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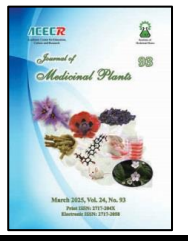

**Research Article**

# *Citrus sinensis*: Antithrombotic potential and safety concerns involving possible interactions based on in vitro coagulometric tests

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ABSTRACT

**Background:** *Citrus sinensis*, popularly called sweet orange, is widely used in Brazil for its calming properties, particularly in treating anxiety and insomnia, even among patients using warfarin. However, there is no scientific data supporting the safe concurrent use of both. **Objective:** To access the in vitro activity of *C. sinensis* ethanolic extract (EtEXT) and its fractions on blood clotting. **Methods:** The study used activated partial thromboplastin time (aPTT), prothrombin time (PT) and plasma fibrinogen measurement (PF), and thrombin generation test (TGT) to evaluate the effects. TGT, a highly sensitive assay, investigates overall changes in the hemostatic system and can evaluate the qualitative micromolecular chemistry of *C. sinensis* EtEXT and its fractions. **Results:** The EtEXTs of sweet orange leaves and their fractions were added to plasma pools at concentrations of 1.67 mg/ml, 2.26 mg/ml, and 2.86 mg/ml. Presence of phenolics as coumarins, flavonoids and tannins, as well as triterpenes was confirmed. At all concentrations, the extract increased PT and aPTT while decreasing PF and TGT, except for the hexane fraction. **Conclusion:** Our findings provide scientific evidence supporting both patient care in the context of warfarin use and the potential development of new antithrombotics. The leaves of sweet orange exhibited in vitro anticoagulant effects, likely due to interference in both extrinsic (EXT-path) intrinsic (INT-path) coagulation pathways. The function of the identified substances in these effects was also discussed, as well as the potential for using this extract in developing antithrombotic agents.

*Abbreviations:* aPTT, Activated Partial Thromboplastin Time; CS, Citrus Sinensis; DCM, Dichloromethane; EtAc , Ethyl Acetate; EtOH, Ethanolic extract; HEX, Hexane; HTF , High Tissue Factor; LTF, Low Tissue Factor; MG, Minas Gerais; ANOVA, One-Way Analysis Of Variance; PF , Plasma Fibrinogen Measurement; PT, Prothrombin Time; SD , Standard Deviation; SPSS, Statistical Package For The Social Sciences; TLC, Thin Layer Chromatographic; ETP, Thrombin Generation; TGT, Thrombin Generation Test; UPLC-FR-DAD, Ultra Performance Liquid Chromatography; WARF, Warfarin; H20, Water

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## 1. Introduction

*Citrus* genus, within Rutaceae family, encompasses several species of important fruit trees worldwide [1]. This genus, characterized by trees and bushes rich in vitamin C, includes species of significant nutritional and medicinal importance such as *Citrus sinensis, C. aurantium, C. limon, C. paradisi* and *C. reticulata* [1, 2]. *Citrus sinensis* (L.) Osbeck, popularly named sweet orange, represents the greatest citrus cultivar group, being responsible for 70% of the total citrus production. Although native to Asia, this plant has spread extensively in tropical, subtropical, and high-temperature regions [3].

The chemical composition of this species is complex, involving carotenoids, steroids, flavonoids, coumarins, phenylpropanoids, and other volatile compounds [1, 4]. Flavonoids, however, are particularly significant both quantitatively and qualitatively. Examples include flavanones such as hesperidin and naringenin, flavones like apigenin and rutin, and polymethoxylated flavones like nobiletin and tangeretin [4-7] (Supplementary Material 1).

In traditional/popular medicine, *C. sinensis* is applied to stimulate the immune system due to its high vitamin C content, and to treat conditions such as cold, bronchitis, anxiety, stress, constipation, colic, and even hypertension [1]. Experimental evidence has demonstrated pharmacological activities including antioxidant, antilipidemic, cardioprotective, antiproliferative and anticancer effects [3]. This species is one of the most used in Brazil; the tea of *Citrus* leaves is employed as a sedative for insomnia and anxiety [8].

Another study conducted in Brazil indicated that this species was frequently used by patients using warfarin (WARF) with atrial fibrillation diagnosis, in the form of leaf tea [9]. It is known that anticoagulation pharmacotherapy is complex and poses several challenges, including the potential for interactions with herbs [10-12]. Plants of the genus *Citrus* have already been implicated in interactions with WARF, such as *C. paradisi* (grapefruit) [13–16] and *C. limon* (lemon) [17]. Additionally, the sweet orange fruit has demonstrated in vitro antiplatelet activity [18], indicating a potential interaction with anticoagulants [19, 20].

From this perspective, studying the anticoagulant activity of *C. sinensis* can provide scientific evidence to support its potential interaction with anticoagulants and evaluate its potential as an antithrombotic agent, given its established antioxidant, anti-inflammatory, and antiplatelet activities. This study aimed to investigate the anticoagulant potential of the ethanolic extract (EtEXT) of *C. sinensis* leaves and their fractions through in vitro coagulometric tests, correlating the results with the chemical composition.

## 2. Methods

### *2.1. Obtention and preparation of the dried plant*

*Citrus sinensis* was collected in March 2017 in Pará de Minas (MG), Brazil, situated at 19°83'S and 44°60'W, at an elevation of 790 meters. A voucher specimen of *C. sinensis* was botanically identified in the Herbarium of UFMG and deposited with the identification number BHBC 178194.

#### *2.1.1. Elaboration of the Extract*

Ethanolic extract (EtEXT) of *Citrus sinensis* was dried and obtained as described by Leite et al., 2019 [19]. It was stored, until use, in a freezer. This extract was employed in coagulometric tests, chromatographic analyses, and fractionation. The fractions of EtEXT were elaborated using liquid-liquid extraction. In the first fractionation, 1 g of the EtEXT was added to 50 mL of water and extracted 3 times with dichloromethane. In the second fractionation, 1 g

of EtEXT was mixtured to 50 mL of water and extracted with solvents of increasing polarities: hexane and ethyl acetate. The fractions obtained were biomonitored by the in vitro TGT assay and analyzed by UPLC-FR-DAD.

*2.2. Chemical composition evaluation*

The existence of phenolic substances (coumarins, flavonoids, tannins) and sterols/triterpenes was evaluated by thin-layer chromatography (TLC) applying reagents with selectivity for each class of compounds: potassium hydroxide NP/PEG, ferric chloride, and Liebermann-Burchard, respectively [21]. The chromatographic profiles were acquired using ultra-performance liquid chromatography (UPLC) as described in the methods section of Leite et al. (2019) [19].

*2.3. Plasma sampling of participants*

Recruitment of participants as well as blood collection and processing happened in April 2017. The volunteers individually signed the the form giving free and informed consent, and all ethical guidelines were followed as per the approval of the institution's ethics committee. Participants were healthy women and men; aged 18 years or more; students and professors of the Faculty of Pharmacy, UFMG. They were not users of any drugs with the potential to modify the hemostatic system (e.g., anticoagulants, antiplatelets, non-steroidal anti-inflammatory drugs, and contraceptives). Twelve people were included in the study, as they met the eligibility criteria.

For all tests performed, low-platelet plasma was used. Plasma samples were obtained from the collection of 20 mL of whole blood in tubes containing sodium citrate (0.109 mol/L). These samples were centrifuged at 3000 g for 15 minutes until plasma separation. The 12 samples were mixed in a pool, and aliquots of 2 mL of the plasma pool (PP) were transferred to microtubes (stored frozen at -80°C). The control was the pure plasma pool, while the test samples included the plasma pool with the addition of the extract.

*2.4. Coagulometric Tests*

Sample preparation involved the addition of 5 mg of the dried EtEXT to 1 mL (NaCl 0.9%). The ultrasound bath was used for ten minutes to aid in dissolution. Aliquots of these preparations were then mixtured in 1 mL of the PP to obtain concentrations of 1.67 mg/mL, 2.26 mg/mL, and 2.86 mg/mL. The control was prepared using the same procedure with NaCl 0.9% replacing the extract. The activity on coagulation was measured by activated partial thromboplastin time (aPTT), prothrombin time (PT), plasma fibrinogen measurement (PF), and thrombin generation test (TGT). The employed methodology can be accessed in Leite et al., 2019 [20]. The intra-assay variation coefficients for PT, aPTT, PF, and TGT were 1.29%, 1.49%, 2.52%, and 0.34%, respectively.

TGT was executed using high tissue factor (HTF) and low tissue factor (LTF), reagents that vary in the amount of tissue factor. For analysis, only the ETP measure was used, representing the thrombin produced during the coagulation process and thus providing better clinical insights. Due to the robustness of TGT, this assay was used to biomonitor the fractionation of the EtEXT of *Citrus sinensis*.

*2.5. Ethical issued*

All procedures involving human participants were performed following the ethical standards of the institutional research committee (Ethical Committee of Universidade Federal de Minas Gerais, under the number 60904316.6.0000.5149).

*2.6. Test statistics*

The Statistical Package for the Social Sciences (SPSS), in its 13.0 version, was employed to data analysis. Tables 1-3 contain the experimental data demonstrated as mean ± standard deviation (SD), and one-way analysis of variance (ANOVA) with post-hoc Tukey HSD was used to evaluate significant differences between the control and the samples considering $P < 0.05$. All experiments were conducted in quadruplicate, yielding consistent results.

The EtEXT of *C. sinensis* showed in vitro anticoagulant activity in all (TGT, aPPT, PT and PF) the coagulometric tests conducted (Tables 1-3). In addition, all fractions obtained in fractionations 1 and 2 were effective in reducing thrombin generation (ETP) in TGT compared to control (Tables 2 and 3). Thrombin generation curves showing the difference in thrombin formation between control and samples of EtEXT of *C. sinensis* (CS) and fractions obtained in the fractionation 2 are shown in Figure 1.

## 3. Results

*3.1. Anticoagulant activity*

**Table 1.** The anticoagulant activity of *C. sinensis* in coagulometric tests: activated partial thromboplastin time (aPTT), prothrombin time (PT) and fibrinogen plasma measurment (PF)

| Coagulometric test | Concentration (mg/mL) | Mean | SD | p value |
|---|---|---|---|---|
| Prothrombin time (PT) (Control = 15.18 ± 0.34 s) | 1.67 | 17.13 | 0.95 | 0.000* |
| | 2.26 | 18.16 | 0.60 | 0.000* |
| | 2.86 | 10.88 | 0.34 | 0.000* |
| Activated partial thromboplastin time (aPTT) (Control=28.45 ± 0.39 s) | 1.67 | 35.81 | 2.52 | 0.000* |
| | 2.26 | 39.66 | 1.97 | 0.000* |
| | 2.86† | 40.43 | 1.32 | 0.000* |
| Fibrinogen plasma measurment (PF) (Control=11.97 ± 1.02 s) | 1.67 | 13.03 | 0.74 | 0.415 |
| | 2.26† | 14.07 | 0.94 | 0.015* |
| | 2.86† | 14.61 | 1.49 | 0.000* |

*Significative difference; †There is no significant difference between this and the previous concentration tested

**Table 2.** The anticoagulant activity of *C. sinensis* showed by ETP parameter (calculated as the area under the curve) using high tissue in high concentration as a trigger

| Thrombin generation parameters | Tested sample | Concentration (mg/mL) | Mean | SD | p value |
|---|---|---|---|---|---|
| **Ethanolic extract (EtEXT) in different concentrations** | | | | | |
| ETP (Control = 1503 ± 61 nM/min) | EtEXT | 1.67 | 712 | 149 | 0.000* |
| | EtEXT | 2.26 | 501 | 120 | 0.000* |
| | EtEXT | 2.86 | 216 | 214 | 0.000* |
| **Fractionation 1: water ($H_2O$) and dichloromethane (DCM)** | | | | | |
| ETP (Control = 1678 ± 92 nM/min) | EtEXT | 2.26 | 898 | 18 | 0.000* |
| | H2O | 2.26 | 1175 | 77 | 0.000* |
| | DCM | 2.26 | 739 | 15 | 0.000* |
| **Fractionation 2: water ($H_2O$), ethyl acetate (EtAc) and hexane (HEX)** | | | | | |
| ETP (Control = 1678 ± 92 nM/min) | EtEXT | 2.26 | 898 | 18 | 0.000* |
| | H2O | 2.26 | 1072 | 20 | 0.000* |
| | EtAc | 2.26 | 941 | 21 | 0.000* |
| | HEX | 2.26 | 1904 | 107 | 0.000* |

*Significative difference

**Table 3.** The anticoagulant activity of *C. sinensis* showed by ETP parameter (calculated as the area under the curve) using high tissue in low concentration as a trigger

| Thrombin generation parameters | Tested sample | Concentration (mg/mL) | Mean | SD | p value |
|---|---|---|---|---|---|
| **Ethanolic extract (EtEXT) in different concentrations** | | | | | |
| ETP (Control = 1487 ± 99 nM/min) | EtEXT | 1.67 | 732 | 49 | 0.000* |
| | EtEXT | 2.26 | 706 | 124 | 0.000* |
| | EtEXT | 2.86 | 596 | 76 | 0.000* |
| **Fractionation 1: water ($H_2O$) and dichloromethane (DCM)** | | | | | |
| ETP (Control = 1678 ± 2 nM/min) | EtEXT | 2.26 | 796 | 140 | 0.000* |
| | $H_2O$ | 2.26 | 982 | 21 | 0.000* |
| | DCM | 2.26 | 1148 | 22 | 0.000* |
| **Fractionation 2: water ($H_2O$), ethyl acetate (EtAc) and hexane (HEX)** | | | | | |
| ETP (Control = 1678 ± 2 nM/min) | EtEXT | 2.26 | 796 | 140 | 0.000* |
| | $H_2O$ | 2.26 | 1255 | 21 | 0.000* |
| | EtAc | 2.26 | 1150 | 23 | 0.000* |
| | HEX | 2.26 | 792 | 15 | 0.000* |

*Significative difference

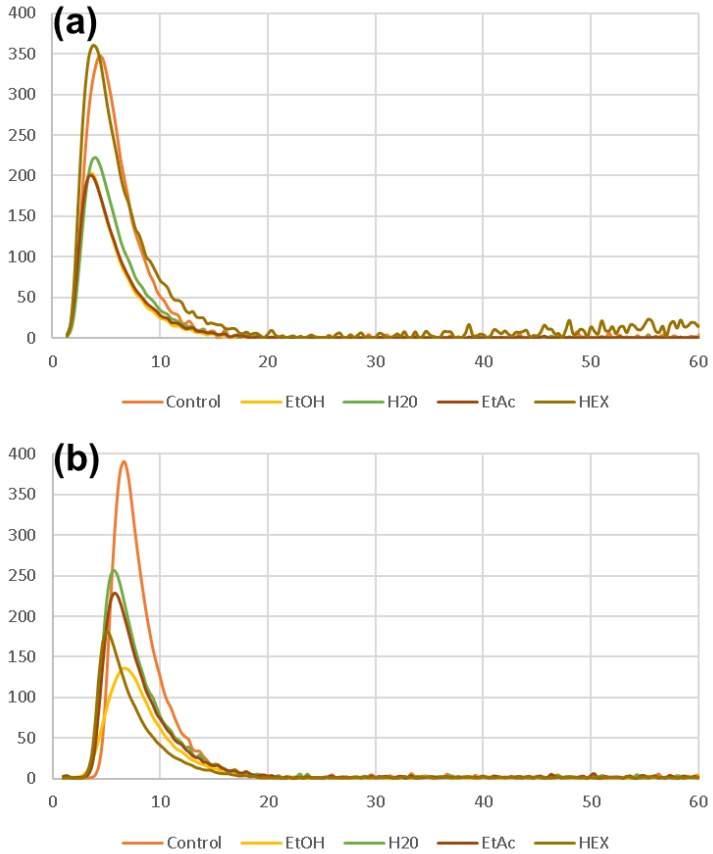


**Fig. 1.** Curves showing thromboin formation for control PP and PP added with ethanolic extract (EtEXT) of *C. sinensis* and its fractions elaborated in the fractionation 2: water ($H_20$), ethyl acetate (EtAc) and hexane (HEX), employing (a) HTF, and (b) LTF.

*3.2. Chemical composition analysis*

Screening obtained for the chemical composition performed by TLC and selective reagents demonstrated that EtEXT of *C. sinensis* has presented flavonoids, coumarins and sterols/triterpenes (Table 4), which corroborates the data of scientific literature [4–7]. In addition, water and ethyl acetate fractions showed coumarins, dichloromethane and hexane fractions showed sterols/triterpenes, while flavonoids were found in all fractions.

The UPLC-DAD analysis showed a predominance of polar compounds and confirmed the existence of flavonoids and coumarins in *C. sinensis* samples, as there were characteristic ultraviolet spectra distributed throughout the chromatogram (Supplementary material 1a) (Mabry, 1970). The fractionations were effective in separating three groups of the substances present in the EtEXT according to polarity, except for the major compound, with retention time (RT) of 7.2 min, which appeared in all fractions. In addition, ultraviolet spectra characteristic of flavonoids also appears in al fractions as well (Supplementary material 1b-d).

**Table 4.** Qualitative analysis obtained TCL showing the main classes of coumpounds (special metabolites) of *C. sinensis* ethanolic extract and fractions

| Special metabolites | Flavonoids | Phenolics/ Tannins | Coumarins | Sterols/ Triterpenes |
|---|---|---|---|---|
| *Citrus sinensis* | + | - | + | + |
| Fractionation 1: water | + | - | + | - |
| Fractionation 1: dichloromethane | + | - | - | + |
| Fractionation 2: water | + | - | + | - |
| Fractionation 2: ethyl acetate | + | - | + | + |
| Fractionation 2: hexane | + | - | - | + |

## 4. Discussion

It is well documented that the pharmacotherapy of anticoagulants has some safety limitations, especially concerning interactions [11, 22]. Additionally, interactions between drugs and medicinal plants are recognized as major clinical and economic issues for the health sector [23]. The most commonly reported interaction in adults is the risk of bleeding resulting from the concomitant use of ginkgo, garlic and ginseng with aspirin, WARF, and other antithrombotic drugs [23].

*C. sinensis* (sweet orange) is largely used by the Brazilian population, mainly as the tea froms the leave to relieve symptoms of anxiety and insomnia [8]. It has been demonstrated that patients using WARF in an anticoagulant clinic in Brazil also use sweet orange leaves for this purpose, posing an extra risk factor: the potential interaction between herbs and WARF [9, 10]. Thus, identifying possible interactions between sweet orange and anticoagulants is crucial to manage the patients on anticoagulation therapy with more effectiveness. Furthermore, preliminary knowledge about the anticoagulant potential of this plant may open avenues for evaluating *C. sinensis* as a new antithrombotic drug.

*4.1. Anticoagulation activity*

*4.1.1. Conventional coagulometric tests*

The combined analysis of PT, aPTT, and PF led to the conclusion that EtEXT of *C. sinensis* inhibited both coagulation pathways in vitro and reduced fibrinogen levels. These tests,

nevertheless, provide information only about the beginning of the coagulation complex process, as the end point happens with just 5% of all the thrombin formed [24–27]. Thus, TGT presents a broader analysis of the hemostatic system over 60 minutes, assessing hemostatic changes with greater sensitivity through the thrombin generated and providing more accurate information about the clotting process as a whole [28-30].

*4.1.2. Thrombin generation test*

TGT uses the Thrombinoscope software to generate a curve depicting the initiation, propagation, and inhibition of coagulation, demonstrating thrombin formation over 60 minutes. The software calculates five parameters, enabling a comprehensive assessment of blood coagulation [30]. Between the calculated parameters, ETP represents the thrombin produced during the entire coagulation process and is more clinically relevant, showing better the balance between anticoagulant and procoagulant forces [31, 32].

TGT has been applied to study the anticoagulant activity of medicinal plants, showing superiority over conventional coagulometric tests due to its informativeness, robustness, and sensitivity [19, 20]. In the consideration of the different concentrations of tissue factor employed, with HTF, coagulation initiates more quickly, focusing on modifications in the EXTpath. With LTF, coagulation initiation takes longer, showing greater sensitivity to alterations in the intrinsic pathway.

The addition of EtEXT of *C. sinensis* to plasma resulted in a reduction of ETP at all concentrations using both HTF and LTF. This indicates that adding EtEXT to plasma resulted in lower formation of thrombin, in a dose-dependent manner (Table 2, Table 3, and Figure 1). Furthermore, this shows inhibition of the EXTpath, supporting the PT results, and the INTpath, supporting the aPTT results.

Fractions of EtEXT obtained in fractionations 1 and 2 were also tested by TGT. Using HTF, all fractions, except the hexane fraction, significantly reduced ETP values and thrombin formation. In fractionation 1, the DCM fraction was even more effective in reducing ETP than *C. sinensis* EtEXT. In fractionation 2, the EtAc fraction most significantly reduced ETP, but EtEXT still showed higher activity than the fractions.

When using LTF, all fractions significantly reduced ETP and thrombin generation. In fractionation 1, the aqueous fraction was more effective in reducing ETP than the DCM fraction, but not compared to *C. sinensis* EtEXT. In fractionation 2, the HEX fraction most significantly reduced ETP, with activity similar to EtEXT.

Analysis of ETP values suggests that EtEXT of *C. sinensis* and its fractions demonstrated considerable in vitro anticoagulant activity by decreasing thrombin formation, apparently due to the inhibition of both coagulation pathways. These in vitro results suggest a potential interaction between sweet orange and anticoagulants, such as WARF, thereby increasing the risk of bleeding in patients using both concomitantly.

*4.2. Fractionation of C. sinensis*

The biological and pharmacological activities of a plant species directly depend on its constituent substances. Therefore, the chemical composition of the EtEXT and fractions of *C. sinensis* were studied to identify substances reffered to the observed activity. The complexity of plants chemistry, and therefore their extracts, often promotes a synergistic effect among the compounds, producing a better therapeutic effect

compared to substances used in isolation [33]. Although classes of chemical substances have been discussed in isolation, it is likely that the phytocomplex (a group of interacting substances) promotes the observed anticoagulant activity in vitro [11, 13].

Analyzing the TLC and UPLC chromatographic profiles for the EtEXT and its fractions revealed that coumarins were detected in the EtEXT and the most polar aqueous fractions (Table 4). Sterols/triterpenes were found in the extract and in the less polar fractions: dichloromethane, ethyl acetate, and hexane (Table 4). Flavonoids were present in the EtEXT and all of the fractions (Table 4) due to their intermediate polarity, which may vary based on the ligands around the basic skeleton. This result was expected as fractionation separates substances according to their affinity for the solvent.

Relating the separation of substances by fractionation with the anticoagulant activity of each fraction, it is possible to speculate on the substances responsible for the observed activity. Considering the extrinsic coagulation pathway, assessed using HTF (Table 2), it is clear that aqueous fractions 1 and 2, rich in more polar compounds such as coumarins and some flavonoids, were active but less so than the ethanol extract. Fractions with medium to low polarity solvents (EtAc and DCM, respectively) were closest to EtEXT in anticoagulant activity in the extrinsic pathway and are rich in flavonoids and sterols/triterpenes. However, the hexane fraction, containing fewer flavonoids and sterols/triterpenes, increased thrombin generation potential. Thus, flavonoids seem to play a crucial part in the anticoagulant activity shown in the extrinsic pathway.

Considering the intrinsic pathway evaluated using LTF (Table 3), the highest activities were observed in aqueous fractions 1 and the hexane fraction, suggesting the participation of both more and less polar compounds. This result clearly shows that the observed activity for EtEXT in the intrinsic pathway is due to the collective substances present in the extract.

In conclusion, the results indicate that flavonoids are primarily responsible for inhibiting the extrinsic pathway, while the phytocomplex appears to be more active in the intrinsic pathway. Flavonoids appear to be essential in the observed anticoagulant activity in vitro, while coumarins and sterols/triterpenes play a more secondary role. However, a more elaborate fractionation could better identify the specific compounds related to the activity.

*4.2.1. Coumarins*

Coumarins were identified by TLC, which was also supported by UPLC analyses. They are o-hydrocinnamic acid lactones from the shikimate pathway [34] (Figure 2) and have been associated with various pharmacological properties [35], and both coumarins and furanocoumarins are well distributed in the genus *Citrus* [36].

As WARF is a coumarin derivative, there is speculation that this class possesses anticoagulant effects. However, this effect would not be detected in the tests performed since WARF acts in the liver. Nevertheless, coumarins may inhibit clotting factors similarly to flavonoids, as both classes share a structural portion derived from the shikimate pathway, enabling similar chemical interactions with receptors [19]. Therefore, despite the lack of scientific information supporting the direct effect of coumarins on the coagulation cascade, flavonoids themselves have been extensively studied and implicated in the direct effect on

blood clotting [37] and will be discussed in more detail below.

*4.2.2. Flavonoids*

Flavonoids are considered to be of mixed biosynthetic origin, formed by three-membered rings [34]. In the chemistry analysis, they are the predominant substances in *C. sinensis* extract and fractions. The major compound in all samples presents a retention time (RT) of 7.2 minutes and a characteristic UV spectrum of a flavanone (λMAX = 283 nm referring to band II and low-intensity band I), possibly hesperidin or naringenin (Malby, 1970) [38] (Supplementary Material 1).

This class has significant anticoagulant potential. Flavonoids are known to be anti-inflammatory, as the complex inflammation pathway shares a path with platelet aggregation/coagulation through the arachidonic acid pathway [11, 12]. Flavonoids like luteolin and apigenin have also been associated with antiplatelet activity by inhibiting platelet adhesion, aggregation, and secretion [39, 40], that promotes blood coagulation through the mechanical pathway. Additionally, flavonoids have demonstrated anti-thrombin activity in vitro, and there are studies that evaluated the structure-activity of flavonoids as thrombin inhibitors. Some, like luteolin, have shown antithrombotic activity in vitro and in vivo [41, 37]. Given the large amount of flavonoids in sweet orange and the various possible mechanisms of coagulation inhibition, this class is likely related to the observed in vitro anticoagulant activity.

*4.2.3. Sterols/Triterpenes*

Sterols and triterpenes are compounds that belong to the class of terpenes and are formed of five-carbon units called isoprenes. Triterpenes contain 30 carbon atoms, and steroids are modified triterpenes [34]. These compounds are less polar and do not absorb at the wavelength used in UPLC analysis, being detected only by TLC. Nonetheless, there is substantial scientific evidence supporting their participation in the observed anticoagulant activity. Studies indicate that both sterols and triterpenes present anti-inflammatory activity [42, 43]. In vitro, and also in vivo, tests have shown that some triterpenes affect phospholipase A2 and COX-2, which are part of the pathway involving arachidonic acid and thromboxane A2, responsible for platelet aggregation [44, 45]. Triterpenes from *Ilex rotunda* inhibited ADP-induced platelet aggregation in rats [46], while triterpenes from *Melaleuca bracteata* showed antiplatelet and anticoagulant activity in rats [47]. Additionally, a triterpene from *Protiterhus longifolia* increased bleeding time in rats and exhibited anti-inflammatory activity [48].

*4.3. Use of Citrus in anticoagulated patients*

There are no studies that contraindicate the use of *C. sinensis* leaf tea, even in patients on anticoagulants. This is likely because its use is primarily associated with folk medicine, and there are no commercial herbal medicines derived from this plant [8]. However, given the high use of this plant by anticoagulated patients and evidence showing that other *Citrus* species can interact with WARF, it is crucial to be conscious of the potential interaction amongst *C. sinensis* and anticoagulants [13, 16, 17].

Because WARF pharmacotherapy is complex, it is recommended that patients be assisted in anticoagulation clinics, places where clinical management and dose adjustments are performed [49, 50]. However, investigating interactions between drugs and medicinal plants is uncommon, mainly due to a lack of professional training and scientific evidence. Studies

evaluating the anticoagulant activity of certain plants can provide useful information to health professionals in anticoagulation clinics, guiding patients on the non-concomitant use of plants like sweet orange that may interact with anticoagulants [19, 51].

Clinical management of interactions significantly improves the quality of patient treatment. Regardless of the lack of scientific information about herbal-drug interactions, potential interactions may be initially based on in vitro studies, case reports, and the herb's chemical composition [11, 51]. This enables health professionals to be more vigilant about the utilization of herbal medicine with potential interactions with anticoagulants [19, 20, 52].

*4.4. Antithrombotic agent: The potential of C. sinensis*

Thromboembolic disorders are characterized by the formation of clots in blood vessels, which is considered a major cause of cardiovascular diseases. In 1856, Virchow suggested a triad of factors leading to thrombosis including stasis, modifications in the vessel wall, and blood coagulability (Figure 2) [53, 54]. Cardiovascular diseases are, however, preventable through lifestyle changes. Additionally, drugs with anti-inflammatory, antiplatelet, antioxidant, and anticoagulant activities can help prevent thrombus formation [54, 55].

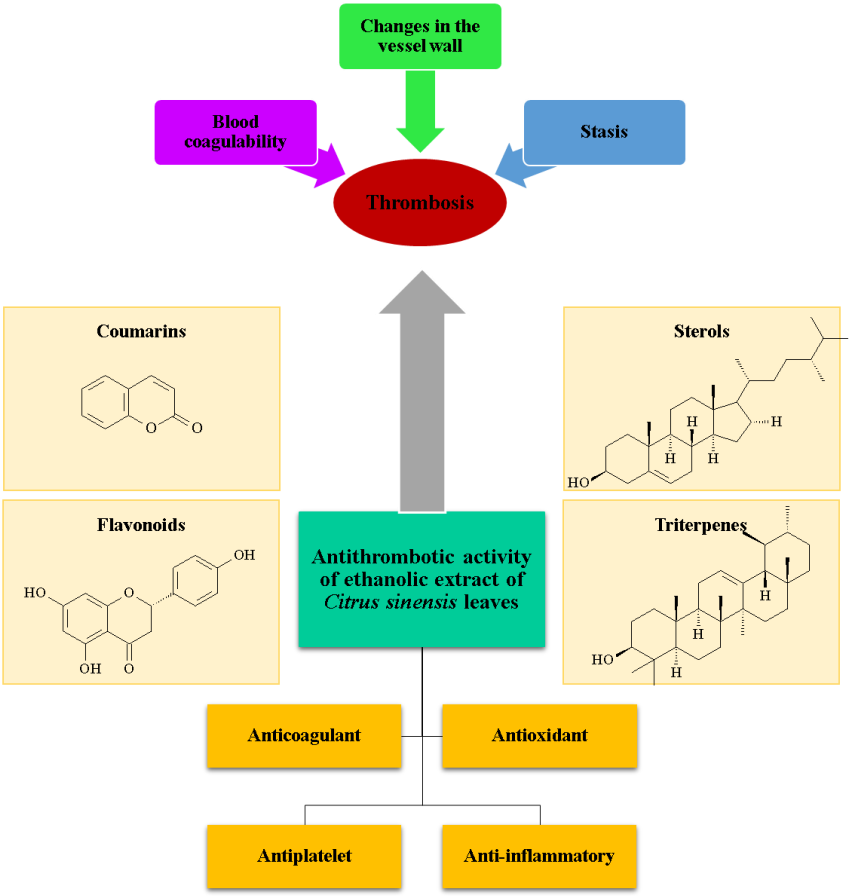


**Fig. 2.** Scheme demonstrating the possible sinergism between *C. sinensis* phytocomplex compounds for antithrombotic activity

As discussed, sweet orange has the chemistry with potential to perform the described activities, primarily due to the large amount of flavonoids in its chemical composition (Figure 2). Coumarins show evidence of anticoagulant activity [19]. Sterols have proven anti-inflammatory activity [42], while triterpenes have demonstrated anti-inflammatory, anticoagulant and antiplateletactivities [44, 47]. Finally, flavonoids are konown to present highly antioxidant and anti-inflammatory chemical substances and they also have already been described as antiplatelet and anticoagulant agents [56–58]. Additionally, plant sterols have been shown to lower cholesterol levels, which is beneficial in preventing cardiovascular disease [43].

Given that *C. sinensis* leaves are widely used in tea form and that this plant, according to its chemical composition, has great capability to exert anti-inflammatory, antioxidant, antiplatelet, and anticoagulant activities, further studies could lead to the development of an easily accessible antithrombotic for the population [54, 55]. In vitro models are excellent for guiding research efforts, but they have some limitations, making it essential to conduct studies in humans. Furthermore, it is important to delve deeper into the chemical composition of *C. sinensis* and the relationship of these compounds with each stage of blood coagulation. This study is a first step towards developing new antithrombotics.

## 5. Conclusion

The integrated analysis of the results demonstrates that the ethanolic extract (EtEXT) of *C. sinensis* leaves exhibited anticoagulant (in vitro) activity by increasing activated partial thromboplastin and prothrombin time, and thrombin formation and also fibrinogen levels. Evidence from the literature, combined with the results of coagulometric tests, supports the theory that the observed effect may be the result of coagulation factors inhibition. This inhibition is likely a result of the synergistic effects of various chemical substances found in sweet orange, such as flavonoids, coumarins, and sterols/triterpenes. Consequently, the intake of sweet orange by patients undergoing anticoagulation therapy needs to be approached with caution due to the risk of interactions enhancing the anticoagulant activity and increasing the risk of hemorrhage. Additionally, evidence indicates that *C. sinensis* also exhibits antioxidant, anti-inflammatory, and antiplatelet activities. Therefore, further study of these activities could lead to the development of drugs with multiple functions, including antithrombotic properties.

## Author contributions

PML, APNM, JMA and RCFD performed the experiments and analyzed its data. PML, AAF, MGC and ROC designed the article; extracted, analyzed data and drafted the manuscript. PML extracted and analyzed data. All authors critically reviewed the manuscript, contributed to its revision, and approved the final version submitted.

## Conflict of interest:

The authors declare that there is no conflict of interest.

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