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Antiplatelet and Antithrombotic Properties of 2-Geranyl-2',3,4,4'-tetrahydroxydihydrochalcone, A Chalcone from *Artocarpus altilis* (Park.) Fosberg Leaves

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ABSTRACT

Thrombosis is a major contributor to the pathophysiology of cardiovascular diseases. The involvement of platelets in the formation of thrombus has been studied extensively. Platelet aggregation inhibition is a promising strategy for the treatment of thrombosis. *Artocarpus altilis* is a medicinal plant containing 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (GTDC) with antiplatelet activity. A previous study showed that the plant extract and GTDC demonstrated antiplatelet activity in ADP-induced platelet aggregation. However, no data are available regarding its efficacy in platelet aggregation induced by other agonists. In addition, the antithrombotic activity of GTDC is unknown. This study evaluates the antithrombotic activity of GTDC and determines the antiplatelet specificity action of GTDC. Antithrombotic activity assay was done using $FeCl_3$-induced arterial thrombosis in rats, whereas the antiplatelet assays were performed in vitro utilizing human platelet induced by several platelet receptor agonists. One-way ANOVA followed by the Tukey post-hoc test (p = 95%) in GraphPad Prism 8 software were used to analyze the data statistically. We found that GTDC (30 and 60 mg/kg) demonstrated antithrombotic activity by prolonging occlusion time due to thrombosis. GTDC showed its highest selectivity in the platelet aggregation pathway induced by ADP compared with thrombin, arachidonic acid, and epinephrine.

Keywords: Cardiovascular disease, Coronary thrombosis, Platelet aggregation, P_2Y_{12} purinoceptor antagonists, Breadfruit, Dihydrochalcone.

Introduction

Currently, cardiovascular diseases are the top cause of death in the world, and is responsible for 17.9 million deaths in 2019, or 32% of deaths.[1, 2]. This high mortality rate presents a challenge for researchers trying to find new pharmaceutical agents to combat cardiovascular diseases. One of the events causing cardiovascular diseases is a blockage in blood vessels that interfere with blood flow and homeostatic mechanisms. Platelet aggregation and thrombus formation play a vital role in this blockage.[3-5]

Platelet aggregation is initiated by the activation of platelet receptors by their respective agonists, such as thrombin, adenosine diphosphate (ADP), arachidonic acid (origin of thromboxane A2), and epinephrine, and at PAR1 (protease-activated receptors 1), P_2Y_{12}, thromboxane prostanoid (TP), and α2A receptors, respectively.

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The activation of these receptors causes a transient increase of cytoplasmic calcium ion and protein phosphorylation in downstream signaling, leading to platelet aggregation.[6, 7] Malfunction in the platelet aggregation can contributes to thrombus formation leading to vascular blockage.[5] The involvement of platelet aggregation in thrombus formation was reviewed in several previous studies.[8-10] Therefore, thrombosis prevention through platelet aggregation inhibition (antiplatelet activity) is a promising approach.[11, 12]

Some antithrombotic drugs have been approved and are on the market. However, the efficacy and safety effects of these drugs need to be optimized, as the risk of bleeding remains high. Thus, the discovery of new antithrombotic agents is challenging. Plants provide abundant secondary metabolites with high structural diversity that could be potentially developed as bioactive agents.[13, 14] *Artocarpus altilis* (Park.) Fosberg or breadfruit is an Indonesian medicinal plant traditionally used to treat various diseases, including cardiovascular diseases. Previous studies have shown that *A. altilis* leaves demonstrated cardioprotective-related activities, such as antiatherosclerotic,[15] antihyperlipidemic,[16] antioxidant,[17] and antiplatelet[18] activities. The leaves of *A. altilis* contain 2-geranil-2′,3,3,4′-tetrahydroxy dihydrochalcone (GTDC; Figure 1) as the main constituent. This compound demonstrated strong antiplatelet activity (half maximal inhibitory concentration [IC_{50}]: 9.09 µM) on platelet aggregation induced by ADP.[18] As platelet aggregation is triggered by several pathways involving activation of platelet

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receptors by their respective agonists, the antiplatelet effect of GTDC on platelet activation pathways other than ADP is worth investigating to determine the selectivity of action. Platelet aggregation is involved in thrombosis development. Therefore, determining the antithrombotic activity of GTDC is a research topic of interest in antithrombotic agent development. This study characterizes the antiplatelet specificity action of GTDC and investigates its antithrombotic activity. This study might contribute to the development of pharmaceutical agents targeting thrombosis from natural resources.

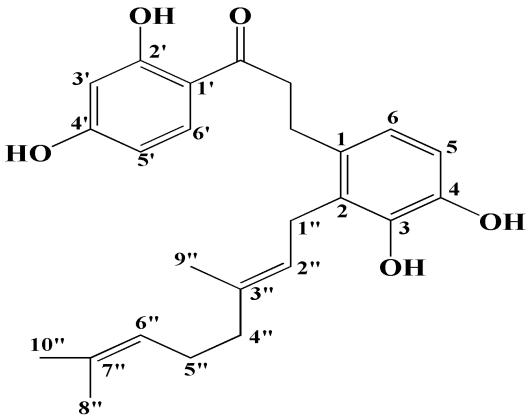

Figure 1: Chemical structure of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone

Materials and Methods

Plant material and chemicals

GTDC was obtained from the previous study,[18] whereas ADP, epinephrine, arachidonic acid, thrombin, ticagrelor, and clopidogrel were purchased from Sigma Aldrich (Missouri, USA). Ketamine was from PT Ikapharmindo Putramas (Jakarta, Indonesia), dimethyl sulfoxide (DMSO), $FeCl_3$, sodium carboxymethyl cellulose, and sodium chloride was purchased from Merck (New Jersey, USA).

Subjects

In line with the previous study, this study utilized human platelets obtained from the blood of healthy participants who met the inclusion as well as the exclusion criteria.[19] The protocols used in this study were reviewed and approved by the institutional ethics committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (number KE/FK/0636/EC/2019) and fulfilled the Helsinki Declaration. The participants (blood donors) have signed informed consent forms.

Animals

Male Wistar rats aged eight weeks (150–250 g) were obtained from the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada. The healthy animals were placed in the animal room at a temperature (25 °C), 70% humidity, and a dark-light cycle of 12 hours, water *ad libitum*, and fed with a standard diet. Throughout the study period, the animals were monitored for their growth and health status to ensure that they were healthy. The protocol was approved by the institutional ethics committee of the LPPT Universitas Gadjah Mada (number: 0064/04/LPPT/I/2021).

Antiplatelet assay

The antiplatelet activity was measured using the Born method as described in previous articles.[20, 21] Platelet-rich plasma (PRP) was obtained from healthy subjects by centrifugation of the blood at 2000 rpm for 10 min and then at 3500 rpm for 15 min. The PRP (492.5 µL) was put in a siliconized cuvette, and different concentrations of GTDC solution (2.5 µL in DMSO) were added. Cuvettes with DMSO (2.5 µL) were used as negative controls (reference). The cuvettes were incubated at 37°C for 3 min. The respective inductors of platelet aggregation (thrombin, epinephrine, and arachidonic acid as PAR1, α2A, and TP receptors agonists, respectively) were then added (5 µL). The platelet aggregation was measured in an aggregometer (Chronolog 490-2D) for 10 min. Platelet poor plasma (PPP; 500 µL) was used as a measurement background. The antiplatelet activity was calculated based on the ability of the GTDC to inhibit platelet aggregation compared with the solvent (DMSO) group.

Antithrombotic assay

The antithrombotic activity was done according to the previous research.[22] The protocol has been approved by the Research Ethics Commission of the Research and Testing Laboratory, Universitas Gadjah Mada, with certificate number 0064/04/LPPT/I/2021. The rats (n = 24) were divided into four groups and treated orally with GTDC (30 mg/kg and 60 mg/kg), clopidogrel (30 mg/kg), or the solvent (Na-CMC 0.5%), 3 hours prior to the thrombosis induction using $FeCl_3$ 5% (0.5 mL). The rats were anesthetized by subcutaneous injection of 100 mg/kg ketamine, dissected, and the blood flow was measured using a Doppler flowmeter (Transonic T106 Module) and ultrasound Doppler transonic flow (TS420 model 0.5 VB). The induction of thrombosis was performed by applying $FeCl_3$-impregnated paper to the carotid artery for three minutes. The blood flow was measured every 15 seconds for 40 minutes, and the occlusion time (time required for thrombus formation that completely blocked blood flow) was recorded.

Statistical analysis

Statistical analysis was performed using SPSS version 25 software. Shapiro-Wilk test was used to evaluate the data distribution; whereas the statistical differences between groups was analyzed with one-way ANOVA followed by the Tukey post-hoc test (p = 95%).

Results and Discussion

Platelet aggregation is activated by multiple pathways. This activation is dictated by the binding of platelet receptors to their agonists. A previous study showed that the extract of *A. altilis* leaves and its main compound GTDC demonstrated antiplatelet activity on platelet aggregation induced by ADP (an agonist of P_2Y_{12} platelet receptor). Here, we determined the antiplatelet specificity action of GTDC on another pathway of platelet aggregation. We used thrombin, epinephrine, and arachidonic acid as platelet receptor ligands/agonists to induce platelet aggregation by activating PAR1, α2A, and TP receptors, respectively. The antiplatelet activity of GTDC was determined in an aggregometer utilizing human platelets induced by several platelet agonists in accordance with their respective receptors. The antiplatelet activity profiles and IC_{50} are presented in Figure 2. Figure 2A–C shows that GTDC had weak antiplatelet activity on platelet aggregation induced by thrombin (PAR1 agonist), epinephrine (α2A receptor agonist), and arachidonic acid (TP receptors agonist) with IC_{50} of 140.27 ± 13.78, 30.48 ± 1.48, and 39.07 ± 8.57 µM, respectively. Strong antiplatelet activity of GTDC was observed in the adenosine diphosphate (ADP)-induced platelet aggregation assay (IC_{50}: 9.09 ± 0.99 µM).[18] GTDC demonstrated antiplatelet activity with the highest selectivity in the platelet aggregation pathway induced by ADP compared with other agonists. This suggested that GTDC might act as a P_2Y_{12} receptor antagonist. A previous in silico study showed that GTDC irreversibly interacts three amino acid residues (Tyr109, Arg256, and Lys280) of the P_2Y_{12} receptor.[18] The binding pattern is identical to clopidogrel (an antiplatelet drug targeting P_2Y_{12} receptor).[23] The IC_{50} comparison of GTDC with the reference platelet receptor antagonists on different platelet receptors was presented in Table 1. To confirm the interaction of GTDC with the ligand binding domain of the P_2Y_{12} receptor, further studies involving a receptor binding assay are required. Apart from the dominant effect of GTDC on the ADP-activated pathway of platelet aggregation, GTDC also showed weak effects on other pathways. Thus, GTDC might also partially target other receptors and pathways downstream of platelet receptor activation by agonists. They share a conserved activation pathway involving cytoplasmic calcium ion production and protein phosphorylation.[6, 7]

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Table 1: Comparison of in vitro antiplatelet activities (IC_{50} values) of GTDC and reference compounds against several platelet receptors.

Platelet receptor	Agonists	IC_{50} (µM)				
		GTDC	FR171113	Yohimbine	Aspirin	Ticagrelor
PAR1	Thrombin	140.27±13.78	6.96±0.88	-	-	-
α2A	Epinephrine	30.48±1.48	-	2.09±0.08	-	-
TP	Arachidonic acid[a]	39.07± 8.57	-	-	11.70±1.23	-
P_2Y_{12}	ADP	9.09±0.99	-	-	-	0.22±0.01[b]

[a]origin of thromboxane A2; PAR1 (Protease-activated receptor 1); TP (Thromboxane prostanoid); -not determined. [b]Reference[18].

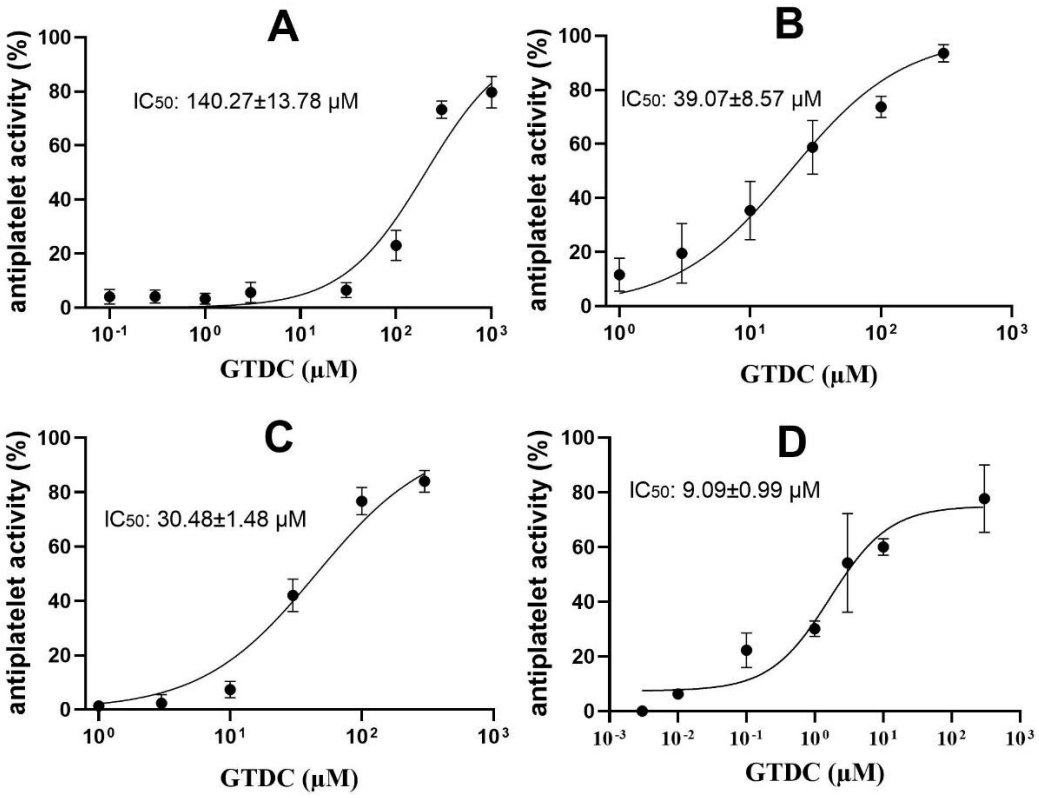

Figure 2: The antiplatelet activity of GTDC in different platelet aggregation pathways induced by thrombin, epinephrine, arachidonic acid, and ADP as PAR1, α2A, TP, and P2Y1 receptors agonists, respectively.
The platelet was pre-treated with GTDC or solvent (DMSO) three min before the induction of platelet aggregation using the respective agonists: thrombin (A), epinephrine (B), arachidonic acid (C), and ADP (D)16. Data are means ± standard error (n = 3).

Three prenylflavonoids from the cortex of the *A. altilis* root, namely artochamins B, dihydroartomunoxanthone, and artocommunol CC have been identified as having antiplatelet activity in the platelet aggregation induced by epinephrine.[24] Here, we provided additional scientific evidence of the antiplatelet activity of *A. altilis*, especially GTDC as its main compound. As platelet aggregation plays a pivotal role in thrombosis pathophysiology, the antithrombotic activity of GTDC was also investigated.[11, 25] The antithrombotic activity was evaluated on $FeCl_3$-induced vascular injury in rats. This vascular endothelial injury-based method is simple and sensitive to antiplatelet agents.[26, 27] The evaluation of the antithrombotic activity of GTDC was performed using $FeCl_3$-induced vascular injury in rats. We found that the induction of thrombosis using $FeCl_3$ dramatically decreased the blood flow rate over time in the solvent group. The treatment of rats with GTDC (30 and 60 mg/kg) or clopidogrel (30 mg/kg) inhibited this decrease (Figure 3A).

Furthermore, the blood occlusion time in rats treated with GTDC was measured. Compared with the solvent group, GTDC could prolong the blood occlusion time dose dependently (Figure 3B). These results showed that GTDC acts as an antithrombotic agent indicated by its ability to increase the blood flow and prolong occlusion time in thrombotic rats. The potency of GTDC as an antithrombotic agent is half that of clopidogrel, the reference drug. Interestingly, previous publications also showed that the extract of *A. altilis* leaves exerted cardio protective-related activities, such as antiplatelet,[18] antihyperlipidemic,[16] antioxidant,[17] and anti-inflammatory activity[28]. In summary, this is the first study showing that GTDC possesses antithrombotic activity. This study provides scientific evidence for the traditional use of *A. altilis* leaves as a cardiovascular disease remedy.

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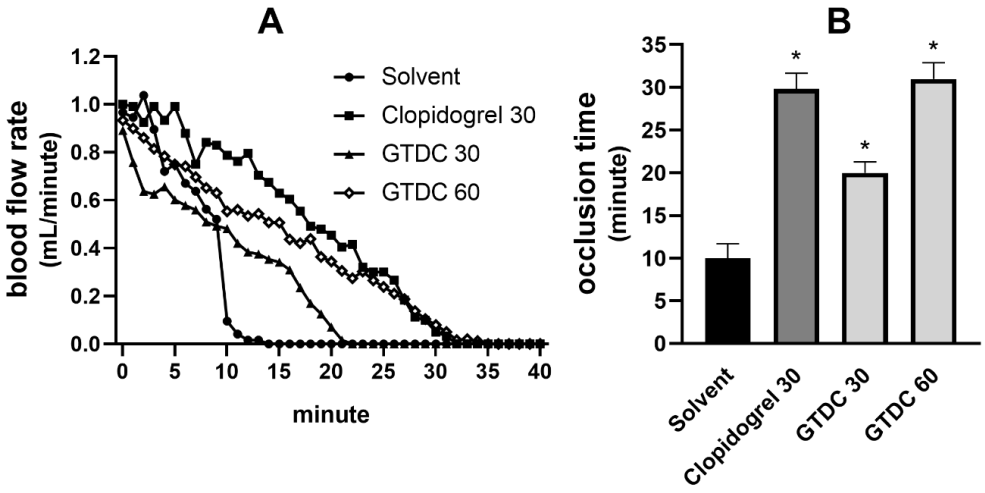

Figure 3: Antithrombotic activity of GTDC in FeCl3-induced arterial thrombosis in rats. A. The blood flow rate was measured every 15 seconds in the carotid aorta. B. Occlusion time (minute required to stop blood flow).
Data are means ± standard error (n = 6); * significant ($p < 0.05$; ANOVA followed by Tukey's post-hoc test.

Conclusion

In this study, GTDC showed antiplatelet and antithrombotic activities. The antiplatelet activity seems to target the P2Y1 receptor as it showed the highest selectivity in the platelet aggregation induced by ADP compared with thrombin, arachidonic acid, and epinephrine. GTDC could be developed further as a lead antithrombotic agent derived from natural resources.

Conflict of Interest

The authors declare no conflicts of interest in this study.

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Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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