IN VITRO ANTIPLATELET AND ANTICOAGULANT ACTIVITY OF INDIGENOUS VEGETABLES FROM SOUTHERN THAILAND

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ABSTRACT

Objective: Epidemiological studies have indicated that diets rich in fruits and vegetables help reduce the risk of cardiovascular diseases (CVDs). However, data about the antithrombotic activity of local vegetables is rare. The objective of this study was to evaluate antiplatelet and anticoagulant activity in indigenous vegetables with high phenolic compounds collected from Southern Thailand.

Methods: Five selected indigenous vegetables were crudely extracted by distilled water and 80% methanol. The extracts were screened for in vitro antiplatelet and anticoagulant activity at a concentration of 10 µg/µl. The antiplatelet activity was measured by inhibition of platelet adhesion to collagen and thrombin-induced platelet aggregation, while the anticoagulant activity was assessed by the prothrombin time (PT) and activated partial thromboplastin time (APTT) tests.

Results: Among the selected vegetables, the extracts of mon-pu (Glochidion perakensse Hook.) and young cashew leaves (Anacardium occidentale L.) showed high antithrombotic properties. The highest antithrombotic activity was observed in the methanol extract of mon-pu, which showed 92.79±0.78% of platelet adhesion inhibition, 102.9±1.53% of platelet aggregation inhibition, and a prolonged APTT assay (48.92±0.94 s). The prolonged APTT but normal PT results suggested that the extract could affect factors VIII, IX, XI, and XII of the intrinsic coagulation pathway.

Conclusion: Our findings demonstrated antiplatelet and anticoagulation properties of indigenous vegetables from Southern Thailand. The multipotential effects of mon-pu extracts on antithrombosis evidently suggest that mon-pu can be considered as an excellent nutraceutical option in the prevention of thrombosis-related CVDs caused by different mechanisms.

Keywords: Indigenous vegetables, Antiplatelet activity, Anticoagulant activity, Southern Thailand.

INTRODUCTION

Cardiovascular diseases (CVDs), a group of disorders of the heart and blood vessels, are the most common cause of death globally. Heart attacks and strokes are common events that are caused by sudden blockage of blood flowing to the heart or brain. Its pathogenesis involves fatty deposits inside the wall of the blood vessels and an increased risk of blood clots. It is very well known that thrombus formation after atheroma plaque rupture can be an important step in the pathophysiological mechanisms underlying CVDs, involving activated platelet adhesion, platelet aggregation, the coagulation system, and the fibrinolytic system. In individuals with risk factors such as an unhealthy diet, hyperlipidemia, hypertension, and diabetes, there is an increased risk of developing CVDs [1].

In recent years, the prevention of chronic diseases has mainly focused on fruit and vegetable consumption. Epidemiological data have indicated that a regular diet rich in fruits and vegetables can promote cardiovascular health as well as reduce the risk of CVDs [2]. The effect of fruit and vegetable consumption on health is related to phytochemical diversity; for example, dietary fiber, folate, vitamins, and phenolic compounds. Among these substances, phenolic compounds have been suspected to prevent vascular dysfunction and to have effects on platelets, endothelial functions, and the coagulation system [3].

In the southern region of Thailand, the climate is tropical and humid throughout the year. Thus, this area has indigenous vegetable diversity that is essential to local foods. In addition, these indigenous vegetables have been used for the supply of local medicine, for instance, in the treatment of diabetes, arthritis, hypertension, and gastrointestinal diseases [4,5]. Thai indigenous vegetables have been reported to be useful sources of antioxidants, and many also possess anti-diabetic, anti-bacterial, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties [6,7]. On the other hand, information about the antithrombotic activity of southern Thai indigenous vegetables is limited. Some fruits and vegetables have demonstrated properties to inhibit platelet activation and coagulation activity. Antiplatelet activity has been described for garlic (Allium sativum L.) [8], onion (Allium cepa L.) [9], tomato (Solanum lycopersicon Mill.) [10], and green bean (Phaseolus vulgaris L.) [11]. Anticoagulant activity has been observed in pineapple (Ananas comosus (L.) Merr.) [12], grape (Vitis vinifera L.) [11], and raspberry (Rubus idaeus L.) [11]. Thus, if people have daily diets with the proper amounts of these fruits or vegetables, it would help to prevent thrombembolism.

In this study, we have selected five indigenous vegetables from Southern Thailand with high phenolic contents and antioxidant activity to investigate their antithrombosis activity [13]. We have evaluated these vegetables for in vitro antiplatelet adhesion, antiplatelet aggregation, and anticoagulant activity. It was anticipated that the results of antithrombotic activity in Southern Thai indigenous vegetables could promote Thai vegetable consumption.

METHODS

Sample collection
Indigenous vegetables used in this study were purchased from three regional supply markets in the southern Thai provinces of Nakhon Si
Vegetable extracts
The vegetables were individually washed with water and rinsed with distilled water. For the extraction preparation, the vegetables were sliced into 50 g each, and then they were homogenized using a homogenizer at maximum speed for 1 min in 150 ml of either methanol (80%) or distilled water. The aqueous extraction of the vegetables was performed using previously described procedures with some modifications [14]. Briefly, the extraction was performed by heat-assisted extraction conditions, and then shaken in a water bath at 50°C for 15 h. The methanolic extraction (methanol:water, 80:20 v/v) was performed using previously described procedures with minor modifications [11]. Briefly, the suspensions were placed at 25°C under stirring for 15 h. The two suspension types were sedimented by centrifugation at 700 g for 10 min, and then filtrated by using a filter paper (Whatman No. 1). The filtrated suspension of the aqueous extracts was lyophilized to obtain a dried form. The solvent of the methanolic extract was removed from the extract using a rotary evaporator (Yamato, Rotary Evaporator, Model-RE 801, NY) at a temperature below 40°C. Both extracts were kept at −70°C. Stock solutions of the crude extracts were prepared by thoroughly mixing with 100% dimethyl sulfoxide (DMSO). After preparing the extraction suspension, the final concentration of 10 μg/μl was filtrated with a sterilized 0.22 μm syringe filter before using in the experiment.

Human blood samples
For the experiments, 10 ml of venous blood samples was collected from 30 healthy volunteers, both men and women between the age group of 18–40 years old, who have no medication history at least 10 days before the experiments and no history of bleeding or thrombosis before blood sampling collection. In addition, the volunteers were informed about the research and asked to sign an informed consent form. Protocol No. WUEC-18-024-01 was authorized by the Office of the Human Research Ethics Committee of Walailak University with the Declaration of Helsinki.

Platelet isolation and platelet-poor plasma (PPP) preparation
Venous blood was drawn by venipuncture and then transferred into a centrifuge tube containing 3.2% trisodium citrate solution at a ratio of 9:1 v/v. Platelet isolation was performed according to a previously described method with minor modifications [15]. Briefly, platelet-rich plasma (PRP) was isolated by centrifugation at 270 g for 10 min. PRP was then centrifuged further for 10 min at 2300 g to sediment platelet pellet. PPP was separated and stored at -70°C for coagulation tests. The pellet was suspended in a Tyrode HEPES buffer (50 mM NaCl, 5 mM KCl, 10 mM glucose, and 10 mM HEPES, pH 7.3) and washed 2 times to remove other cellular debris. The platelets were suspended in a Tyrode HEPES buffer at a final concentration of 2×10^9/ml and immediately used for platelet adhesion and aggregation assays.

Platelet adhesion assay
Adhesion of platelets to collagen was evaluated according to a previously described method with minor modifications [16]. Briefly, the suspension was pre-incubated with vegetable extracts at 37°C for 10 min, and 1% DMSO and 75 μg/μl of aspirin served as a vehicle control and a positive control, respectively. Using a collagen-coated 96-well plate (Millipore Human Collagen Type IV Coated Strip, Merck Schuchardt OHG, Hohenbrunn, Germany), the wells were incubated with 200 μl of phosphate buffer saline (PBS) containing 1% BSA for 1 h and then washed 3 times with 200 μl of PBS. Next, 50 μl of platelet suspension was pipetted into each coated well, and 40 μl of the platelet activator, thrombin (0.25 U/ml), was added. The plate was incubated for 60 min at room temperature and then washed 3 times with 200 μl PBS to eliminate unattached platelets. After that, 140 μl of the substrate solution (0.1 M citrate buffer containing 5 mM p-nitrophenyl phosphate, and 0.1 % (w/v) Triton X-100, pH 5.4) was added to each well. The experiment reaction was stopped after 10 min of incubation at 25°C by adding 100 μl of NaOH (2 N). The absorbance of the reaction was measured at 405 nm using a Multiskan™ GO UV/Vis microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Oy, Finland).

Platelet aggregation assay
Platelet aggregation was determined using a microrotter plate according to a previously described method with minor modifications [17]. Platelet suspension was pretreated with extracts at 37°C for 10 min, and 75 μl of suspension was then pipetted into 96-well plates. Next, 10 μl of thrombin (0.25 U/ml), agonist, was added to the wells. The optical density at 600 nm was read immediately and after incubation for 20 min using a Multiskan™ GO UV/Vis microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific Oy, Finland). Platelet aggregation was calculated by subtracting the absorbance value at 20 min from the initial absorbance of the same well followed by normalization with the vehicle control (1% DMSO). Here, 75 μg/μl of aspirin was used as a positive control.

Prothrombin time (PT) activity assay
To evaluate anticoagulation activity of the vegetable extracts against the extrinsic pathways of coagulation, we measured the PT. The PT assay was carried out following the protocols of a previously described method with minor modifications [18]. The tests were conducted in a Coagulogram analyzer (Humaclot Duo Plus, Wiesbaden, Germany). Here, 1% DMSO was employed as a vehicle control and 3 IU/ml of heparin (Cristalia®, Itapira, SP, Brazil) was used as a positive control. The results of the PT were expressed as clotting time in seconds (s).

Activated partial thromboplastin time (APTT) activity assay
To assess the anticoagulation activity of the indigenous vegetable extracts toward the intrinsic pathway of coagulation, the APTT activity assay was performed following the protocols of a previously described method with minor modifications [18]. The sample of 90 μl of plasma collected from human volunteers was mixed with 10 μl of extract at 37°C for 5 min. After 5 min, a 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 0.025 mol/l CaCl₂ solution (Siemens Healthcare Diagnostics Products GmbH, Germany) was added to initiate the reaction assay, and the clotting time was measured. Here, 1% DMSO was employed as a vehicle control and 3 IU/ml of heparin (Cristalia®, Itapira, SP, Brazil) was used as a positive control. The results of the PT were expressed as clotting time in seconds (s).

Table 1: The names of the selected vegetables used in the study and their percentage (w/w) of extraction yields

| English name                        | Thai name                  | Scientific name                      | Aqueous (%) | Methanol (%) |
|----------------------------------|----------------------------|-------------------------------------|-------------|--------------|
| No English name young leaves     | Mon-pu                     | Glochidion perakens Hooikf.         | 1.61        | 2.93         |
| Cashew young leaves              | Yot mamuang himphaphan     | Curcuma longa L.                    | 2.67        | 7.00         |
| Turmeric rhizomes                 | Kh-a-ni-oan                 | Citrus reticulata Blanco.           | 2.53        | 4.60         |
| Tangerine young leaves            | Bai-som-paan               | Alpinia officinarum Griff.          | 3.23        | 3.53         |
| Joint-whip ginger young fruits   | Kha-ing                     |                                    | 1.64        | 3.13         |

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was used as a positive control. The results of the APTT were expressed as clotting time in seconds (s).

**Statistical analysis**

The results were expressed as means ± standard error of the mean. Statistical comparisons of the data were achieved by a one-way analysis of variance (ANOVA) using GraphPad Prism Version 6.0 (San Diego, California, USA). p<0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Extract yields**

The list of the five selected indigenous vegetables from Southern Thailand and their percentage of extraction yields are shown in Table 1. The yields ranged from 1.61% to 7.00%. Comparatively, the methanol extracts had a higher yield than the aqueous extracts. The highest percent yield was from the young cashew leaves (Anacardium occidentale L.) at 7.00% by methanolic extraction. In contrast, the aqueous extract of the young mon-pu leaves (Gliricidia sepium (Jacq.) Hook.f.) showed the lowest percentage yield at 1.61%. It is generally known that phenolic compounds are widespread constituents of plant diets. In addition, they are more soluble in polar organic solvents due to the presence of a hydroxyl group [19].

Based on a previous study from Kongkachuichai et al., these vegetables were shown to be rich in polyphenol compounds (mon-pu: 4,762.76, cashew: 4,075.79, turmeric: 1,037.31, tangerine: 609.22, and joint-whip ginger: 571.88 GAE/100 g) [13]. Hence, methanol could be more efficient in the extraction of major constituents from these vegetables than water.

**Antiplatelet activity**

Platelet adhesion and aggregation are essential for the formation of a platelet plug at damaged blood vessels. Interfering, the process of platelet activation is one of the therapeutically strategies for the treatment of platelet-related thrombosis [20]. We screened antiplatelet activity of the aqueous and methanolic extracts from the selected vegetables at a concentration of 10 µg/µl. According to the experiments with the five indigenous vegetables, both the aqueous and methanolic extracts of four of the vegetables including mon-pu, cashew, turmeric, and joint whip ginger revealed significant antiplatelet adhesion (17.82–92.79%) and antiplatelet aggregation (16.25–102.9%). In contrast, the methanolic extract of young tangerine leaves (Citrus reticulata Blanco) presented only antiplatelet adhesion by approximately 29%. The results of the platelet adhesion assay demonstrated that the methanolic extract of mon-pu and young cashew leaves showed high antiplatelet adhesion activity at 92.79% and 81.10%, respectively. Among the aqueous extracts, the highest inhibitory effect on platelet adhesion was observed in mon-pu (90.08%), as shown in Table 1. In addition to antiplatelet adhesion activity, we found that the methanolic extracts of mon-pu and young cashew leaves presented high antiplatelet aggregation properties by approximately 100% (Table 2). Aspirin at 75 µg/µl concentration was used as a positive control, having percent inhibition of 87.4±3.14 and 56.7±4.06 against platelet adhesion- and thrombin-induced platelet aggregation, respectively.

We had selected the extracts from mon-pu to study the inhibitory effect toward platelet adhesion and platelet aggregation at various concentrations (1, 2, 3, 4, and 5 µg/µl). The analysis results demonstrated that both the aqueous and methanolic extracts of mon-pu showed dose-dependent platelet adhesion inhibition (Fig. 1) and platelet aggregation inhibition (Fig. 2). However, the methanolic extract had a higher effect toward platelet function than the aqueous extract. The methanolic extract of mon-pu at 1 µg/µl showed approximately 70% inhibition of platelet aggregation (Fig. 2). This dose amount of the extract showed a potentially similar inhibitory effect on platelet aggregation when compared to a previous study on crude rice (Oryza sativa L.) at 7.00% by methanolic extraction. In contrast, the aqueous extract of the young mon-pu leaves showed 29% inhibition of platelet aggregation when compared to a previous study on crude rice (Oryza sativa L.) at 7.00% by methanolic extraction.

**Table 2: The percentage of platelet adhesion inhibition on collagen and thrombin-induced platelet aggregation of the extracts**

| Vegetables            | Platelet adhesion inhibition (%) | Platelet aggregation inhibition (%) |
|-----------------------|----------------------------------|-------------------------------------|
|                       | Aqueous | Methanol | Aqueous | Methanol |
| Mon-pu                | 90.08±0.71*# | 92.79±0.78*# | 100.5±0.34* | 102.9±1.53*# |
| Cashew young leaves   | 71.85±3.36*h  | 81.10±4.30*# | 96.20±4.96* | 98.16±5.93*# |
| Turmeric              | 53.84±3.16*# | 30.22±4.37*# | 16.25±4.01* | 50.12±4.12*# |
| Tangerine young leaves| 5.81±5.19*a  | 29.19±4.24**| 2.09±1.62* | 15.48±3.31 |
| Joint-whip ginger fruit | 25.28±3.12*a| 17.82±1.27*b | 56.81±9.19*d | 93.08±4.08** |

Values are expressed in mean ± standard error of the mean for each antiplatelet activity from duplicate analysis of ten samples (n=10); *p<0.05, compared to the vehicle control (1% dimethyl sulfoxide); **p<0.05, compared to the aqueous extract from the same vegetable. Mean values with different subscript letters within the same column of each antiplatelet activity are significantly different at p<0.05.

**Fig. 1:** The percentage of platelet adhesion inhibition of mon-pu extracts at various concentrations. *p<0.05, compared to the vehicle control (1% dimethyl sulfoxide); **p<0.05, compared to the aqueous extract from the same vegetable. Values are expressed in mean±standard error of the mean for each antithrombin adhesion from duplicate analysis of three samples (n=3).

**Fig. 2:** The percentage of platelet adhesion inhibition of mon-pu extracts at various concentrations. *p<0.05, compared to the vehicle control (1% dimethyl sulfoxide); **p<0.05, compared to the aqueous extract from the same vegetable. Values are expressed in mean±standard error of the mean for each antithrombin adhesion from duplicate analysis of three samples (n=3).
Table 3: Effects of the vegetable extracts on anticoagulant activity based on the prothrombin time and activated partial thromboplastin time of normal human plasma

| Vegetables               | Prothrombin time (s) | Activated partial thromboplastin time (s) |
|--------------------------|----------------------|------------------------------------------|
|                          | Aqueous              | Methanol                                 | Aqueous                  | Methanol                  |
| Mon-pu                   | 12.74±0.35           | 12.94±0.35                               | 42.92±1.75*              | 48.92±0.94*               |
| Cashew young leaves      | 12.99±0.18           | 12.85±0.17                               | 27.27±0.64               | 28.37±0.89                |
| Turmeric                 | 14.43±0.17           | 14.42±0.15                               | 27.32±0.54               | 27.86±0.61                |
| Tangerine young leaves   | 13.80±0.15           | 14.20±0.22                               | 26.64±0.67               | 28.31±0.77                |
| Joint-whip ginger fruit  | 1.35±0.27            | 1.40±0.15                                | 26.90±0.92               | 26.49±0.59                |

Values are expressed in mean ± standard error of the mean for each clotting time from triplicate analysis of ten samples (n=10). *p<0.05, compared to the vehicle control (1% dimethyl sulfoxide)

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Fig. 3: Anticoagulant activity of the mon-pu extracts with various concentrations using activated partial thromboplastin time assay. *p<0.05, compared to the vehicle control (1% dimethyl sulfoxide); †p<0.05, compared to the aqueous extract from the same vegetable. Values are expressed in mean ± standard error of the mean for each clotting time from triplicate analysis of five samples (n=5)

APTT by the extracts from the mon-pu treatment suggested inhibition of coagulation factors VIII, IX, XI, or XII of the intrinsic pathway. Simple chelation of Ca2+ by the extracts, inhibiting coagulation factor activation, could be excluded because the PT was normal. Various plants showed anticoagulant activity against the coagulation factors of the intrinsic pathway, such as Zingiber cassumunar Roxb. [28], Syzygium cumini L. [29], and raspberry (R. idaeus) L. [11]. It was evident that the phytochemicals of S. cumini L. such as limonene, flavonoids, and phenolic could play an important role in interfering with the coagulation factors of the intrinsic pathway, resulting in prolonged APTT [29]. In a previous study regarding the effects of phenolic compounds on coagulation factors, Kuntic et al. demonstrated that quercetin-3-rutinoside could inhibit factors VIII and IX of the intrinsic coagulation pathway [30].

Collectively, our results suggested that young mon-pu leaves and young cashew leaves are good sources of bioactive compounds with antiplatelet properties, especially mon-pu which has both antplatelet and anticoagulation activity. In addition, these two vegetables contain a high amount of phenolic contents and a great amount of antioxidant capacity among Southern Thai indigenous vegetables [13,14]. Therefore, the consumption of these vegetables can be beneficial in the prevention of CVDs related to thrombotic events.

CONCLUSION

These findings illustrated some antiocoagulant activity in indigenous vegetables from Southern Thailand. Particularly, young mon-pu leaves can be used as a supplementary antithrombotic agent to improve and/or prevent CVDs and, therefore, have an important impact on human health. Further studies are essential to advance in the knowledge of the bioactive phytochemicals of these vegetables and their mechanisms of action.
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CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

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