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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 µM quercetin showed potent prolong APTT with 37.43±1.60 s, followed by 500 µM of kaempferol and luteolin (34.63±1.29 s and 4.83±1.56 s, respectively). Furthermore, a combination of 500 µM 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 µM 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 compere 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

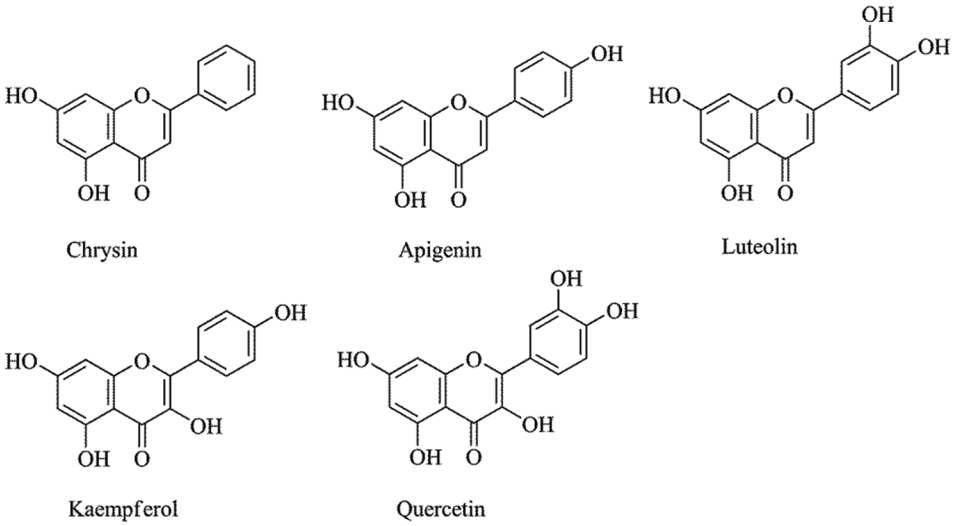

Fig. 1: The chemical structures of test compounds

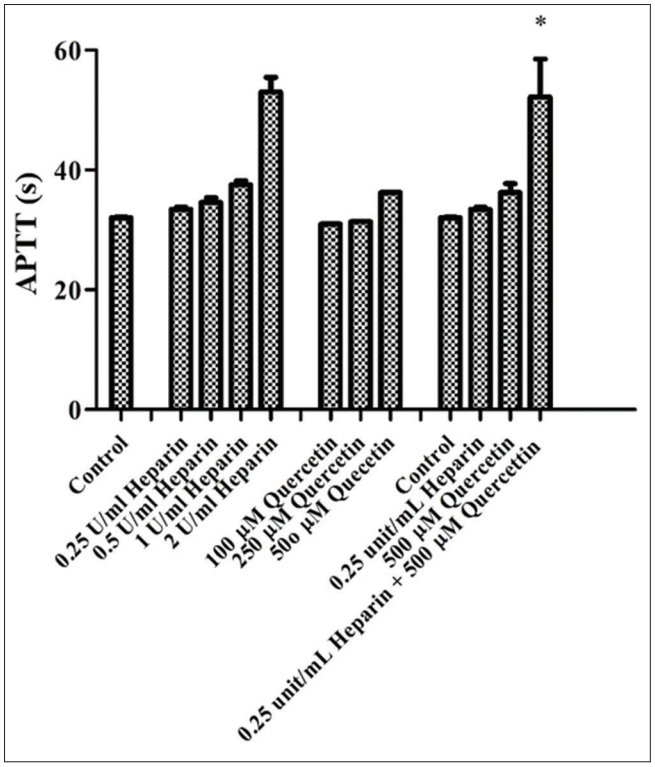

Fig. 2: Combined effect of quercetin and heparin on APTT assay. *p<0.05, compared to individual effects (0.25 U/ml heparin and 500 µM quercetin)

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 sassy 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*

[a]Mean±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 $CaCl_2$ 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