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Regular Article

Inhibitory effects of sulphated flavonoids isolated from *Flaveria bidentis* on platelet aggregation

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Abstract *Flaveria bidentis* is a plant species that has as major constituents sulphated flavonoids in the highest degree of sulphatation. Among them, quercetin 3,7,3′,4′-tetrasulphate (QTS) and quercetin 3-acetyl-7,3′,4′-trisulphate (ATS) are the most important constituents. Both showed anticoagulant properties. The objective of the present study was to evaluate the effects of these flavonoids on human platelet aggregation in comparison with the well-known inhibitor quercetin (Qc) by using several agonists. Platelet-rich plasma (PRP) or washed human platelets (WP) were incubated with different concentrations of the flavonoids to be tested (1 to 1000 μM, final concentration), and the platelet aggregation was induced by using adenosine 5′-diphosphate (ADP), epinephrine (EP), collagen, arachidonic acid (AA) and ristocetin as agonists. QTS (500 μM) and Qc (250 μM) markedly inhibited platelet aggregation with all the aggregant agents, except ristocetin, whereas ATS (1000 μM) showed only slight antiplatelet effects. In addition, QTS and Qc antagonized the aggregation

Abbreviations: ADP, adenosine 5′-diphosphate; TxA_2, thromboxane A_2; Qc, quercetin; QTS, quercetin 3,7,3′,4′-tetrasulphate; ATS, quercetin 3-acetyl-7,3′,4′-trisulphate; UV-V, ultraviolet-visible; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; ^{13}C-NMR, carbon nuclear magnetic resonance spectroscopy; MS, mass spectroscopy; DMSO, dimethylsulfoxide; EP, epinephrine; AA, arachidonic acid; PRP, platelet-rich plasma; PPP, platelet-poor plasma; WP, washed human platelets; TxB_2, thromboxane B_2; EIA, enzyme-immuno assay.

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of PRP or WP induced by U-46619, a mimetic thromboxane A2 (TxA_2) receptor agonist. Challenged with collagen or arachidonic acid, the thromboxane B_2 (TxB_2) formation was also inhibited by the flavonoids, mainly by QTS and Qc, in WP. These results demonstrate that QTS and in minor extension ATS induce a deleterious effect on the production of TxA_2, as judged by TxB_2 formation, in stimulated WP and a marked interference on the TxA_2 receptor according to the profile of inhibition of the agonist-induced platelet aggregation when using ADP, EP, AA and collagen and confirmed with U-46619.

Introduction

Activation of platelets plays a key role in hemostasis and in circulation; platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adhered platelets release some biologically active constituents and aggregate themselves. After activation, platelets provide a catalytic membrane surface for thrombin generation, which accelerates the formation of fibrin necessary to stabilize thrombi. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, strokes and peripheral vascular disease [1]. Key to these events is the presence on the platelet surface of receptors that can respond rapidly to soluble agonists, including collagen, thrombin, adenosine 5′-diphosphate (ADP) and thromboxane A2 (TxA_2), which are typical members of the superfamily of G-protein-coupled receptors [2]. TxA_2 is a powerful platelet agonist, and its formation by stimulated platelets represents an important amplifying signal for platelet activation by binding to a specific receptor and causing an increase in intraplatelet calcium and phospholipase C and protein kinase C activation [3,4]. Therefore, regulation of platelet activity by using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. The most commonly used antiplatelet agents in this situation are aspirin, clopidogrel and abciximab, which act by different mechanisms [5]. In addition, platelet activation may be inhibited by a number of dietary components such as alcohol [6], some dietary fats [7] and polyphenols [8]. Flavonoids are polyphenolic compounds with a wide distribution in plants, including plant foods. Several in vitro studies showed that polyphenols such as resveratrol, the flavonoids quercetin (Qc) and catechin inhibit platelet aggregation [9—11]. Results obtained by the incubation of human or animal platelets with isolated flavonoids suggest that the antiplatelet properties may be attributed to the inhibition of TxA_2 formation [12], thromboxane receptor antagonism [13], protein kinase C activation [14] and phosphoinositide synthesis [15].

Sulphated esters of flavonoids represent an interesting group of sulphured compounds that have been found in a number of plant species especially in the Asteraceae family. Among them, *Flaveria bidentis* (L.) Kuntze is the only species that synthesize Qc derivatives with the highest degree of sulphatation known so far: the fully sulphated quercetin 3,7,3′,4′-tetrasulphated (QTS) and quercetin 3-acetyl-7,3′,4′-trisulphate (ATS) [16,17]. Both were studied by us in relation to their biological activities, and they demonstrated to possess different properties such as inhibition of human lens aldose reductase [18] and anticoagulant effects by acting as heparin cofactor II agonists [19]. However, the effect of sulphated flavonoids on platelet function has not yet been investigated. Thus, the purpose of the present study was to investigate the effects of ATS and QTS isolated from *F. bidentis* on platelet function stimulated by different agonists. We carried out the study in comparison with Qc, with a view to explore the possible mechanism(s) in the modulation of the platelet activity.

Material and methods

Extraction, purification and identification of flavonoids

ATS and QTS: Both were isolated, purified and identified as reported previously [19].

Qc: This flavonoid has been isolated from the leaves of *F. bidentis* as well [20], but, as it occurs in a very low concentration in relation with the other constituents, we decided to obtain the flavonoid by acidic hydrolysis of QTS. Thus, to develop the present studies, QTS was treated with ClH 0.1%, heated on a steam bath for 45 min then cooled and centrifuged. After separation of the supernatant,

the precipitate was washed several times with distilled water until neutral pH and finally dried at 100 °C/5 mm for 12 h. By using spectroscopic techniques [ultraviolet-visible (UV-V), proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR) and mass spectroscopy (MS)], identification was attained, and data were coincident with those in literature [21—23].

Dissolution

QTS and ATS were dissolved in water and Qc in dimethylsulfoxide (DMSO).

Reagents

Collagen (type I from calf skin), epinephrine (EP), adenosine 5′-diphosphate (ADP), arachidonic acid (AA) and ristocetin were purchased from Biopool (Medica-Tec, Argentina). U-46619 was obtained from Biomol (Plymouth Meeting, PA, USA). Bovine serum albumin, EDTA and indomethacin were purchased from Sigma Chem (St Louis, MI, USA). Thromboxane B_2 (TxB_2) kits were a generous gift from Neogen (Lexington, KY, USA).

Platelet preparation

Blood samples were taken from healthy volunteers who assured not to have taken any drugs during the 2 weeks prior to the blood sampling. Blood was collected into buffered sodium citrate (3.2% w/v) pH 6.5 as the anticoagulant at a ratio of 1:9 v/v. Platelet-rich plasma (PRP) was obtained on the same day by centrifugation of the blood at 200 $\times g$ for 20 min. Isolated platelets were adjusted to $2.5—3.0\times10^8$/ml using autologous platelet-poor plasma (PPP). Washed human platelets (WP) were prepared as previously described [24] and only used within 2 or 3 h after PRP preparation and adjusted as indicated above with Tyrode buffer.

Platelet aggregation

Aggregation was measured turbidimetrically at 37 °C, with constant stirring at 1000 rpm in a Chrono-Log aggregometer (Chrono-Log, PA, USA). The absorbance of PPP or Tyrode's solution was assigned as 100% aggregation and the absorbance of PRP or WP as 0% aggregation, respectively. Briefly, PRP or WP were incubated with different concentrations of the flavonoids to be tested (1 to 1000 μM, final concentration) and incubated at 37 °C under periodic stirring. The reaction was allowed to proceed from 0 to 90 min. Every 30 min, aliquots were taken, the platelet aggregation was initiated by the addition of the agonists, and the curves obtained were registered and always compared to normal PRP, without flavonoids incubated in the same conditions. To evaluate the effects of ATS, QTS and Qc, the percentages of inhibition of platelet aggregation were calculated from the increases in transmission observed with the flavonoids on the basis that no inhibition was observed in the incubated controls with sole vehicle. In our experimental conditions, final concentrations of the platelet aggregants using normal PRP [24] were the following: collagen 1 μg/ml; ADP 2.5 μM; EP 50 μM; AA 0.5 mM; ristocetin 1.0 mg/ml; and U-46619 1 μM.

Thromboxane B_2 assay

Washed human platelet suspensions ($2.5—3.0\times10^8$/ml) were preincubated for 30 min in the presence or absence of ATS, QTS or Qc (1 to 1000 μM) before the addition of collagen (1 μg/ml) or AA (0.5 mM). Six minutes after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the suspensions to stop the reactions. The vials were then centrifuged for 3 min at 12,000 $\times g$. The thromboxane B_2 (TxB_2), the stable metabolite of TxA_2, levels of the supernatants were measured using a competitive enzyme-immuno assay (EIA) kit according to the manufacturer's instructions.

Statistical analysis

Experimental results are expressed as the means± S.E.M. and are accompanied by the numbers of observations. Statistical significance ($p<0.05$) was assessed by using Student's t-test.

Results

Preincubation of the platelet suspension with flavonoids here tested caused a time-dependent inhibition of platelet aggregation when different agonists to reach a plateau at 30 min of incubation were used (data not shown). For this reason, this period of incubation was used for the subsequent experiments.

Qc has previously shown to inhibit platelet aggregation in both PRP and WP, with a number of different agonists, including collagen, thrombin, ADP or AA. On the other hand, the effects of sulphated group substituents in the Qc molecule such as in ATS and QTS on platelet aggregation have not been studied. In our experimental system, Qc and QTS were observed to cause a concentration-

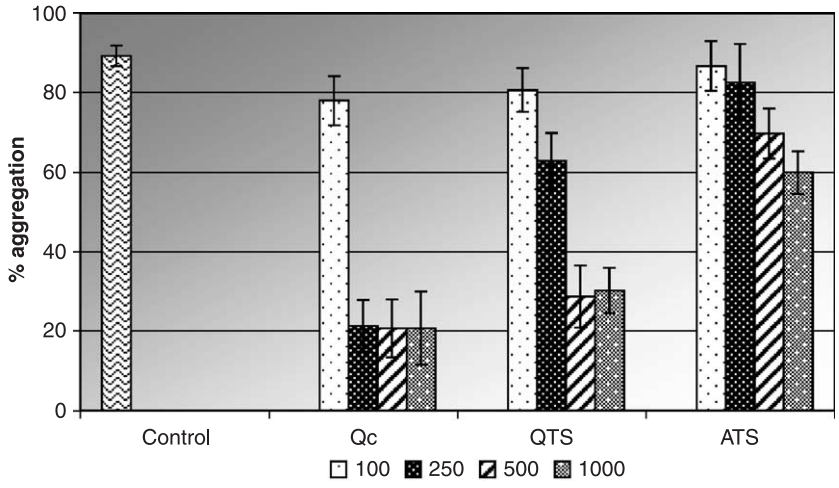

Figure 1 The concentration-dependent inhibitory effects of flavonoids on platelet aggregation (expressed as % of aggregation) induced by collagen 1 μg/ml at different concentrations of Qc, QTS and ATS in μM. The other agonists showed a similar pattern. Percentages of aggregation are presented as means±S.E.M. (n=3—4).

dependent inhibition of platelet aggregation induced by different agonists, whereas ATS showed a weak response on the platelets. The concentration in which we observed the maximum inhibition of the platelet aggregation with PRP were 250 μM of Qc, 500 μM of QTS and 1000 μM of ATS. Higher concentrations of the three flavonoids studied did not demonstrate to exercise a major inhibitory effect when collagen (1 μg/ml) was used as stimulant agent (Fig. 1). Similar results were obtained when other aggregant agents were used (results not shown).

In PRP, ADP (2.5 μM) and EP (50 μM) cause a biphasic aggregation. Preincubated platelets with Qc significantly inhibited ADP-induced aggregation by 19.5±5.3 vs. 70.2±3.1 and EP-induced aggregation by 14.5±5.6 vs. 74.5±2.5 compared to normal control, respectively. QTS at a concentration of 500 μM inhibited ADP- and EP-induced aggregation in PRP in a similar order of magnitude to 250 μM of Qc, and, for both compounds, the effect was more marked on the second wave of aggregation (Table 1). By contrast, ATS (50 to 1000 μM) did not significantly affect platelet aggregation when using two different concentrations of ADP (2.5 and 10 μM) or EP (50 and 100 μM) (data not shown).

Collagen (1 μg/ml) and AA (0.5 mM) cause approximately a 90% aggregation of PRP, but, in the presence of Qc or QTS, a marked inhibition of platelet aggregation was observed. In fact, Qc significantly inhibits collagen (21.3±6.5 vs. 89.3±5.4, p<0.001) and AA (9.2±3.4 vs. 86.6±5.4, p<0.001) compared to normal control. QTS inhibits 28.7±7.8 and 20.3±4.3 for collagen and AA, respectively, being both values statistically significant compared to the value obtained in absence of flavonoids (p<0.001). On the other hand, ATS showed a lower response when both inducers were tested on PRP (59.9±8.4 and 49.8±8.9 p<0.05, respectively) compared to normal control (Table 1).

Given the marked effect of Qc and QTS on platelet aggregation, we investigated if the interaction between the platelet glycoprotein Ib—IX and the factor von Willebrand are affected in presence of these flavonoids. Qc (250 μM), QTS (500 μM) and ATS (1000 μM) did not cause any significant alteration of ristocetin-induced aggregation (Table 1). Higher concentrations of the three flavonoids

Table 1 Effects of Qc, QTS and ATS on platelet aggregation induced by different agonists

Flavonoids	% Platelet aggregation				
	ADP	EP	Collagen	AA	Ristocetin
Control	70.2±3.1	74.5±2.5	89.3±5.4	86.6±5.4	88.7±3.3
Qc	19.5±5.3**	14.5±5.6**	21.3±6.5**	9.2±3.4**	79.6±4.4
QTC	23.3±4.5**	19.7±6.7**	28.7±7.8**	20.3±4.3**	75.4±5.6
ATS	62.8±9.8	58.5±10.7	59.9±8.4*	49.8±8.9*	80.4±10.3

Values are expressed as means±S.E.M. of the % aggregation (n=2-5).
*p<0.05, **p<0.001 compared to normal controls.

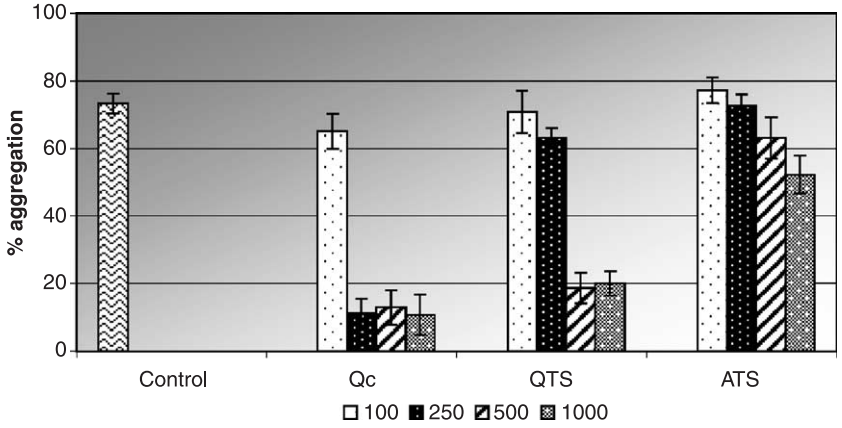

Figure 2 Effects of flavonoids on the platelet aggregation (expressed as % of aggregation) induced by U-46619 (1 μM) at different concentrations of Qc, QTS and ATS in μM. Percentages of aggregation are presented as means±S.E.M. (n=3—4).

tested were also ineffective on platelet aggregation (results not shown). Moreover, to evaluate a possible protein binding capacity of the flavonoids that could affect the final concentrations of these compounds in the PRP, some assays of platelet aggregation were performed using WP. The results obtained in this condition assay were similar to the results found in PRP except that the Qc concentration necessary to obtain the same percentage of platelet inhibition was slightly inferior (data not shown). To support the robustness of the experiments indicated above, these were performed in

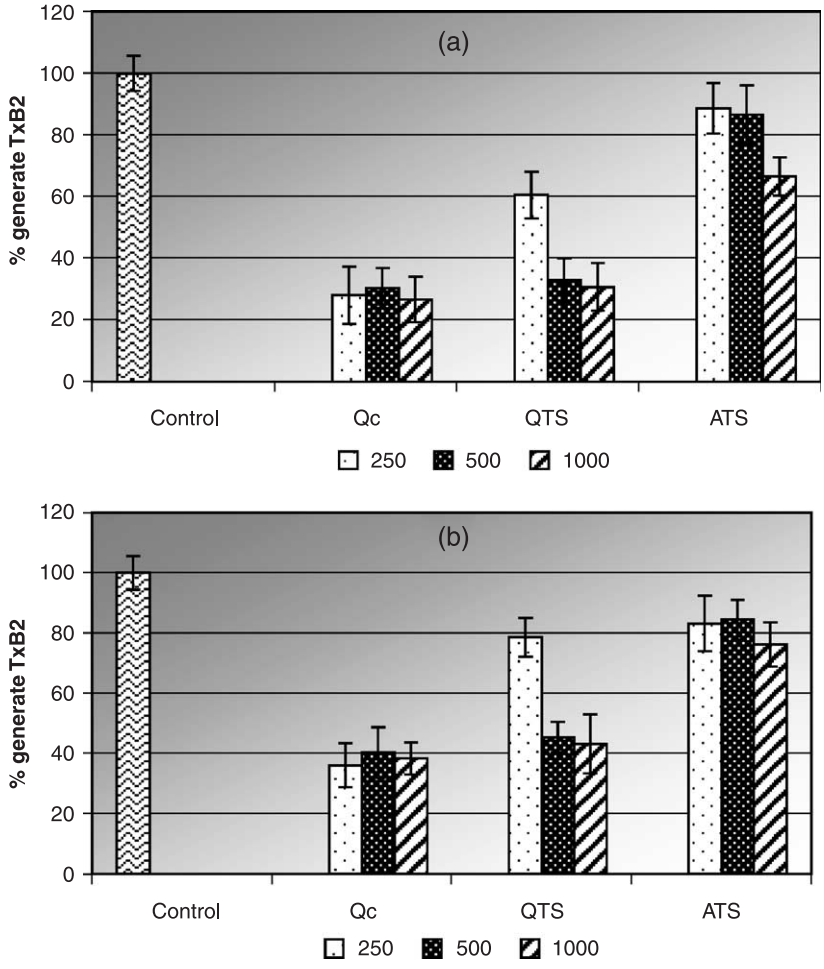

Figure 3 Thromboxane B_2 production in washed platelet incubated with Qc, QTS and ATS and induced by collagen (a) and AA (b).

triplicate and repeated at least twice, and S.E.M. values of the averages were below 10%. DMSO (0.5%) was used as solvent control when Qc was evaluated and had no effects on platelet aggregation at these concentrations.

The above finding suggests that the antiplatelet effects may be due to the interaction between flavonoids and TxA_2 receptors and/or an interference in the thromboxane pathway. Therefore, we used U-46619 (a TxA_2 analogue) as the aggregation inducer. Qc (250 μM) and QTS (500 μM) significantly inhibited platelet aggregation, and ATS (1000 μM) was almost not able to inhibit U-46619-induced platelet aggregation (Fig. 2).

Often, the measurement of TxB_2, a stable metabolite of TxA_2, in supernatant of platelet suspension is used as index of TxA_2 generation. As it can be seen in Fig. 3, resting platelets produced relatively little TxB_2 but in the presence of AA (0.5 mM) or collagen (1 μg/ml) caused a significant elevation of TxB_2 formation. Furthermore, results obtained by the use of various concentrations of Qc, QTS and ATS (1 to 1000 μM) indicated that the two former significantly inhibited TxB_2 formation in platelets when the platelets were stimulated with both AA or collagen. On the contrary, ATS showed a low capacity of TxB_2 formation (Fig. 3).

Discussion

The present study demonstrates that QTS, a sulphated flavonoid isolated from *F. bidentis*, inhibits platelet aggregation in vitro in a similar order of magnitude to Qc. According to the results of our assays, the mechanisms of action appear to involve the combination of cyclooxygenase/TxA_2 synthetase inhibition and TxA_2 receptor blockage. On the other hand, the other sulphated flavonoid, ATS, did not significantly inhibit platelet aggregation. In fact, QTS was very effective in inhibiting aggregation induced by ADP, EP, collagen, AA and U-46619, but it was ineffective in ristocetin-induced platelet aggregation. Although the action mechanism of various platelet aggregation agonists differs, QTS and Qc significantly inhibit the platelet aggregation that was stimulated by all of them. This implies that these compounds may block a common step shared by these inducers.

We observed in PRP that the shape change, and the primary wave induced by ADP were not affected in the presence of either QTS or Qc, but the second wave was markedly abolished. A similar pattern was observed when EP was used as platelet aggregation agonist. The results obtained and bearing in mind that the second wave, which is strictly dependent on thromboxane production, let us conclude that the inhibition of TxA_2 production appears to be the mechanism involved in the antiaggregatory effects of QTS.

Qc also inhibits platelet aggregation by the use of ADP or EP as activating agents of platelet aggregation in a similar mode to QTS although in a lower concentration (250 vs. 500 μM) (Table 1). Moreover, in previous studies, Qc (1000 μM) was able to induce inhibition of platelet aggregation in PRP in agreement with our results [8].

Collagen-induced platelet aggregation is mainly due to the presence of a specific receptor on platelet membrane, the glycoprotein VI. Recently, Hubbart et al. [13] provided evidence that Qc inhibits platelet activation induced by collagen through inhibition of multiple components of the glycoprotein VI signaling pathways. In our study, QTS inhibits platelet aggregation induced by collagen, like Qc (Table 1). However, this assay did not allow us to draw any other conclusions or to infer a similar action mechanism between both as we would be only speculating.

To study whether the flavonoids herein studied interfered in the generation of arachidonic acid mediated by phospholipases, we used AA as platelet agonist in the following assays. The results show an important inhibition of the platelet aggregation in presence of QTS and Qc, suggesting that this pathway is not involved (Table 1). Similar results were reported for Qc by other authors although in a variable range of concentration. These discrepancies can be attributed to the different experimental procedures and reagents [7,12,25].

The results hitherto obtained allow us to assume that a possible mechanism of QTS on the inhibition of platelet aggregation might be a break of the TxA_2 formation or a blockage of TxA_2 receptors, so we used the mimetic agonist of TxA_2, U-46619, as inducer in PRP. Fig. 2 depicts a marked inhibition of aggregation of the PRP incubated in presence of QTS and Qc, but it is practically absent in presence of ATS. These results suggest that Qc, QTS and ATS (in this order) produce a blockade in the receptors of the TxA_2. To test a possible interference in the formation of TxA_2, we assayed the TxB_2 (a stable metabolite of TxA_2) in the supernatant of washed platelet incubated with Qc, QTS or ATS and stimulated with collagen and AA. We used washed platelets instead of PRP because recently it was demonstrated that QTS and in minor extension ATS inhibit thrombin by activation of heparin cofactor II [19], so, in this assay, the thrombin generation, an important stimulus for platelet activation and TxA_2 production, is totally excluded. Production of TxB_2 was inhibited by Qc, QTS and in minor extension by

ATS (Fig. 3). These results are indicating that the thromboxane pathway is affected.

In contrast with the above results, ristocetin-induced platelet aggregation of PRP incubated with flavonoids did not induce changes in the profile of aggregation even when suspensions of washed platelets were used. Ristocetin differs from other aggregant agents because it induces platelet "agglutination" more than "aggregation" in the presence of plasma von Willebrand factor and an intact platelet receptor glycoprotein Ib. This reaction is distinct from platelet aggregation induced by the previously mentioned agonists, all of them induce the binding of fibrinogen to the platelet receptor glycoprotein IIb—IIIa complex.

The results show that these compounds do not affect the interaction of the von Willebrand factor and glycoprotein Ib—IX, a central key of the platelet adhesion.

In conclusion, the analysis of each result suggests that QTS inhibits agonist-induced human platelet aggregation in a similar order of magnitude to Qc. This inhibitory effect would involve at least the following two mechanisms: (1) QTS initially induce a deleterious effect on the production of TxA_2, as judged by TxB_2 formation in stimulated washed platelets incubated with flavonoids, and (2) QTS markedly inhibits the TxA_2 receptors according to the profile of inhibition of the agonist-induced platelet aggregation when using ADP, EP, AA, collagen and then confirmed with U-46619.

However, further studies such as intraplatelet NO pathways or cyclic GMP and tyrosine phosphorylation of multiple components of signaling pathway will be necessary to increase our understanding of the action mechanism of these flavonoids on platelet function.

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