A geranylated chalcone with antiplatelet activity from the leaves of breadfruit (*Artocarpus altilis*)

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Abstract

Platelet plays a crucial role in cardiovascular diseases (CVDs) development. Abnormalities in platelet aggregation provokes thromboembolism, eventually leading to death. In Indonesia, breadfruit (*Artocarpus altilis*) leaf is traditionally used to treat CVDs. This study aimed to evaluate the antiplatelet activity of *A. altilis* leaf extract (AAE) and to identify its active compound. *A. altilis* leaves were extracted with ethanol, and the antiplatelet activity was assessed using ADP-induced platelet aggregation. The major compound was isolated with column chromatography followed by preparative TLC, and the structure was determined on the basis of UV, MS, IR, and NMR spectra. The binding mode of the active compound to platelet receptors was characterized in *in silico* study. AAE exhibited an antiplatelet activity (IC₅₀ of 252.23 µg/mL). A geranylated chalcone, 2-geranyl-2',3,3,4'-tetrahydroxydihydrochalcone (GTDC) was identified as the antiplatelet compound (IC₅₀ of 9.09 µM). GTDC actions with P2Y12 platelet receptor involving three amino acid residues.

Keywords

*Artocarpus communis*, 2-geranyl-2',3,3,4'-tetrahydroxydihydrochalcone, thrombosis, P2Y12, P2Y1

Introduction

The cardiovascular diseases remain the main cause of death globally (Benjamin et al. 2019). The development of cardiovascular diseases and blood hemostasis involves platelets, which are unucleated blood components. During a vascular injury, platelets are recruited, and aggregated upon the binding of platelet surface receptors to their respective agonists. These agonists and receptors are ADP for P2Y1 and P2Y12 receptors, thrombin for PAR-1 and PAR-4 receptors, epinephrine for α2a receptor, thromboxane A2 for TPα and TPβ receptors, serotonin for 5HT-2A receptor, and collagen for GPⅡa/Ⅲa, GPⅡb/Ⅲa, and GP VI receptors. Their binding initiates the change in the shape of platelets and their aggregation, leading to blood coagulation and clotting (thrombus). Uncontrolled platelet aggregation can trigger circulatory disorders, including stroke, myocardial infarction, and thrombosis (Mackman 2008; Yeung et al. 2018).
Among agonists, ADP plays a significant role because it not only induces platelet aggregation through the activation of P2Y1 and P2Y12 receptors; but it is also released when platelets degranulate even when they are activated by other agonists. ADP induces the initial and late phases of platelet aggregation (Puri and Colman 1997; Murugappa and Kunapuli 2006). In the initial phase, the activation of the P2Y1 receptor by ADP causes the mobilization of intracellular calcium ions; as a result, platelet shape changes, thromboxane A2 forms, and platelets start to aggregate. Conversely, in the late phase of aggregation, the activation of the P2Y12 receptor stabilizes platelet aggregation and maintains platelets in their aggregated state by intensifying platelet degranulation and thromboxane A2 production (Puri and Colman 1997; Murugappa and Kunapuli 2006; Koupenova and Ravid 2018). With this advanced understanding, ADP receptors are considered potential therapeutic targets in cardiovascular diseases (Storey 2001) and important components of the development of antiplatelet drugs, such as prasugrel and clopidogrel, which target the P2Y12 receptor.

The discovery of antiplatelet agents is a promising approach to treat cardiovascular diseases (Mackman et al. 2020). Antiplatelet agents are beneficial to patients with a high risk of thromboembolism. These agents control the function of platelets by preventing platelet adhesion, activation, and aggregation (Lopes 2011). Although some antiplatelet agents, such as aspirin, thienopyridine derivatives (clopidogrel and ticagrelor), and GP IIb/IIIa receptor antagonists (abciximab and tirofiban), have been developed, their efficacy, and safety profiles should be improved. Furthermore, medicinal plants have inspired drug discovery and provided abundant structures of bioactive molecules (Thomford et al. 2018). Artocarpus altilis has been traditionally used by folks in the west part of Central Java, Indonesia, as a herbal remedy to treat cardiovascular diseases. It is composed of promising natural compounds beneficial to human health (Jagtap and Bapat 2010).

This work aimed to investigate the antiplatelet activity of A. altilis leaf extract and identify the active compound responsible for its antiplatelet activity. This study provided a scientific basis for promoting the traditional usage of A. altilis to treat cardiovascular diseases.

Materials and methods

Plant material

A. altilis leaves were collected from Mlati, Sleman District, Yogyakarta Province of Indonesia. The plant was identified by Dr. Djoko Santosa, a botanist at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia (number BF/192/Ident/Det/V1/2013).

Subject

Platelet-rich plasma (PRP) was obtained from 10 healthy participants who met the criteria described previously (Cattaneo et al. 2013). The inclusion criteria were as follows: aged 18–45 years and platelet number of 15 × 10^4/µL–40 × 10^4/µL. The exclusion criteria were as follows: pregnant women, those who smoked at least 30 min before blood withdrawal, those who consumed caffeine at least 2 h before the experiment, those who consumed drugs affecting hemostasis (e.g., NSAIDs, anticoagulant, or anti platelet agents) at least a week before the experiment, and participants with hemophilia or bleeding disorders. The protocol for this study was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Universitas Gadjah Mada-DR. Sardjito General Hospital (Ref: KE/FK/0320/EC/2017).

Extraction, fractionation, and isolation

The dried leaf powders of A. altilis (2 kg) were extracted with 20 L of ethanol. The extract was concentrated using a vacuum rotary evaporator, left at room temperature until it was dry (262 g), and partitioned with a mixture of n-hexane : ethyl acetate (2:1) to yield 2.84 g of pure active compound. The structure of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (GTDC) was elucidated on the basis of its spectral data. Spectral UV and IR were recorded on a spectrophotometer (Hitachi U-2800) and an FTIR spectrometer (Perkin Elmer Spectrum 100). LC-MS data were obtained from a Mariner Biospectrometry (Cambridge Scientific, USA) connected to an HPLC instrument (Hitachi L 6200) by using a Q-TOF mass spectrometer (ESI). NMR spectra were obtained using a DELTA 2500M Hz spectrometer (Jeol) running at 500 M Hz (1H-NMR) and 125.76 M Hz (13C-NMR) by using CDCl_3 as a solvent and TMS as an internal standard.

Structural identification of the compound

The structure of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (GTDC) was elucidated on the basis of its spectral data. Spectral UV and IR were recorded on a spectrophotometer (Hitachi U-2800) and an FTIR spectrometer (Perkin Elmer Spectrum 100). LC-MS data were obtained from a Mariner Biospectrometry (Cambridge Scientific, USA) connected to an HPLC instrument (Hitachi L 6200) by using a Q-TOF mass spectrometer (ESI). NMR spectra were obtained using a DELTA 2500M Hz spectrometer (Jeol) running at 500 M Hz (1H-NMR) and 125.76 M Hz (13C-NMR) by using CDCl_3 as a solvent and TMS as an internal standard.

Evaluation of antiplatelet activity

The platelet aggregation assay was done using turbidimetry-based method (Koltai et al. 2017) in an aggrego-
meter (Chrono-log 490-2D, Chrono-log Corporation, Pennsylvania, USA) in accordance with the protocol described previously (Fakhruridin et al. 2019). Blood was collected in a tube containing sodium citrate and centrifuged at 2000 rpm for 10 min at room temperature and further at 3500 rpm for 15 min to obtain platelet-rich plasma (PRP) and platelet-poor plasma, respectively. The tested samples were added to the siliconized cuvettes containing PRP and stirred for 2 min at 37 °C prior to the induction of platelet aggregation with 10 μM ADP (Sigma Aldrich; Missouri, USA). The percentage of platelet aggregation was compared with that of the solvent-treated group and determined as a decrease in the amplitude after 10 min of incubation with ADP. GraphPad Prism 8 was used to generate a dose–response curve and a nonlinear regression curve for IC50 calculation. Ticagrelor was utilized to confirm the responsiveness of the bioassay to the antiplatelet drug.

### Molecular docking study

A docking study was conducted to examine the affinity of GTDC to the ADP receptor (P2Y12) by using MOE-Dock 2015.10 (Chemical Computing Group Inc.) on a computer with Intel Core i5-4460 3.20 GHz, 4.00 GB memory, and a Windows operating system. The crystal structures of the protein complex were taken from the Protein Data Bank (PDB id:4NTI). Ticagrelor (a selective P2Y12 receptor agonist), ethyl 6-[4-(benzylsulfonyl)amoyl] piperidine-1-yl]-5-cyano-2,3,4,4'-tetrahydroxydihydrochalcone or AZD 1283 (a native ligand), and ADP (a purinergic P2Y12 agonist) were used in this study. In addition, the Triangle Matcher function and London DG were used as the placement method and scoring function, respectively.

### Results

#### Structure identification of the active compound

The isolated active compound was identified as a geranylated chalcone, 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (GTDC). GTDC appeared as yellowish oil with ESI-MS 433 m/z [M+Na+] (calc. for C30H26O5; UV:342 nm; IR vmax in cm⁻¹: 1450 (CH), 1496 (C–C), 1628 (C=O), 2360 (C≡C). ¹H-NMR (400 MHz, CDCl₃): δ 6.37 (1H, dd, 2.4 Hz, H-3'), 6.36 (1H, dd, 2.4 Hz, H-5'), 7.57 (1H, d, 8.4 Hz, H-6'), 3.10 (2H, t, H-α), 2.97 (2H, t, H-β), 6.73 (1H, dd, 8 Hz, H-5), 6.71 (1H, dd, 8 Hz, H-6), 3.41 (2H, d, H-1'), 5.17 (1H, t, H-2'), 2.04 (2H, m, H-4'), 2.06 (2H, m, H-5'), 5.00 (1H, t, H-6'), 1.65 (3H, s, H-8'), 1.79 (3H, s, H-9'), 1.57 (3H, s, H-10'). ¹³C-NMR (400 MHz, CDCl₃): δ 17.81 (C-10), 58.88 (1H, s, 2'-OH), 54.44 (1H, s, 3'-OH), 5.33 (1H, s, 4-OH). ¹H-NMR (400 MHz, CDCl₃): δ 113.86 (C-1'), 165.30 (C-2'), 103.66 (C-3'), 162.63 (C-4'), 107.81 (C-5'), 132.27 (C-6'), 203.87 (C-7), 39.81 (C-8'), 27.78 (C-9β), 132.32 (C-7'), 26.38 (C-5'), 25.81 (C-8'), 16.35 (C-9'), 17.81 (C-10'). The NMR spectra were compared with those of a previous study (Table 1). The data from the 2D HMBC and HMQC experiments are provided in Suppl. material 1. The obtained spectra were consistent with those in a previous study (McLean et al. 1996), and the chemical structure is presented in Fig. 1.

The antiplatelet activities of the extract and the compound were assessed using the ADP-induced platelet aggregation.

### Table 1. ¹H and ¹³C NMR spectroscopic data of GTDC in CDCl₃.

| H No. | GTDC Data | GTDC (McLean et al. 1996) |
|-------|-----------|--------------------------|
|       | ¹H-NMR Δ ppm (2H, m, j) | ¹H-NMR Δ ppm (2H, m, j) | ¹³C-NMR Δ ppm |
| 1     | - | 113.86 | - | 113.61 |
| 2     | - | 165.30 | - | 165.18 |
| 3     | 6.37 (1H, dd, 2.4Hz) | 103.66 | 6.38 | 103.57 |
| 4     | - | 162.63 | - | 163.17 |
| 5     | 6.36 (1H, dd, 2.4Hz) | 107.81 | 6.36 | 108.01 |
| 6     | 7.57 (1H, d, 8.4Hz) | 132.27 | 7.57 | 132.25 |
| C=O   | - | 203.87 | - | 204.02 |
| A     | 3.10 (2H, t) | 39.81 | 3.10 | 39.68 |
| B     | 2.97 (2H, t) | 27.78 | 2.97 | 27.81 |
| T     | - | 131.17 | - | 132.09 |
| 2     | - | 126.05 | - | 126.12 |
| 3     | - | 142.51 | - | 142.52 |
| 4     | - | 142.98 | - | 142.74 |
| 5     | 6.73 (1H, dd, 8Hz) | 112.97 | 6.71 | 112.92 |
| 6     | 6.71 (1H, dd, 8Hz) | 121.55 | 6.65 | 121.34 |
| 7     | 3.41 (2H, d) | 25.99 | 3.40 | 25.84 |
| 2'    | 5.17 (1H, t) | 121.80 | 5.17 | 121.86 |
| 3'    | - | 139.03 | - | 138.50 |
| 4'    | 2.04 (2H, m) | 39.69 | 2.04 | 39.63 |
| 5'    | 2.06 (2H, m) | 26.38 | 2.06 | 26.40 |
| 6'    | 5.00 (1H, t) | 123.76 | 5.02 | 123.79 |
| 7'    | 1.65 (3H, s) | 25.81 | 1.65 | 25.69 |
| 9'    | 1.79 (3H, s) | 16.35 | 1.78 | 16.30 |
| 10'   | 1.57 (3H, s) | 17.81 | 1.57 | 17.71 |
| 2-OH  | 12.75 (1H, s) | - | 12.84 | - |
| 4-OH  | 5.88 (1H, s) | - | 5.81 | - |
| 3-OH  | 5.44 (1H, s) | - | 5.81 | - |
| 4-OH  | 5.33 (1H, s) | - | 5.57 | - |

### Figure 1. Structure of 2-geranyl-2',3,4,4'-tetrahydroxydihydrochalcone (GTDC).

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Fig. 2A indicates that AAE strongly inhibited platelet aggregation in a concentration-dependent manner with IC50 of 252.23 ± 6.46 µg/mL. The antiplatelet activity of AAE at a concentration of 500 µg/mL was equal to that of ticagrelor at 0.01 µM; while at 750 and 1000 µg/mL AAE demonstrated a stronger activity. This finding suggested that AAE might contain the antiplatelet compound. Further separation of the extract via liquid–liquid partition followed by Sephadex LH-20, silica gel column chromatography, and preparative TLC revealed GTDC as the active compound. This compound inhibited platelet aggregation in a dose-dependent manner in micromolar ranges with IC50 of 9.09 µM. By contrast, ticagrelor had IC50 of 0.22 µM (Fig. 2B), which is almost similar to that in a previous study (0.32 µM) (Ahn et al. 2016). This result indicated that the antiplatelet activity of GTDC was lower than that of ticagrelor.

In vitro antiplatelet activity

To obtain further details regarding the antiplatelet activity of GTDC, we assessed the platelet aggregation profile of GTDC in the late phase of aggregation (10 min). Fig. 3 shows the platelet aggregation profile of GTDC in ADP-induced platelet aggregation until 10 min of observation. GTDC moderately inhibited the initial platelet aggregation, as indicated by its lower aggregation peak (black and red line peaks) than that of the solvent-treated group (blue line peak). In the late phase of aggregation (10 min), the platelet aggregation level remained high in the absence of GTDC (A). The presence of GTDC lowered the aggregation peak in the initial phase (red and black lines in B compared with the blue line). However, GTDC strongly induced platelet disaggregation, as indicated by the regressed curves (black and red lines) after the aggregation peak. This result implied that GTDC not only inhibited platelet aggregation but also induced disaggregation to destabilize the aggregated platelets. The antiplatelet activity profile of GTDC mimicked that of ticagrelor (green line).

Figure 2. Antiplatelet activities of AAE and GDTC in ADP-induced platelet aggregation. Sigmoidal dose–response curves showing the antiplatelet activity of AAE (A), GTDC, and ticagrelor (B). The percentage of platelet aggregation was calculated on the basis of the decrease in the aggregation peak. Data were mean ± SD (n = 3); *p < 0.05 relative to the solvent-treated group (set as 100% aggregation).

Figure 3. Curve showing the antiplatelet activity profiles of GTDC in ADP-induced platelet aggregation. A Baseline or peak of platelet aggregation (solvent treatment); B Peak of platelet aggregation in GTDC or ticagrelor treatments; C Platelet disaggregation in GTDC or ticagrelor treatments. Blue line, solvent; black line, 0.1 µM GTDC; red line, 1 µM GTDC; and green line, 0.1 µM ticagrelor.
In silico experiment

A molecular docking study was performed to obtain insights into the interaction between GTDC and the ADP receptors responsible for platelet aggregation. The binding of GTDC to the platelet receptors (P2Y1 and P2Y12) was compared with that of ticagrelor. The table in Fig. 2 shows that in the P2Y1 receptor, the docking scores of GTDC and ticagrelor (−13.4361 and −15.6871, respectively) were higher than that of ADP (−23.6418). This result indicated that they did not effectively antagonize the P2Y1 receptor in the presence of ADP. Conversely, in the P2Y12 receptor, the docking scores of GTDC and ticagrelor (−11.9185 and −11.6145, respectively) were lower than that of ADP (−11.3436). This finding suggested that GTDC and ticagrelor could antagonize the P2Y12 receptor but not P2Y1 in the presence of ADP. The analysis of the binding mode (Fig. 4A, B) revealed that GTDC interacted with the P2Y12 receptor through three amino acid residues (Tyr105, Tyr109, and Cys194); while the interaction of ticagrelor with the receptor involving four

Figure 4. Putative interactions of ligands and the binding pockets of P2Y12 receptor. The binding modes of GTDC (A), and ticagrelor (B) at P2Y12 receptor. (C) The overlay-complexes of GTDC (blue) and AZD 1283 (red); and (D) Ticagrelor (blue) and AZD 1283 (red) at the binding sites of P2Y12 receptor. The table shows the docking score of the compounds to P2Y12 receptor.

| Compounds  | P2Y1  | P2Y12 |
|------------|-------|-------|
| ADP        | -23.6418 | -11.3436 |
| GTDC       | -13.4361 | -11.9185 |
| Ticagrelor | -15.6871 | -11.6145 |
Discussion

Preventing cardiovascular diseases by providing promising therapeutic agents is a challenge in biomedical sciences. A. altilis leaves have been traditionally used in Central Java, Indonesia, to treat cardiovascular-related diseases, which are correlated with platelet aggregation. In this study, an antiplatelet in vitro assay was conducted to assess the effectiveness of AAE, and the active compound was characterized. AAE inhibited platelet aggregation with IC50 of 9.09 μM. The active compound was identified as GTDC, which represented a major compound in the extract. Its chromatography profile is provided in Suppl. material 1: Fig. S2. Although the presence of GTDC in A. altilis leaves was described previously (McLean et al. 1996; Wang et al. 2007), the antiplatelet activity of GTDC has not been described in previous studies. Another study has identified three antiplatelet prenyllavonoid compounds (dihydroartomunoxanthone, artocamins B, and artocammunol CC) from A. altilis roots. These compounds demonstrate an antiplatelet activity in epinephrine-induced platelet aggregation (Weng et al. 2006). Additionally, Jantan et al. (Jantan et al. 2010) showed that prenylchalcones and prenyllavonoids (isobavachalcone, 2',4'-dihydroxy-4-methoxy-3'-prenylidihydrochalcone, cycloartobiloxanthone, artonin E, and artonin E triacetate) from plants belonging to the genus of Artocarpus (A. lowii, A. scortechinii, and A. teysmanii) have antiplatelet activities in ADP-induced platelet aggregation. Here, we demonstrated that GTDC isolated from A. altilis leaves exerted an antiplatelet activity in ADP-induced platelet aggregation. This finding provided additional scientific evidence regarding the antiplatelet potential of geranylated or prenylated chalcone and flavonoid as typical compounds present in Artocarpus plants (Hakim et al. 2006).

In this study, we found that GTDC inhibited platelet aggregation and induced the disaggregation of aggregated platelets. This finding was interesting because the efficacy assessment of the antiplatelet agent in ADP-induced platelet aggregation might rely not only on the inhibition of platelet aggregation in the early phase (measured by a decrease in the aggregation peak) but also on the disaggregation activity after the aggregation peak (late phase) (Labarthe et al. 2005; Lordkipanidze et al. 2009). ADP-induced platelet aggregation involves two platelet surface receptors: a P2Y1 receptor, which mediates the initial phase of platelet aggregation, including platelet shape change, aggregation, and initial thrombi formation; and P2Y12, which participates in the late phase of platelet aggregation involving the stabilization of aggregates (Jarvis et al. 2000). Although the simultaneous activation of both receptors is required for normal platelet aggregation in the presence of ADP, the activation of the P2Y12 receptor is a key element for the stabilization of platelet aggregates and the amplification of other platelet responses that contribute to a major platelet aggregation leading to thrombosis (Cattaneo 2015; Benjamin et al. 2019).

In addition to the inhibitory effect on platelet aggregation, GTDC demonstrated a strong platelet disaggregation activity similar to that observed in ticagrelor in the late phase of platelet aggregation. Considering the antiplatelet curve profile of GTDC in the late phase of platelet aggregation as illustrated in Fig. 3, we found that GTDC showed a platelet disaggregation activity with IC50 of 0.34 µM (Suppl. material 1: Fig. S3). Previous studies also proposed this alternative way of assessing antiplatelet activities. The assessment of platelet disaggregation or aggregation in the late phase of aggregation following the treatment of an antiplatelet agent antagonist of ADP receptors should be considered as an antiplatelet measure because it matches with clinical efficacy (Labarthe et al. 2005; Lordkipanidze et al. 2009). Interestingly, the platelet disaggregation activity (measured in the late phase of aggregation) of GTDC was stronger than that of the inhibitory activity of platelet aggregation (measured in the initial phase of platelet aggregation or based on the peak of aggregation). As P2Y1 and P2Y12 receptors are responsible for the initial and late aggregation, respectively, GTDC may antagonize P2Y12 more strongly than the P2Y1 receptor. Nevertheless, further studies employing a receptor–ligand binding assay are needed to confirm this effectiveness.

The in silico approach revealed that the interaction between GTDC and P2Y12 receptor involved more amino acid residues compared with that of P2Y1. Thus, the binding of GTDC was more favorable to P2Y12 than to the P2Y1 receptor. The molecular docking study showed that the docking score of GTDC was lower (−11.9185) than that of ADP (−11.3436), suggesting that GTDC could prevent the binding of ADP to P2Y12. This finding explained the stronger binding affinity of GTDC to P2Y12 rather than to the P2Y1 receptor. The structure–activity relationship of chalcone-derived compounds demonstrated that the 4-OH phenolic group and the adjacent geranyl group are essential for the antiplatelet activity in ADP-induced platelet aggregation (Jantan et al. 2009; Jantan et al. 2010). Coincidentally, both functional groups are present in GTDC and may contribute to antiplatelet activities.

Recent studies and clinical evidence indicated that antagonizing the P2Y12 receptor remains the main target in coronary artery thrombosis as it shows a prolific efficacy (Nylander and Schulz 2016; Li et al. 2020; Mansour et al. 2020). Additionally, this receptor functions in platelet aggregation and aggregate stability. GTDC inhibited platelet aggregation and destabilized platelet aggregates, which might correspond to the antagonistic effect of the P2Y12 receptor. A similar effect was observed in ticagrelor used in this study. Similar to its predecessor clopidogrel, ticagrelor is an antiplatelet drug that shows clinical efficacy by selectively antagonizing the P2Y12 receptor (Anderson et al. 2010). However, both agents can cause serious bleeding and dyspnea as common adverse effects that limit their use (Nawarskas and Snowden 2011). Further investigation on the binding
mode and mechanistic characterization of P2Y12 ligands in platelet aggregation cascades is challenging because of the diverse chemical structure of the ligands with potentially distinct side effects. GTDC may lead the way for the discovery of a promising natural antiplatelet agent.

Conclusion

We found that GTDC, a geranylated chalcone isolated from A. altiss leaves demonstrated a promising antiplatelet activity. It exerts an antiplatelet activity by inhibiting platelet aggregation and inducing platelet disaggregation in the initial and late phases of aggregation, respectively. The docking study indicates that GTDC interacts with the P2Y12 receptor via three amino acid residues.

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**Supplementary material 1**

**Supplementary Figures**

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Data type: **Figure S1.** 2D HMBC and HMQC observation of the correlations in the NMR spectral experiments of GTDC. **Figure S2.** TLC profile of *A. altilis* extract and the isolated compound GTDC. **Figure S3.** Sigmoidal dose–response curve showing the platelet disaggregation activity of GDTC in ADP-induced platelet aggregation.

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