

ANTI-COAGULANT PROPERTIES OF FLAVONOID COMPOUNDS: POTENTIAL STRUCTURE-FUNCTIONAL RELATIONSHIP

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ABSTRACT

Objective: Flavonoids, naturally-occurring compounds in fruits and vegetables, possess anti-coagulant property. However, a very few studies were attempted to understand how flavonoid structure influences its anti-coagulation property, such as clotting time. In this study, we investigated structurally similar flavonoid compounds which differ in the number of hydroxyl groups and compared their anti-coagulation properties.

Methods: We selected and evaluated five flavonoid compounds, that is, chrysin, apigenin, luteolin, kaempferol, and quercetin, for their anti-coagulant properties using *in vitro* prothrombin time (PT) assays and activated partial thromboplastin time (APTT) assay.

Results: Our findings suggested that quercetin, kaempferol, and luteolin showed a significant anti-coagulant effect on APTT ($p < 0.05$) in a dose-dependent manner. The dose of 500 μ M quercetin showed potent prolong APTT with 37.43 ± 1.60 s, followed by 500 μ M of kaempferol and luteolin (34.63 ± 1.29 s and 4.83 ± 1.56 s, respectively). Furthermore, a combination of 500 μ M of quercetin with 0.25 U/ml of heparin demonstrated prolong APTT (52.16 ± 5.18 s) when compared with individual effects of either 0.25 U/ml heparin (33.4 ± 0.50 s) or 500 μ M quercetin (37.43 ± 1.62 s) alone.

Conclusion: Our results demonstrated that numbers of the hydroxyl group on flavonoid compounds influence anti-coagulation properties. In addition, the prolonged APTT assay results suggested that quercetin, kaempferol, and luteolin could affect factors VIII, IX, XI, and XII of intrinsic pathway. Moreover, the synergistic effect of quercetin further enhances the heparin anti-coagulation effect. Based on our findings, we recommend that the consumption of vegetables and fruits rich in quercetin, luteolin, and kaempferol could help prevent thrombotic stroke in high-risk patients.

Keywords: Flavonoids, Quercetin, Activated partial thromboplastin time assay, PT assay, Anti-coagulant.

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INTRODUCTION

Non-communicable diseases, including diabetes mellitus (DM) and hypertension (HT), have been increased in both developing and developed countries [1]. DM and HT are well-established risk factors for stroke, wherein pathological conditions worsen blood vessel damage and stimulate excessive blood clotting [2-4]. In contrast, blood clotting is an important event during trauma and other vascular damages, where it plays pivotal role to stop bleeding and consequently seals up vascular damage, thus prevent blood loss. Two primary blood clotting pathways are (1) intrinsic pathway (factors VIII, IX, XI, and XII) and (2) extrinsic pathway (factor VII). Both pathways induce fibrin clot formation [5-7]. Patients with DM and HT are being treated with warfarin, heparin, and aspirin to prevent blood clotting and thus avoid life-threatening stroke condition [8]. In contrast, long-term use of these medical drugs on anti-coagulant treatment caused hemorrhagic risk [9]. This study was conducted to explore anti-coagulant ability of natural flavonoid compounds, and their potential being a safer alternative to prescribed anti-coagulant drugs.

Flavonoids, a natural-occurring compounds in fruits and vegetables, have been known for anti-platelet and anti-coagulant properties, both *in vitro* and *in vivo* [10]. However, the structure-functional relationship and possible mechanistic role of flavonoids as an anti-coagulant agent are not well studied. Our objective of this study was to investigate and compare the effect of flavonoids (chrysin, apigenin, luteolin, kaempferol, and quercetin) on the hemostasis through extrinsic and intrinsic pathways using *in vitro* prothrombin time (PT) assays and activated partial thromboplastin time (APTT) assay, respectively. In this study,

we investigated anti-coagulant properties of flavonoids with different numbers of hydroxyl groups. We also investigated potential synergetic effect of flavonoids and a low dose of heparin, which can confirm if eating a diet rich in fruits and vegetables containing flavonoids could promote stroke prevention in hypertension and diabetic patients [11].

MATERIALS AND METHODS

Chemicals and reagents

Chrysin, apigenin, luteolin, kaempferol, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures are shown in Fig. 1. Dimethyl sulfoxide (DMSO) was obtained from Merck (MA, USA).

Blood sampling and plasma preparation

Ten milliliters of venous blood samples were obtained from healthy volunteers without a history of bleeding or thrombosis ($n=10$, aged 18–30 years) according to human blood collection which was approved by the Office of the Human Research Ethics Committees of Walailak University (protocol no. WUEC-18-024-01). Venous blood samples were transferred to blood collection tube (BD Vacutainer® sodium citrate tubes, Becton, Dickinson and Company, Franklin Lakes, NJ) containing 0.105 M sodium citrate (9:1 v/v, blood: anti-coagulant) and then subjected to centrifugation (800 g, 10 min, and 25°C). The supernatant plasma was transferred to a new tube and stored at -80°C until use.

PT activity assay

PT assays were carried out following the coagulometer protocols as previously described [12] with modifications for each sample using

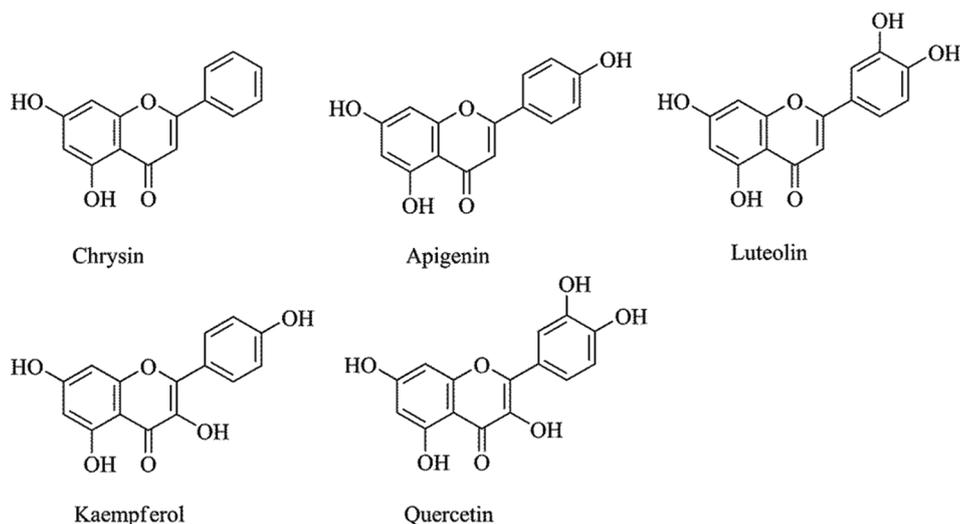


Fig. 1: The chemical structures of test compounds

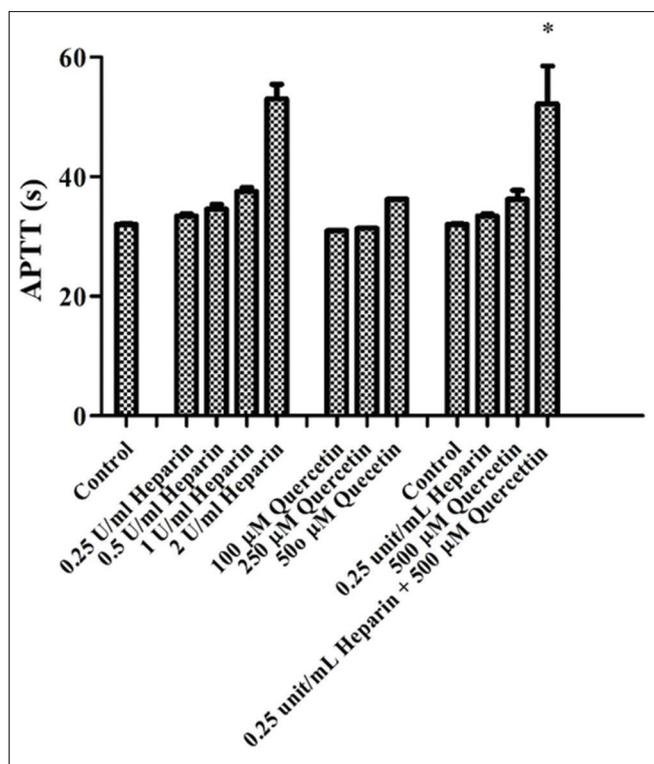


Fig. 2: Combined effect of quercetin and heparin on APTT assay. *p<0.05, compared to individual effects (0.25 U/ml heparin and 500 µM quercetin)

an automatic coagulation instrument (Humaclot Duo Plus, HUMAN, Wiesbaden, Germany). Briefly, 90 µl of plasma sample was incubated with 10 µl of test compounds (Chrysin, apigenin, luteolin, kaempferol, and quercetin) diluted in normal saline (final concentration of 250 and 500 µM, 37°C). After 5 min pre-incubation, 100 µl of PT assay reagent (Thromborel® S, Siemens Healthcare Diagnostics Products GmbH, Germany) were added to initiate the reaction assay and the clotting time was measured. One percent DMSO was used as vehicle control and heparin (2 IU/ml) (Cristalia®, Itapira, SP, Brazil) was used as a positive control.

Table 1: Effect of flavonoids on anti-coagulant activity based on PT and APTT of normal human plasma

Compounds	Concentrations	PT (s) ^a	APTT (s) ^a
Vehicle control	0.5% DMSO	15.23±0.05	32.03±0.20
Heparin (Positive control)	1 IU/ml	14.60±0.57	37.50±0.96
	2 IU/ml	15.70±0.37	53.00±3.48
	3 IU/ml	17.53±0.24	>200
Chrysin	250 µM	15.07±0.41	31.87±0.67
	500 µM	14.50±0.00	32.40±0.21
Apigenin	250 µM	14.83±0.65	32.03±0.20
	500 µM	14.40±0.36	33.06±0.95
Luteolin	250 µM	13.83±0.05	29.83±1.01
	500 µM	14.10±0.57	34.83±1.56*
Kaempferol	250 µM	14.25±0.05	31.20±0.28
	500 µM	13.90±0.50	34.63±1.29*
Quercetin	250 µM	14.70±0.20	31.37±0.21
	500 µM	6.30±0.85	37.43±1.60*

^aMean±SD (n=10). *p<0.05, compared with the vehicle control group. PT: Prothrombin time, APTT: Activated partial thromboplastin time

APTT activity assay

In APTT activity assay, 90 µl of plasma sample collected from human volunteers were mixed with 10 µl of test compound (final concentration of 250 and 500 µM) at 37°C. After 5 min, pre-warmed aPTT reagent (Actin® FS APTT, Siemens Healthcare Diagnostics Products GmbH, Germany) was added to the mixture. Clotting time was measured after the addition of 50 µl of CaCl₂ solution using automated coagulometer (Humaclot Duo Plus, HUMAN, Wiesbaden, Germany). One percent DMSO was used as vehicle control and 1 IU/ml heparin (Cristalia®, Itapira, SP, Brazil) was used as a positive control.

Statistical analysis

All data were expressed as means±SD of three measurements. Statistical comparisons of the prolonged time between test compounds and vehicle control (1% DMSO) were achieved by non-parametric indecent t-test using GraphPad Prism version 5.0 (San Diego, California). p-value <0.05 was considered statistically significant.

Anti-coagulation assays

Although flavonoids have been reported to prolong anti-coagulant effect on human blood sample – both *in vitro* and *in vivo*, the structure-functional relationship of flavonoids (e.g., chrysin, apigenin, luteolin, kaempferol, and quercetin) has not been studied. We first determined

whether the prolongation of APTT and PT was due to the structure-functional relationship of chrysin, apigenin, luteolin, kaempferol, and quercetin on healthy volunteer blood samples. All these flavonoids share the same backbone structure and differ in numbers of hydroxyl groups primarily. To differentiate the anti-coagulant mechanistic properties of flavonoids through extrinsic and/or intrinsic pathways, PT and APTT assays were performed, respectively. In PT and APTT assays, each flavonoid was incubated with a human blood sample for 5 min with different final concentrations of 250 and 500 μM and prolonged PT and APTT time were measured. As shown in the Table 1, an average PT and APTT on healthy volunteers were 15.23 ± 0.05 s and 32.03 ± 0.20 s, respectively. Heparin was used as a positive control in this study. The results of PT and APTT assays demonstrated that flavonoids contained the higher anti-coagulant effect on APTT compared to PT assay, thus indicating that these flavonoids might interfere with intrinsic clotting cascade. Among test compounds, luteolin, kaempferol, and quercetin showed a significantly prolonged time of APTT assay in a dose-dependent manner. The dose of 500 μM quercetin showed the potent prolong APTT (37.43 ± 1.60 s), followed by 500 μM of kaempferol (34.63 ± 1.293 s) and luteolin (4.83 ± 1.56 s).

The combined anti-coagulant effect of quercetin and heparin

Among flavonoids tests, quercetin exhibited the potent anti-coagulant activity against APTT assay. It is thus, we further determined the combined anti-coagulant effect of quercetin and heparin (a prescribed drug for anti-coagulant), whether a reduced dose of heparin in blood clotting formation. We then combine the low dose of heparin (0.25 U/ml) with the various concentration of quercetin (100, 250, and 500 μM) in healthy blood plasma before the addition of anti-coagulant agent. The dose of 0.25 U/ml of heparin and 500 μM of quercetin could prolong aPTT with 52.16 ± 5.18 s when compared to individual effects (0.25 U/ml heparin (33.4 ± 0.50) and 500 μM quercetin (37.43 ± 1.62)) (Fig. 2) suggesting that quercetin and heparin might have synergistic effects against blood clotting.

DISCUSSION

In the present study, we first determined the anticoagulant effect of flavonoids including chrysin, apigenin, luteolin, kaempferol, and quercetin, (all are naturally occurring flavonoid compounds found abundantly in the fruits in vegetables) on blood samples collected from healthy volunteers. Our results demonstrated anti-coagulant effect of luteolin, kaempferol, and quercetin on blood clotting through APTT assay, but not PT assay. These contrasting outcomes indicated that flavonoids could affect the intrinsic coagulation pathway and not extrinsic pathway.

On the beginning of the 21st century, the number of patients with non-communicable diseases, including DM, HT, and stroke, has been increased from time to time [1-3]. These diseases could damage blood vessel and finally, blood clotting formation, leading to morbidity and mortality associated with stroke and cardiovascular [13]. Alternative medicines, such as herbal medicines, have been considered as a good source of anti-coagulant agents [14]. At present, anti-coagulation research community is focused on identifying plant-based materials with anti-coagulant activities, to prevent early stroke recurrence during blood vessel damage in DM and HT patients [14]. Flavonoids, natural-occurring substances in fruits and vegetables, have been focused and previously been reported containing anti-coagulant effect both *in vitro* and *in vivo*.

Based on our results, where potent inhibitory effect of quercetin on clotting formation was observed, we further studied and determined that the combined effect of quercetin and heparin was more effective. As shown in Fig. 2, at low concentration of heparin 0.25 IU/ml contained low anti-coagulant activity; however, when we mixed 0.25 IU/ml heparin together with 500 μM quercetin, the prolonged clotting time is significantly increased compared to either 500 μM quercetin or 0.25 IU/ml heparin alone. The synergistic effect could be contributed due to differences in the mechanism of action of quercetin and heparin. Heparin reversibly binds to antithrombin III in the intrinsic pathway, resulting in the inactivation of blood clotting [15], whereas quercetin

potentially interferes with complex VIII and XI activity. Complex VIII and XI play a role in the intrinsic blood clotting cascade [16]. Therefore, we believe that the different anti-coagulation activation targets of heparin and quercetin potentially increase the prolonged time of APTT assay.

As shown in Table 1, chrysin and apigenin, which contain 2 and 3 hydroxyl groups, respectively, did not confer anti-coagulant activity. However, luteolin and kaempferol, containing 4 hydroxy groups, have shown increased prolonged clotting time, and quercetin containing 5 hydroxy groups exhibited the most potent anti-coagulant effect in our findings. The data clearly suggested that the number of hydroxyl groups in the flavonoids could potentially contribute to anti-coagulation properties. Our current finding is supported by previous study, where Manolove *et al.* demonstrated that the position of the hydroxyl group in the other non-flavonoid molecules confers anti-coagulant activity of the compounds [15]. The results of prolonged APTT implied that luteolin, kaempferol, and quercetin may affect factors VIII, IX, XI, and XII in the intrinsic pathway. This result is similar to the previous report of Kuntic *et al.*, 2011 that rutin (quercetin-3-rutinoside) has been shown to inhibit factors VIII and IX of the intrinsic pathway [17].

CONCLUSION

We demonstrate that quercetin possesses the highest anticoagulant properties among tested flavonoids. We also demonstrated that the numbers of hydroxyl group in flavonoid compounds could affect their anti-coagulant properties. Based on our data and findings, we propose that consumption of vegetables and fruits containing flavonoids (including quercetin, luteolin, and kaempferol) could potentially prevent thrombotic stroke, especially in the DM or HT patients. Moreover, eating flavonoid-abundant fruits and vegetables might help lower the use of prescribed drugs for stroke prevention in DM and HT. The *in vivo* study of combining quercetin with chemotherapeutic drugs, for example, heparin needs to be further elucidated.

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CONFLICTS OF INTEREST

All authors have none to declare.

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