# Modulation of Platelet Function and Signaling by Flavonoids

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**Abstract:** Blood platelets play a crucial role in the primary hemostasis and vessel wall repair. However; platelet hyperactivation is implicated in the pathogenesis of cardiovascular diseases such as thrombosis, atherosclerosis and stroke. Epidemiological data have suggested that regular consumption of fruits and vegetables, which are rich in flavonoids, is associated to a reduction in cardiovascular events. The cardioprotective effect of flavonoids is partly due to the inhibition of platelet function. However; the mechanisms underlying the anti-platelet effect of these compounds remain unclear. The aim of this review is to discuss the role of platelets in cardiovascular disease and to provide an overview of the potential anti-platelet effect of flavonoids, focusing on the various platelet signaling pathways modulated by flavonoids, including oxidative stress, protein tyrosine phosphorylation, calcium mobilization and nitric oxide pathway. The understanding of these mechanisms will be helpful in the development of new anti-platelet agents based on flavonoids with fewer or no adverse effects.

**Keywords:** Platelets, flavonoids, cardiovascular diseases, nitric oxide, oxidative stress, tyrosine phosphorylation, calcium mobilization.

## INTRODUCTION

It is known that platelet hyperactivation which is associated to many cardiovascular risk factors (hypertension, stress, hypercholesterolemia, smoking, diabetes mellitus, etc.) plays a crucial role in the development and progression of cardiovascular diseases (CVD) including myocardial infarction, cerebrovascular diseases, atherosclerosis, peripheral artery diseases and stroke [1]. Prevention of these disorders requires the use of antiplatelet agents [2], such as aspirin [3], ticlopidine, clopidogrel or prasugrel, which block different platelet signaling pathways. However, these agents induce different undesirable effects, such as risk of bleeding and gastric ulcers. Given the importance of platelet hyperactivation in the pathogenesis of CVD, many researches worldwide are focusing on the development of new and more effective agents of different origins that could inhibit platelet function and therefore reduce the risk of CVD. Recently, studies concerning prevention and/or treatment of CVD have been focused on natural substances from plants. Flavonoids, a subclass of polyphenols, are widely distributed in vegetables and fruits [4]. These compounds play an important role in several plant functions such as growth, reproduction and protection from pathogen and predators [5]. They are also responsible for the flavor, pigmentation, and the attractive color of flowers, fruits, and leaves [4, 6]. Medicinal plants containing flavonoids have historically been used in traditional medicine to treat various diseases. Epidemiological reports suggest that individuals who consume large amounts of fruits and vegetables have lower rates of CVD, and the beneficial effect of these foods has been attributed to their elevated flavonoid levels [7-9]. Flavonoids are thought to reduce CVD by a variety of mechanisms including, reduction of low-density lipoprotein (LDL) oxidation [10-11], improvement of endothelium relaxation [12-13], modulation of the inflammatory process [14-15], and inhibition of platelet function [16]. Several others biological effects of flavonoids have been reported including antihypertensive effect [17], endothelium-dependent vasodilation action [13], and antioxidant properties [18]. In platelets, flavonoids have been shown to exert inhibitory effects *in vitro* [19-20] and *in vivo* [21-22]. It has been reported that flavonoids inhibit platelet adhesion, aggregation, secretion and recruitment [23-24]. However; the mechanisms underlying these actions in platelets are not fully understood.

In this review article we will discuss the role of platelets in the development of cardiovascular disorders and summarize the potential anti-platelet effect of flavonoids, focusing on the various platelet signaling pathways modulated by flavonoids, which may be helpful in developing new strategies to reduce and/or to treat cardiovascular events.

## EVIDENCE THAT PLATELETS ARE ACTIVATED IN CARDIOVASCULAR DISEASES

According to the world heath organization report for 2005, about 17,5 million people die due to CVD each year worldwide, and this figure will increase to 23,6 millions people by 2030. This dramatic situation depends on different risk factors including stress, diet and lifestyle. It was reported that platelet hyperactivation, which is associated to different pathological situations, such as hypertension, diabetes mellitus and vascular diseases [25-28], plays an important role in the development of thrombosis, and the incidence of cardiovascular disorders [29]. Several factors might induce platelet hyperactivation including stress, hypertension,

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diabetes, hypercholesteromia, and smoking (Fig. **1**). In general, platelet activation occurs after endothelium damage, atherosclerotic plaque rupture or stimulation with platelet agonists [30-31]. Upon activation, platelets change shape, express fibrinogen receptors and release vasoactive and pro-aggregatory agents including ADP, serotonin and thromboxane $A_2$ ($TxA_2$) among others, which further enhance platelet activation. The expression of the glycoprotein IIb-IIIa complexes on the platelet membrane increases platelet aggregation and thrombus formation [32]. It has been reported that in diabetic and hypertensive patients, platelets are more sensitive to agonists and showed increased spontaneous platelet aggregation [33-35] which promotes thrombus formation. The platelet abnormalities described in CVD may be explained by increased production of vasoactive and pro-aggregatory agents and/or reduced formation of anti-aggregatory substances (prostacyclin ($PGI_2$) or NO), resulting in an enhanced platelet activation and aggregation. In addition, it has been shown that enhanced platelet activation is associated with increased levels of platelet-derived microparticles with procoagulant properties [36-38]. Furthermore, CVD are associated to oxidative stress and increased generation of reactive oxygen species (ROS) including $H_2O_2$ and superoxide anion ($O_2^-$) [27-28], which may further enhance platelet activation [39-43] leading to cardiovascular complications.

## FLAVONOIDS

### A. Chemical Structure, Distribution and Absorption

Flavonoids are polyphenolic compounds that are produced by the secondary metabolism of plants. They are found in medicinal plants and many vegetables and fruits including onions, apples, broccoli, tea, grapes and berries [4]. Flavonoids have been known for their beneficial effects on health long before they were isolated. Thus, in 1930, a new substance was extracted from oranges and was designed as vitamin P before researches demonstrated that it was a flavonoid (rutin). The beneficial properties of flavonoids have then stimulated researches in an attempt to isolate new compounds and to study their pharmacological effects. Flavonoids are found either as aglycones or as glycoside conjugates, and the content and the nature of flavonoids vary largely between plant species and varieties of the same species [5]. Their basal structure consists of 15 carbon atoms with two terminal aromatic rings linked through an oxygenated heterocycle ($C_6$-$C_3$-$C_6$) [44]. Over than 6000 varieties of flavonoids have been identified in plant sources [4], and based on their molecular structure, flavonoids can be divided into six main classes (flavanols, flavanones, flavones, isoflavones, flavonols and anthocyanidins) [44-46] (Fig. **2**). The flavonol quercetin and the flavone apigenin are found in many fruits and vegetables such as onions, apples, broccoli, berries and tea. The flavanone naringenin is found in citrus, and catechins are abundants in green tea, whereas anthocyanidins are found in berries, strawberries, grapes, and tea. Genistein is an isoflavone found principally in legumes. Data on the absorption, metabolism, and excretion of flavonoids are contradictory, and the form of flavonoid seems to influence its rate of absorption and metabolism [47-48]. In fact, flavonoids aglycones are passively absorbed in the small intestine; however glycosides require degradation by intestinal and colonic microflora [48]. Other reports have shown that the glycosylated form of quercetin is absorbed more

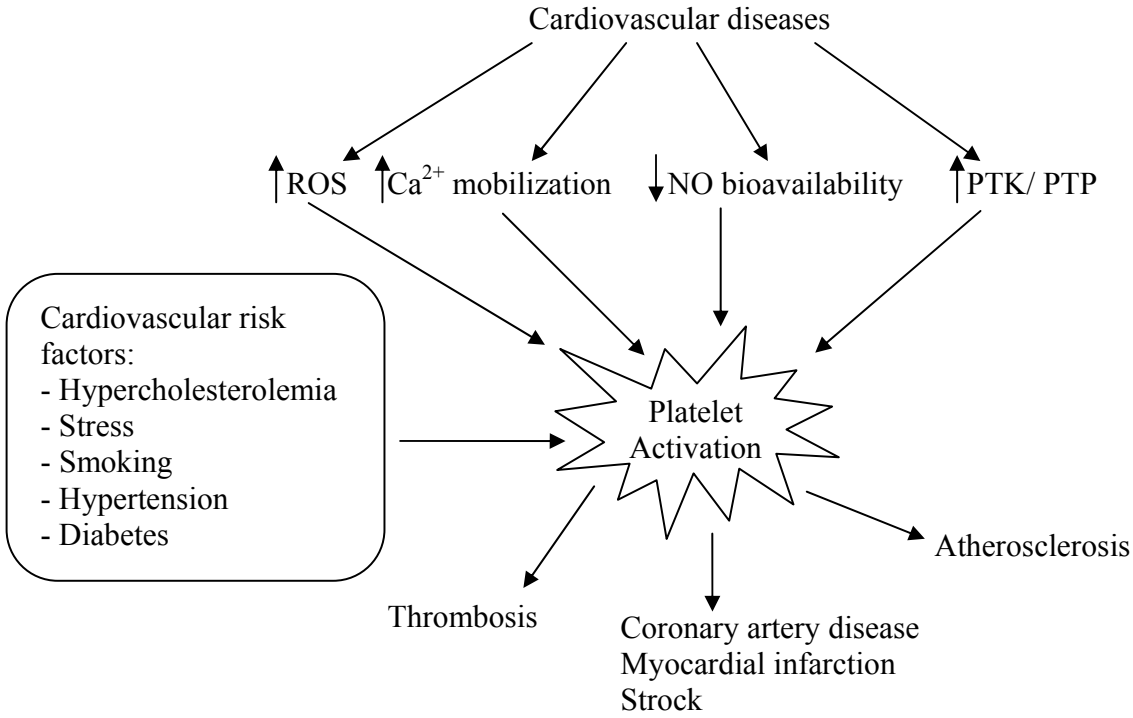


**Fig. (1).** Mechanisms of platelet activation in cardiovascular diseases. ROS: Reactive oxygen species; NO: Nitric oxide; $Ca^{2+}$: Ion calcium; PTK: Protein tyrosine kinase; PTP: Protein tyrosine phosphatase.

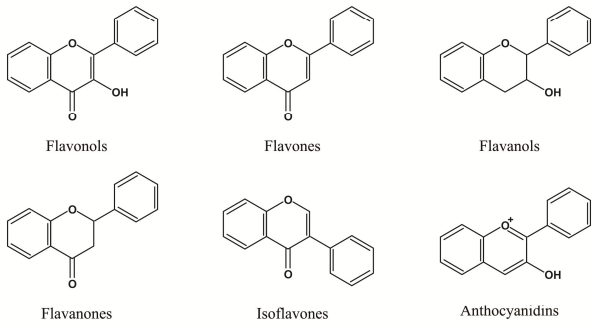


**Fig. (2).** Chemical structures of the main classes of flavonoids.

readily than its aglycone form [49]. However; catechin which is not a glycosylated flavonoid, is absorbed more efficiently [50]. In addition, Koleckar *et al.* [51] have shown that glycosylation of flavonoids decreased their antiplatelet activity. Conjugation of flavonoids plays also an important role in their activities. It implies for example the addition of a methyl group and/or a sulfate group on the flavonoid skeleton, which increases its circulatory elimination and decreases its toxicity. Moreover, conjugation of flavonoids increased their half-lives (23-28h) [52] which further their accumulation resulting in sufficient active flavonoid concentrations. After absorption, flavonoids are subjected to several transformations in the liver [47-48, 53]. Cellular activities of flavonoids are also influenced by their structures such as the number of hydroxyl groups, degree of insaturation or glycosylation. Indeed, flavonoid glycosides have generally no or little effect on platelet function [54], whereas, flavonoids aglycones suppress platelet activation [55].

## B. General Properties

Flavonoids are well known by their role in the prevention from coronary heart diseases, and several studies have been carried out on the pharmacological effects of these compounds [13, 17, 56-59]. Epidemiological studies suggest a cardioprotective role of a diet rich in fruits and vegetables [60-61]. The beneficial effect of fruits and vegetables may be attributed to their flavonoid content [62]. In fact, the lower mortality rates from CVD in Mediterranean countries in comparison with other territories have been attributed to a flavonoid-rich diet. Others actions of flavonoids include anti-inflammatory, anti-allergic, antiviral, antibacterial, anti-thrombotic, anti-carcinogenic and antioxidant activities [5, 63-65]. Several epidemiological studies have reported that dietary flavonoids reduce the risk of coronary heart disease [56, 65-66]. A meta-analysis of prospective cohort studies showed that high dietary intake of flavonoids from different sources is inversely associated with coronary heart disease mortality [8]. Others studies have shown that regular intake of flavonoids is associated with reduced risk and cardiovascular heart disease mortality [7, 9, 67-69]. The protective effects of flavonoids against CVD complications may be related to different mechanisms including anti-platelet properties [16]. In fact, it has been shown that flavonoids inhibit platelet adhesion, secretion and aggregation [23-24, 32, 70]. In addition, flavonoids have been reported to inhibit different enzymes involved in cellular signaling [20, 71-72], to exert anticoagulant activity [73], and to increase NO generation [23]. Moreover, it was reported that there is an inverse correlation between flavonoids intake and total plasma cholesterol concentration [74].

## C. Flavonoids and Platelet Function

In view of the importance of the role of platelet dysfunction in CVD, there has been a large amount of research concentrated on finding compounds that can reduce platelet function and, therefore decrease heart diseases. Platelet function can be modulated by a number of pharmacological agents including aspirin, ticlopidine, clopidogrel or prasugrel. However, these drugs present undesirable effects such as bleeding, gastric ulcers, and liver dysfunction. Natural compounds such as flavonoids, polyphenols and terpenoids have been found to have various biological activities including cardioprotective effects [45]. Epidemiological studies suggest that regular consumption of flavonoids is inversely associated with risk and mortality of cardiovascular diseases [68-69]. Moreover, the beneficial effect of flavonoids may be mainly related to their antioxidant activity [75] and their antiplatelet actions [76]. In humans, a number of studies have investigated the effects of flavonoid-rich foods and drinks on platelet function and revealed a reduction of platelet activation [22, 77]. Moreover, flavonoids have been shown to inhibit platelet adhesion, aggregation, secretion and recruitment [23-24]. In fact, it has been reported that quercetin and catechin inhibited platelet aggregation *in vitro* [21, 78-79]. Selected flavonoids, such as quercetin, kaempferol, and myricetin were shown to be effective inhibitors of platelet aggregation in dogs and monkeys [80]. Furthermore, experimental studies have reported that dietary isoflavonoids such as genistein inhibit rat platelet activation [81], and an *in vivo* study has reported that intravenous injection of myricetin inhibits cat platelet aggregation [82]. In platelets from patients with type 2 diabetes mellitus, cinnamtannin-B, a flavonoid isolated from *L. nobilis* has been shown to reduce platelet hyperactivity and development of apoptotic

events [83-84]. Moreover, in human platelets, it has been shown that quercetin and catechin inhibit *in vitro* platelet aggregation and secretion, as well as platelet procoagulant activity [73]. Previous studies have reported that combination of antiplatelet agents with preparation from herbal origin may be more beneficial [85]. Thus, it was reported that there is a synergism between flavonoids and aspirin in inhibiting platelet function [86]. In addition, Navarro-Núñez and coworkers [87] have shown that the flavonoid apigenin when used in combination with aspirin potentiates its *ex vivo* antiplatelet effect.

On the basis of their flavonoid content, medicinal plants and vegetables may be a potential source of antiplatelet agents. Indeed, it was reported that flavonoids isolated from *Sophora japonica* (Leguminosae) suppress rat platelet aggregation induced by arachidonic acid (AA) and U46619 [88]. In human platelets, it has been shown that flavonoids isolated from *Leuzea carthamoides* inhibited platelet aggregation induced by ADP and collagen [51]. Moreover, Ling *et al*. [89] showed that isolated flavonoids from *Chromolaena odorata* exert an inhibitory effect on platelet activating factor (PAF) receptor binding *in vitro*. We have reported in a previous study that infusions rich in flavonoids obtained from different medicinal plants, including *Arbutus unedo* and *Urtica dioica* exert antiaggregant effects [90-91]. In addition, Mattiello *et al*. [92] have shown that treatment of platelets with either pomegranate (*Punica granatum*) juice or the polyphenol-rich extract (2 μM) from pomegranate fruit inhibits platelet aggregation, calcium mobilization, $TxA_2$ production and $H_2O_2$ formation induced by collagen and AA. In platelets isolated from soy protein-fed rats, it has been shown that soybean isoflavones inhibit thrombin-stimulated serotonin release [81]. Kang *et al.* [93] demonstrated that green tea catechins and epigallocatechin gallate inhibited ADP-, collagen-, epinephrine-, and calcium ionophore A23187-induced human platelet aggregation *in vitro*. Various flavonoids isolated from a methanol extract of *Sophora japonica* have been shown to exert inhibitory effect on collagen- and U46619-induced platelet aggregation, and these compounds were more active than acetylsalicylic acid (ASA) [88]. Furthermore, it has been shown that storage of platelets in cold temperature induces their activation which makes them less functional after transfusion. However; pretreatment of platelets with flavonoids before storage prevents their activation [94]. It is noteworthy to mention that the major investigations on the effects of flavonoids on platelet function are focused on particular compounds, although flavonoids can act synergistically. Indeed, it was reported that catechin and quercetin inhibited platelet adhesion to collagen [95], and this effect was more profound when the two flavonoids were used in combination.

The antiplatelet effect of flavonoids involve different signal transduction pathways including inhibition of $Ca^{2+}$ influx [96], inhibition of lipoxygenase, cyclooxygenase [97], cyclic Adenosine monophosphate (cAMP) phosphodiesterase (PDE) [20, 98] and cyclic guanosine monophosphate (cGMP)-PDE [99]. Among other mechanisms involved in the antiplatelet effect of flavonoids are: reduction of phospholipase C (PLC) activation by scavenging $H_2O_2$ production [95], inhibition of lipid peroxidation and increased nitric oxide (NO) production [100].

## EFFECT OF FLAVONOIDS ON PLATELET SIGNALING

In view of the beneficial effects of flavonoids on cardiovascular diseases, a number of studies have focused on the understanding of the mechanisms underlying the effect of these compounds on platelet function [20, 23, 90, 95]. Thus, different platelet signaling pathways have been shown to be affected by flavonoids including oxidative stress, $Ca^{2+}$ mobilization, the balance of tyrosine phosphorylation/dephosphorylation and NO pathway.

### A. Oxidative Stress

Reactive oxygen species (ROS) such as hydroxyl radical ($OH^-$), $O_2^-$ and $H_2O_2$ are molecules with a free (unpaired) electron that are highly reactive. ROS are produced into cells during oxidation reactions of the normal metabolism. However; under certain circumstances such as stress, diabetes, hypertension, the levels of free radicals is increased, which causes serious cellular damages. In fact, ROS act as second messengers regulating different cellular functions such as platelet aggregation, however; overproduction of ROS plays an important role in cardiovascular morbidity and mortality [101-102]. Oxidative stress is associated with a number of cardiovascular risk factors, such as hypertension, diabetes, dyslipidemias and smoking. It has been shown that oxidative stress plays a pivotal role in the modulation of cellular functions in cardiovascular diseases [101]. In fact, enhanced platelet aggregation in patients with CVD has been associated to reduced platelet antioxidant defenses [103], and oxidant stress increase platelet aggregation [104]. In resting platelets, ROS are continuously produced and scavenged. Platelet stimulation with agonists increase generation of ROS such as $O_2^-$, $H_2O_2$ and others [105], which may play an important role in cellular signaling [106]. For instance, platelet stimulation with thrombin (0.5U/mL) enhanced dichlorodihydrofluorescein fluorescence to a similar extent than 100 μM $H_2O_2$ [107]. The major sources of ROS in human platelets include the NAD(P)H oxidase, superoxide dismutase (SOD), dysfunctional platelet constitutive NO synthase (cNOS), activated cyclooxygenase, and phosphoinositids metabolism [108-110]. In addition to free radical produced by platelets, platelets are exposed to ROS present in blood. It has been shown that in platelets from patients with cardiovascular risk factor such as hypertension or diabetes the production of ROS is higher than in controls [111-112]. Several studies have reported that ROS enhance platelet activation which is involved in atherothrombosis [40]. Furthemore, $H_2O_2$ amplify platelet response to stimulation and enhance platelet cyclooxygenase activation [113-114] and liberation of AA [115]. Lipid hydroperoxides have been reported to stimulate cyclooxygenase [116], which lead to an increased generation of TxA2, whereas, lipid peroxides inhibit the synthesis of PGI2 [117-118], an inhibitor of platelet aggregation [119]. In addition, oxidant stress increase oxidative damage and alters platelet membrane fluidity which augments platelet aggregation [104]. In addition to their direct effect on platelets, *in vivo* and *in vitro* studies suggest that ROS increase platelet adhesion to the endothelium in

different ways [120-122] leading to the devellopment of thrombosis. To protect themselves from ROS, living organisms have developed various effective mechanisms [123] such as enzymatic inactivation (superoxide dismutase, catalase, and glutathione peroxidase). Antioxidants, such as glutathione, ascorbic acid, α-tocopherol and flavonoids represent also an effective mechanism to inactivate ROS in living cells. Because of their hydroxyl groups, flavonoids stabilize ROS by interacting with the reactive compound of the radical according to the following reaction:

$$FOH + R' \longrightarrow FO' + RH$$

where FOH is flavonoid, R' is free radical, and FO' is less reactive free radical.

It has been suggested that the beneficial effect of flavonoids on CVD resides partly in their antioxidant activity which is related to radical scavenging and metal chelation [124]. Flavonoids can interfere with the different free radical-producing systems, and they can also increase the function of the endogenous antioxidants. Indeed, epicatechin and rutin have been shown to be powerful radical scavegers [125]. The scavenging activity of rutin may be due to its inhibitory effect on the enzyme xanthine oxidase which is an important source of ROS. Other flavonoids such as quercetin, silibin and luteolin have been shown to be potent inhibitors of xanthine oxidase [126-129]. In human platelets, the flavonoids catechin and epicatechin have been shown to inhibit platelet aggregation induced by ADP, collagen and epinephrine, and to decrease platelet production of malondialdehyde after stimulation by AA, suggestting that catechins protect platelets from peroxidative stress [130]. In addition, it was reported that flavonoids inhibit various enzymes involved in the production of ROS, including lipoxygenase, cyclooxygenase, monooxygenase, xanthine oxidase, and NADPH-oxidase [126, 128]. Through their ability to reduce the formation of lipid peroxides, antioxidants, such as flavonoids, may decrease the levels of $TxA_2$ relative to $PGI_2$ and thus attenuate platelet hyperactivation. Furthermore, it has been shown that quercetin scavenge lipid alkoxyl and peroxyl radicals and repair tyrosine radicals, superoxide radical anions and α-tocopheroxyl radicals [131]. Collagen-induced platelet aggregation is associated with an increased production of $H_2O_2$ which amplifies platelet activation [41]. It has been shown that dietary isoflavonoids reduce collagen-induced $H_2O_2$ production in rat platelets [81]. Moreover, Pignatelli *et al.* [95] have reported that the flavonoids quercetin and catechin inhibits the release of platelet $H_2O_2$ induced by collagen, and this effect was more profound when the two flavonoids were used in combination. Furthermore, ROS have been shown to cause platelet activation by regulation of protein kinase C (PKC) [132]. Results of experimental studies have reported that quercetin and catechin reduced platelet function by inhibiting the formation of $O_2^-$ through the inhibition of PKC and NADPH oxidase [133]. Pearson *et al.* [86] and Murphy *et al.* [32] showed that epichatechin increase NO synthesis in endothelial cells and platelets, which inhibit platelet function. Others studies have reported that flavonoids maintain proper levels of NO and $PGI_2$ by scavenging free radicals [45]. Blache *et al.* [75] have reported that *in vivo* treatment with catechin inhibit ROS-mediated platelet hyperactivity induced by an acute iron load in a rat model. In addition, polyphenols have been reported to enhance SOD activity leading to increased platelet antioxidant capability [134].

### B. $Ca^{2+}$ Mobilization

$Ca^{2+}$ mobilization controls different aspects of platelet function such as activation, shape change, secretion, and aggregation [135-136]. Activated platelets increase intracellular free $Ca^{2+}$ concentration by $Ca^{2+}$ release from intracellular stores, and $Ca^{2+}$ influx through the plasma membrane channels [137-138]. In fact, interaction of agonists with specific platelet G protein-coupled receptors leads to activation of PLC and formation of inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ releases $Ca^{2+}$ from intracellular stores, while DAG activates PKC. The emptying of the internal $Ca^{2+}$ stores induces $Ca^{2+}$ influx across the plasma membrane by a process called store-operated calcium entry (SOCE) [139-141]. It has been reported that platelets from patients with cardiovascular disorders, such as hypertension, and those observed in diabetic patients showed higher intracellular $Ca^{2+}$ concentration under resting conditions and after stimulation with agonists compared to normal platelets [28, 90-91, 142-145]. Enhanced $Ca^{2+}$ mobilization may account for further platelet activation by different manners such as stimulation of the $TxA_2$ biosynthesis and increase in the phosphotyrosine content [146-148]. Thus, an inhibition of platelet $Ca^{2+}$ increase may have beneficial effect on platelet hyperactivation and CVD. Several studies have investigated the effect of flavonoids on platelet $Ca^{2+}$ signaling. In fact, it has been shown that treatment of platelets with Cinnamtannin B-1, a naturally occurring A-type proanthocyanidin, reduced ROS generation and inhibited SOCE in human platelets by 50% after 1h pretreatment [83]. Guerrero *et al.* [54] have reported that the flavonoids apigenin, genistein, luteolin and quercetin impaired U46619-induced calcium mobilization in human platelets. McNicol and coworkers [149] have reported that genistein inhibited U46619-induced platelet calcium release. Furthermore, it was reported that quercetin inhibited $Ca^{2+}$ mobilization in platelets stimulated by collagen, and this effect was mediated by an inhibition of PLC [94]. In addition, it has been shown that quercetin inhibited cyclic nucleotide PDE, resulting in an increased platelet cAMP levels which decreases intracellular $Ca^{2+}$ concentration and platelet activation [150]. In human platelets, it was demonstrated that *trans*-resveratrol (0.1-10μM) and other phytoestrogens such as apigenin, daidzein and genistein inhibited platelet aggregation to thrombin by an inhibition of store-operated $Ca^{2+}$ channels [96], an effect that is likely attributed to the generation of second messenger that block calcium influx such as cAMP or cGMP [30, 151]. In *in vitro* study [152], the flavonoid epigallocatechin gallate (EGCG) from green tea has been shown to decrease platelet $Ca^{2+}$ mobilization induced by collagen and thapsigargin, a $Ca^{2+}$-ATPase pump inhibitor.

### C. Tyrosine Phosphorylation/Dephosphorylation

Tyrosine phosphorylation is required for different platelet functions including aggregation, calcium mobilization and $TxA_2$ synthesis. Human platelets contain several proteins tyrosine kinase (PTK) such as Syk, MAP kinases (ERK-1,

ERK-2, p38- MAP) and MEK 1 and MEK 2 kinases [153]. The extend of protein phosphorylation depends on the activity of PTK and protein tyrosine phosphatase (PTP). Platelet activation is associated with an enhanced PTK activity, which induces phosphorylation of different proteins such as the Src family, Fak and Syk proteins [154] which act as transducers of the initial signal originated at the plasma membrane after ligand-receptor interaction. PTK activity is increased in activated platelets compared to normal platelets [155]. Many investigations have been carried out on the effects of flavonoids on the balance tyrosine phosphorylation/dephosphorylation in platelets. In fact, a number of studies have investigated the effect of the isoflavonoid genistein on the balance between PTK and PTP in platelets. It has been reported that dietary isoflavonoids inhibit rat platelet activation by reducing PTK and increasing PTP activities [81]. This finding is in line with others researches on the effect of genistein on PTK and protein phosphorylation in stimulated platelets [156]. Furthermore, the flavonoids apigenin, genestein, luteolin and quercetin have been reported to inhibit tyrosine phosphorylation and ERK ½ activation in human platelets [54]. Moreover, it was reported that the anthocyanidin cinnamtannin B-1 reduced thrombin-induced activation of Bruton's tyrosine kinase (Btk), which is involved in SOCE in platelets [157-158] and subsequently, protein tyrosine phosphorylation [159]. Inhibition of platelet PTK has also been demonstrated with the flavonoid apigenin [54]. Furthermore, Hubbard *et al.* [160] have shown that quercetin inhibited intracellular $Ca^{2+}$ mobilization and whole-cell tyrosine protein phosphorylation in platelets. The flavonoids apigenin, genistein, luteolin and quercetin have also been shown to inhibit the increase in phosphotyrosine content in platelets and other cells [161-162]. Moreover, Jin *et al.* [152] have demonstrated that epigallocatechin gallate (EGCG) from green tea inhibited collagen-induced washed rabbit platelet aggregation by blocking the collagen-mediated PLC and protein tyrosine phosphorylation.

### D. Nitric Oxide Pathway

Nitric oxide (NO) is produced by different cells such as endothelial cells, macrophages and platelets by the cNOS. Although the NO produced by the cNOS is important for the dilation of blood vessels and the inhibition of platelet aggregation [163], secretion [164], and adhesion [165], the increased concentrations of NO produced by the inducible NO synthase can result in oxidative damage. It is well known that platelets and megakaryocytes possess their own cNOS and activated platelets release NO and $O_2^-$ [23, 166-167]. NO modulates platelet function by stimulation of the soluble guanylyl cyclase (sGC) and subsequent formation of cGMP [168-169]. cGMP activates protein kinase G (PKG) [170] and protein kinase A (PKA) [169], which modulates platelet calcium mobilization by reducing $Ca^{2+}$ entry [151]. In fact, activation of PKG and PKA induce the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) which inhibits platelet activation [171] by decreasing intracellular $Ca^{2+}$ concentration [151]. The balance between oxidative stress and platelet production of NO plays a key role in platelet aggregation [23, 166]. Indeed, in platelets from patients with CVD, the bioactivity of NO is limited by its reduced biosynthesis and its increased degradation by superoxide, which further enhanced platelet activation. NO can interact with superoxide or lipid peroxyl radicals to form peroxynitrite ($ONOO^-$) or lipid peroxynitrite, respectively which decreases NO bioavailability [172], and enhances platelet aggregation. Furthermore, NO reduces the expression of the complex GPIIb-IIIa however; $O_2^-$ overexpresses this glycoprotein [173]. Flavonoids can scavenge free radicals and prevent them to react with NO resulting in less damage [174]. A previous study reported that polyphenols inhibit platelet recruitment by increasing NO production [23]. It was reported that *in vitro* incubation and oral supplementation with purple grape juice (PGJ), which is rich in flavonoids, inhibited platelet aggregation by increasing NO release, decreasing superoxide production, and inhibiting PKC activity [23]. In agreement with these results, it has been shown that treatment of platelets with quercetin and catechin in combination increased generation of NO, reduced formation of $O_2^-$, and inhibited PKC and NADPH oxidase activation, which results in reduction in platelet function [133]. In addition, Pearson *et al.* [86] and Murphy *et al.* [32] showed that epicatechin increased NO synthesis in both the endothelial cells and platelets which cause vasodilation and inhibition of platelet aggregation.

### E. Other Mechanisms Involved in the Anti-Platelet Effects of Flavonoids

Flavonoids can reduce platelet hyperactivity and associated CVD through a variety of other mechanisms including inhibition of $TxA_2$ formation [175], reduction of cyclooxygenase and lypoxygenase activities [20], and by an accumulative effect of flavonoids in the platelet membrane [176]. $TxA_2$ receptor antagonism and increased formation of $PGI_2$ [175, 177] have also been proposed as possible flavonoid-induced anti-platelet mechanisms. Indeed, in rabbit platelets, quercetin, kaempferol and fisetin (13-22 μM) were shown to antagonize $TxA_2$ receptor [175]. In addition, the flavonoids apigenin and genistein have been shown to compete for binding to the platelet $TxA_2$ receptor and consequently abolish downstream signaling [54, 178]. Furthermore, quercetin has been shown to reduce collagen-induced platelet activation through blockade of the glycoprotein VI signaling pathway [160]. In human platelets, it has been shown that flavonoids isolated from onion (*Allium cepa*) inhibit platelet aggregation induced by collagen and ADP and dissociate the aggregates produced by ADP, events that might be mediated by their interaction with the platelet membrane lipids [179]. In *in vivo* study, it has been shown that micronized purified flavonoids reduced ADP and collagen-induced platelet aggregation, increased platelet disaggregation in rat platelets and inhibited fibrinogen binding to ADP-stimulated platelets [24]. In addition, it has been reported that the flavonoids luteolin and quercetin extracted from olive oil reduce platelet aggregation by inhibiting cAMP-PDE activity [180]. Bucki *et al.* [73] have shown that platelet treatment with the flavonoids quercetin or catechin reduce phosphatidylserine (PS) exposure and phosphoinositide metabolism after platelet stimulation with collagen, thrombin or calcium ionophore. Other study has shown that flavonoids from *Cephalotaxus wilsoniana* and *Justicia procumbens* inhibited platelet aggregation, and the effect of these compounds rely on the inhibition of $TxA_2$ formation and COX-1 activity [181]. Finally,

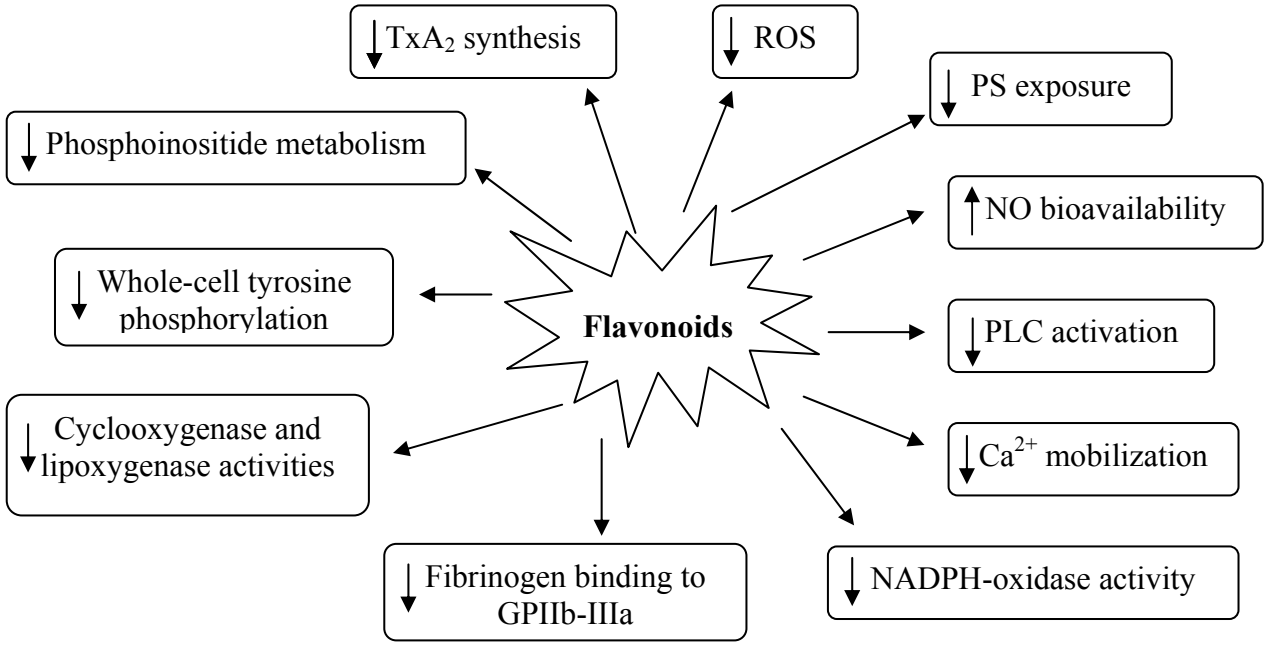


**Fig. (3).** Possible platelet signaling pathways modulated by flavonoids. ROS: Reactive oxygen species; NO: Nitric oxide; $TxA_2$: Thromboxane $A_2$; PLC: Phospholipase C; $Ca^{2+}$: Ion calcium; PS: Phosphatidylserine.

inhibition of actin polymerization can also contribute to the anti-platelet effect of flavonoids [182].

## CONCLUSION

Platelet hyperactivation is involved in the development and progression of cardiovascular diseases. Thus, platelet signaling pathways represent an interesting therapeutic target that could be investigated for the development of novel treatments for cardiovascular events. In this context, flavonoids have been shown to reduce platelet function, and therefore, protect from cardiovascular diseases by different ways. As summarized in Fig. (**3**), different platelet signaling pathways are modulated by flavonoids such as calcium mobilization, ROS generation, NO bioavailability or protein tyrosine phosphorylation. The understanding of these molecular mechanisms may be helpful in discovering new therapeutic strategies for the treatment and/or the prevention of cardiovascular diseases complications associated to platelet hyperactivity.

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## ABBREVIATIONS

CVD = Cardiovascular diseases

ROS = Reactive oxygen species

$TxA_2$ = Thromboxane $A_2$

NO = Nitric oxide

$PGI_2$ = Prostacyclin

$O_2^-$ = Superoxide anion

$H_2O_2$ = Hydrogen peroxide

AA = Arachidonic Acid

SOCE = Store-Operated-Calcium-Entry

cGMP = cyclic Guanosine monophosphate

cAMP = cyclic Adenosine monophosphate

PLC = Phospholipase C

$Ca^{2+}$ = Ion calcium

cNOS = constitutive NO synthase

PKC = Protein kinase C

PTK = Protein tyrosine kinase

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