Quercetin 3, 7, 3´, 4´-Tetrasulphate from *Flaveria bidentis* Inhibits the Plasminogen Activator Inhibitor-1 but not the Tissue-Type Plasminogen Activator Expression in Human Normal Fibroblasts

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ABSTRACT

Sulphated flavonoids quercetin 3, 7, 3´, 4´-tetrasulphated (QTS) and quercetin 3-acetyl-7, 3´, 4´-trisulphate (ATS), obtained from *Flaveria bidentis* have demonstrated some antithrombotic properties. We analyzed whether both compounds affected the expression of tissue-plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1), the components of the fibrinolytic system. Normal human fibroblasts were pretreated with different concentrations of each sulphated flavonoid (0.1 µM to 500 µM), during 2 and 3 h followed by a 24 h incubation with phorbol myristate acetate (PMA). Results of the tPA and PAI-1 expression compared to control, showed different behaviors for the two flavonoids studied. A null inhibitory effect on the tPA expression was detected at all the concentrations for QTS and ATS. In contrast, QTS from 50 µM onwards showed a significant inhibitory effect on PAI-1 expression ($p < 0.05$). The profibrinolytic activity of QTS enhances its antithrombotic properties and encourages us to study these properties in animal models.

Keywords- *Flaveria bidentis*; sulphated flavonoids; tissue-plasminogen activator; plasminogen activator inhibitor-1; human fibroblasts.

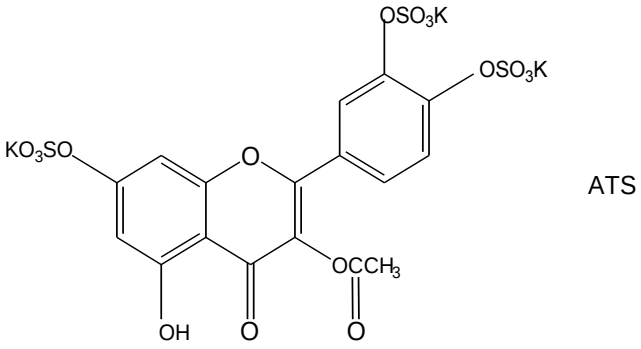
1. INTRODUCTION

Thrombotic disease is the major cause of morbidity and mortality worldwide and therapeutic anticoagulation is widely used to treat and prevent thromboembolic disorders. Effective anticoagulation has formed the basis of treatment for acute venous thromboembolic events for a long time and reduced the mortality rate in this condition from 30% to 3-8%. In the treatment of thrombotic disease, the future is focused on the search of the "ideal-antithrombotic" drugs, i.e. those that can selectively

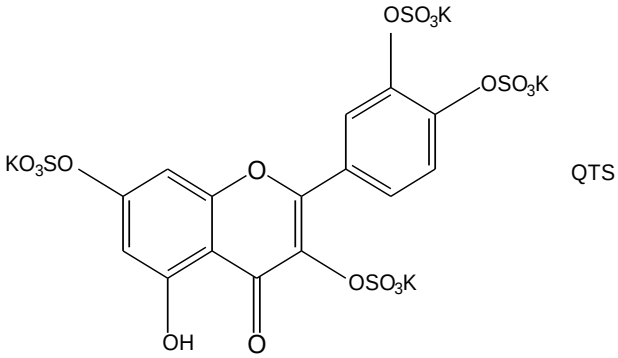
interrupt pathological thrombin activities and the exaggerated platelet responses in order to avoid the subsequent thrombosis seen in atherosclerotic vessels as well as the absence of side effects such as hemorrhagic events [1]. Anticoagulant agents not only prevent new clot formation but also facilitate intrinsic mechanisms of clot lysis by retarding existing clot progression. Fibrinolytic system constitutes a part of the hemostatic system responsible for the degradation of fibrin deposits [2]. Plasminogen is the main component of the fibrinolytic system and is activated into its active enzyme form plasmin by activators. Tissue-type plasminogen activator (tPA) is the most potent activator of plasminogen in plasma and the main regulator of fibrinolysis [3].

After stimulation, tPA is locally released into the circulation from the endothelial cells where it is produced and the plasminogen activation is facilitated by a fibrin surface, which restricts fibrinolysis to the site of thrombus formation. Moreover, once bound to fibrin, tPA is protected from inhibition by plasminogen activator inhibitor 1 (PAI-1), its principal inhibitor in plasma. PAI-1 belongs to the superfamily of serine-protease inhibitors and plays an important role in physiologic processes such as regulation of fibrinolysis and proteolysis, angiogenesis, wound healing, and cell [4]. More recently, PAI-1 has been linked to the development of vascular diseases such as venous thrombosis and atherosclerosis [5].

Flavonoids are polyphenolic compounds with a wide distribution in the plant kingdom. Several flavonoids have remarkable biological activities, such as inhibitory effects on enzymes, modulatory effects on some cell types, and protection against allergies, as well as antiviral, anti-malarial, anti-oxidant, anti-inflammatory, anti-tumor and antithrombotic properties [6], among others. Sulphated esters of flavonoids represent an interesting group of sulphur compounds that have been found in only few plant families, especially in the Asteraceae [7]. *Flaveria bidentis* (L.) Kuntze belongs to this family and is the only species that synthesize quercetin derivates with the highest degree of sulphation known so far such as quercetin 3-acetyl-7, 3´,4´-trisulphate (ATS) and quercetin 3,7,3´,4´-tetrasulphate (QTS).



ATS



QTS

In our laboratory, we demonstrated that QTS and in a lesser extend ATS possesses important anticoagulant and antiplatelet functions and it was recently reported that QTS inhibits tissue factor expression in human monocyte [8-10]. These properties place these compounds as a new group of substances with various antithrombotic characteristics.

In view of the antithrombotic properties of both compounds and the importance of the fibrinolytic system in the progression of atherothrombosis, we designed the present study in order to determine the effects of sulphated flavonoids on the fibrinolytic activity by measuring the expression of tPA and PAI1 in culture supernatants of normal human fibroblasts induced by phorbol myristate acetate (PMA).

2. MATERIAL AND METHODS

2.1. Plant material

Flaveria bidentis was collected by A.M. Agnese and J.L. Cabrera in the surroundings of Córdoba city (Córdoba, Argentina) in March 2012. The plant material was identified by Prof. Dr. Luis Ariza Espinar and voucher specimen nº 2813 is deposited at CORD. In order to obtain ATS and QTS only the leaves were used. They were dried at shadow and the flavonoids were extracted and purified

as previously reported [8] with some modifications. Briefly, plant material was extracted by maceration with H_2O/EtOH (1:1). The filtered extracts were combined, concentrated at reduced pressure until reach a 40% of the initial volume. The aqueous extract was extracted with petroleum ether (60–80°C) in a liquid–liquid extraction apparatus. The aqueous layer was separated and concentrated to a quarter of its volume. An equal volume of EtOH was added to the obtained extract with the aim to precipitate the sulphated flavonoids. The separation of ATS and QTS was accomplished on a Sephadex G-10 (Pharmacia, Uppsala, Sweden) column by using H_2O as mobile phase. QTS was first obtained followed by a mixture of QTS plus ATS and posterior fractions gave ATS alone. The fractions were monitored chromatographically on Whatman number 1 paper and using H_2O as mobile phase.

Flavonoids were recrystallized from water and dried at 100°C/5 mm. The identification was achieved by spectroscopic methods (UV–V) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) in comparison to the data previously published by our laboratory.

It is important to point out that, differently from the majority of the aglycon flavonoids, the presence of several sulphated groups in both flavonoids (Fig. 1) makes them completely soluble in water.

2.2. Fibroblasts preparation

Normal human skin fibroblasts ($2.5x10^5$cel/mL) were cultured in DMEM medium (Sigma, MI, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 40µg/mL of gentamicin at 37ºC, 5% CO_2 and 95% humidity, in 24-well culture plates. After 24 h of incubation, the fibroblasts monolayers (90-95% confluent) were cultured in DMEM medium free of FBS during 24 h before being used for the experiments.

2.3. Preincubation of human normal fibroblasts with flavonoids

To test the effect of QTS and ATS on the expression of tPA and PAI-1, fibroblasts monolayers were incubated with different concentrations of each flavonoid (0.1 µM to 500 µM) for 2 and 3 h at 37°C-5% CO_2. After the incubation, 100 ng/mL of PMA were added and the cells incubated for another 24 h. After culture, the cells were removed by centrifugation and the supernatants were harvested and stored at -80°C for tPA and PAI-1 determinations. As control for 100% and 0% of tPA and PAI-1 expression, the fibroblasts cultured with vehicle instead of flavonoids were treated with and without PMA, respectively.

As positive control and not related to flavonoids, 0.1% EtOH was used as a known inhibitor of enzymes of the fibrinolytic system in culture cells (11).

2.4. Cell viability test

The toxic effect of the flavonoids on the fibroblasts was checked by a cell survival test using a tetrazolium-based colorimetric assay [12]. A value greater than 10 % of disrupted cells was considered as a significant indication of cytotoxicity.

2.5. Determination of tPA and PAI-1

Total tPA and PAI-1 antigen levels were measured in triplicate in fibroblasts culture supernatants according to the manufacturer's instructions using ELISA kits (Asserachrom tPA and PAI-1, Diagnostica Stago, Asnieres, France). The values were expressed in percentages taking into account the 100% control (normal human fibroblasts incubated with vehicle and stimulated with PMA).

2.6. Statistical analysis

Values are expressed as means ± S.D. Statistical significance was determined using ANOVA test, with $p < 0.05$ being considered significant.

3. RESULTS AND DISCUSSION

tPA activity is an important determinant of the coagulation-fibrinolysis balance. Increased PAI-1 inhibits tPA activity, disturbing this balance and leading to a prothrombotic state. Even though increased PAI-1 is mainly responsible for the decreased fibrinolysis it would be important to investigate tPA expression as well.

The present results clearly showed that both sulphated flavonoids do not regulate tPA expression in culture supernatants at any of the concentration tested. In fact, the percentage of tPA antigen did not significantly change with increasing concentrations of ATS and QTS (0.1 µM to 500 µM) (Fig. 2 A and B). On the other hand, results on the PAI-1 protein expression in normal human fibroblasts showed different behaviors for the two flavonoids studied. In fact, ATS did not induce an inhibitory effect in PAI-1 expression at any of the concentrations tested (Fig. 2 C). In contrast, a significant inhibitory effect in PAI-1 expression was detected with QTS concentrations ranging from 50 to 300 µM at 2 h of incubation compared to non-treated cells (Fig. 2 D). Moreover, with the above mentioned concentrations of QTS, a 2.6 fold decrease in PAI-1 expression ($p < 0.05$) compared with vehicle treated cells (100%) was observed.

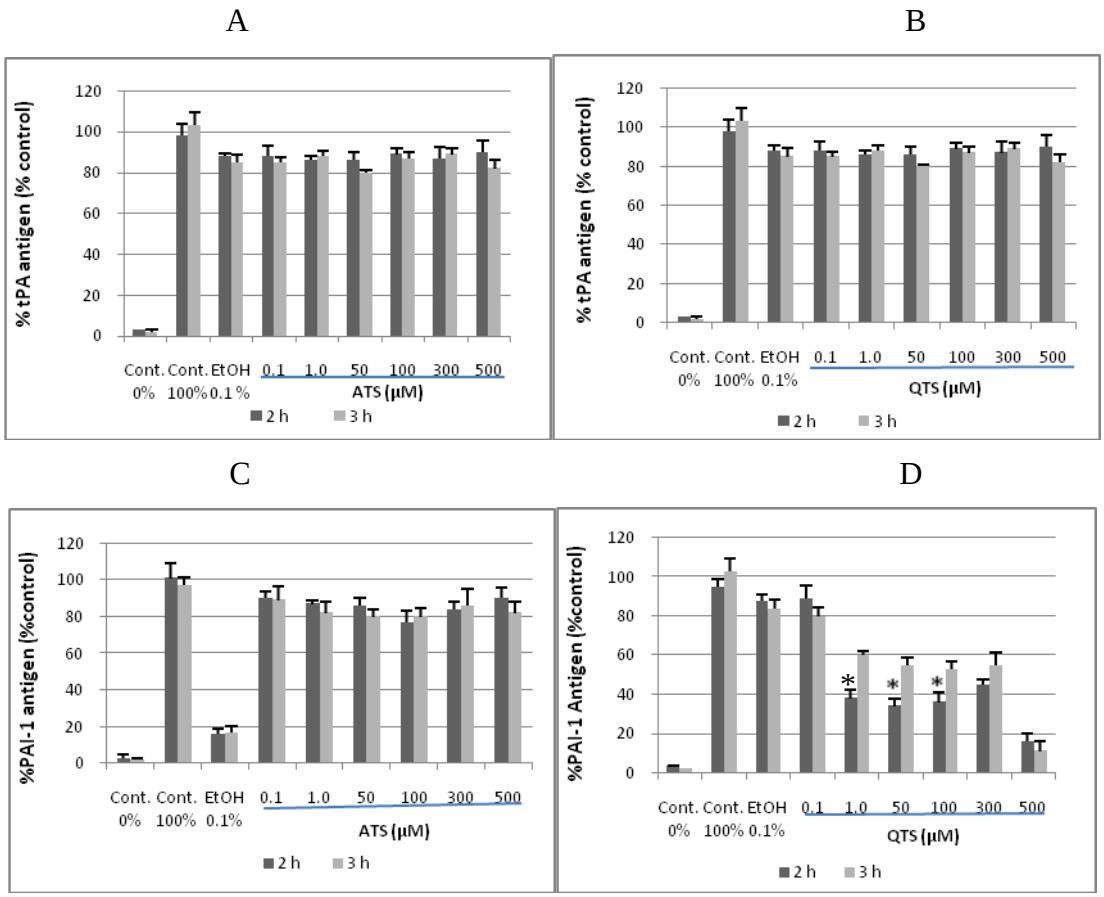


Fig. 2: Normal human fibroblasts pretreated with ATS or QTS for 2 or 3 h followed by a 24 h incubation with PMA (100 ng/mL) to induce tPA (1) or PAI-1 (2) expression, which was measured in culture supernatants using an ELISA assay. Values are expressed as a percentage of those obtained when cells were incubated with PMA (Control 100%) and vehicle. For 0 % of tPA expression (Control 0%) the fibroblasts were treated with vehicle without stimulation with PMA. For comparison purposes, EtOH was used based on its PAI-1 inhibitory capability. Data are representative of two independent experiments in triplicate, and are expressed as means ± S.D. Asterisks denote a statistically significant difference ($p < 0.05$) compared to Control 100%.

The effect of ATS and QTS on cell viability was also investigated since some flavonoids had been previously shown to be cytotoxic and had induced apoptosis [13]. Under our experimental conditions, it was found that only concentrations greater than 500 µM in both flavonoids showed a cytotoxic effect (data not shown).

The difference in the inhibitory activity between QTS and ATS is still unclear. The structural difference between both flavonoids could be the cause for their different activity, specifically the presence in position 3 of a sulphate or acetyl group, respectively, that impede the expression of PAI-1, it could be because their different electronegativity, based on the acid groups, bigger in the sulphate case [14]. Although the molecular basis for this modality of inhibition is currently unknown, the regulation of fibrinolytic system has been reported by other polyphenoles [15,16]. The compound QTS here studied which possesses a high degree of sulphatation is a natural flavonoid chemically related to quercetin. This last flavonoid was reported to induce a decrease of PAI-1 expression in the aortic endothelium in Sprague Dawley rats *in vivo*[17] and another report showed that it is able to increase the tPA and uPA antigen (2- to 3-fold) concomitant with a sustained augment in surface-localized fibrinolytic activity [18]. Although the net effect of QTS and quercetin is the same, i.e. increased fibrinolysis, the action mechanisms for which both compounds achieve this effect, are quite different. Further studies are required to cover this topic in order to determine whether the substituents sulphate group in QTS are responsible for profibrinolytic activity.

To our knowledge, this is the first finding highlighting the effects of sulphated flavonoids on the fibrinolytic system. The combination of antiplatelet aggregation, anticoagulant action, and profibrinolytic activities reported for QTS suggest that this compound may be effective in preventing thrombus formation through several pathways.

In conclusion, the principal findings of this study include, first, that QTS down regulates the expression of PAI-1 in culture supernatants of normal fibroblasts; second, tPA levels do not appear to be regulated by QTS and, third these effects are only observed for QTS but not for ATS. Finally, the profibrinolytic activity of QTS enhances its antithrombotic properties. All these considerations plus the

fact of the high solubility in water of this flavonoid makes QTS a strong candidate as an antithrombotic natural substance. Next steps involving animal models will be developed to progress in our investigations.

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DISCLOSURES

The authors state that they have no conflict of interests.

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