosolic proteins were separated by 10% SDS-PAGE and transferred on to a nitrocellulose membrane. Membranes were incubated with anticytochrome c antibody (1:1000) in TBST for 2 h followed by horseradish-peroxidase (HRP)-conjugated anti IgG antibody (1:10,000) in TBST and exposed to enhanced chemiluminescence. Lane 1 represents control platelets (untreated). Lane-2 represents 2mM H$_2$O$_2$-treated platelets. Lanes -3, -4 and -5 represent platelets pre-treated with 50, 75 and 100 µg/mL of crocin respectively and then treated with H$_2$O$_2$. Histograms represent the expression levels of cytochrome c in respective groups. a*: significant compared to control; b*: significant compared to collagen-treated (p< 0.05).
Fig. 2.8 Effect of crocin on collagen-induced platelet aggregation and adhesion:

(A) Concentration-dependent inhibition of collagen (2 μg/mL) - induced platelet aggregation by crocin in PRP: (i) control (collagen alone), (ii) crocin 2 μg/mL, (iii) 10 μg/mL, (iv) 25 μg/mL. (B) Graphical representation of the above data showing the percentage platelet aggregation. (C) Effect of platelet adhesion on the immobilized collagen type 1 with crocin pre-treated collagen and crocin pre-treated PRP. Values are presented as means ± SEM (n = 5), expressed as percentage increase in platelet adhesion. a*: significant compared to control.
Fig. 2.9 The graphical abstract depicts the protective effect of crocin on platelets:

Crocin protects platelets from oxidative stress, thus preventing them from undergoing apoptosis; it also inhibits agonist-induced platelet aggregation; and therefore crocin has the potential to be developed as an effective anti-platelet drug for CVDs.