Vietnam Journal of Science and Technology ***62*** *(3) (2024) 425-434*
doi:10.15625/2525-2518/19013

Potent inhibitory effect on human platelet aggregation of the aerial part of *Canna edulis*

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Received: 28 September 2023; Accepted for publication: 10 January 2024

Abstract. *Canna edulis* Ker Gawl has been used in Vietnamese traditional medicine for the treatment of heart diseases with very limited scientific evidence. This study aimed to evaluate the inhibitory effect on human platelet aggregation of the aerial part of *C. edulis*. Aerial *C. edulis* was firstly macerated with methanol, and then fractionated with different solvents to obtain 3 fractions: *n*-hexane, ethyl acetate and aqueous extracts. The inhibitory effects on human platelet aggregation of extracts were evaluated via three parameters: percentage inhibition of platelet aggregation (%I), area under the aggregation curve (AUC) and aggregation slope, using two agonists, adenosine diphosphate (ADP) and collagen. The results showed that all extracts significantly inhibited platelet aggregation in a dose-dependent manner for both agonists. Moreover, all extracts significantly reduced AUC and slope, indicating their antiaggregatory effects on both general aggregation and aggregation velocity. Among the extracts, the ethyl acetate fraction exhibited the strongest effect regardless of agonists used (%I at 4 mg/mL, 99.2 % for both ADP and collagen). The *n*-hexane fraction also had a significant inhibitory effect but it was weaker than the others ($p < 0.05$). This is the first study to demonstrate the potent antiplatelet effect of the aerial part of *C. edulis*. This plant could be a potential natural source to search for new antiplatelet agents and to develop dietary supplements for the management of cardiovascular diseases.

Keywords: Antiplatelet, *Canna edulis*, cardiovascular, agonist.

Classification numbers: 1.2.1, 1.2.2, 1.4.8.

1. INTRODUCTION

Platelets play a crucial role in ensuring the integrity of the vascular system. Upon injury, they interact with the exposed extracellular matrix to form a hemostatic plug at the damaged site [1]. Disorders in this process may cause unwanted platelet clots that obstruct the flow in blood vessels, leading to thrombosis. Arterial and venous thrombosis is a severe complication of cardiovascular disease and contributes to morbidity and mortality worldwide [2]. The use of antithrombotic agents, including antiplatelet, anticoagulant, and fibrinolytic agents, is the cornerstone in the management of thrombotic events. Despite the advancement in antithrombotic therapies, their side effects and drug resistance remain significant concerns. Therefore, more

effective and safer treatment options for thrombosis are needed [3, 4]. Searching for new medications from medicinal plants has become a topic of interest to researchers around the world [5, 6].

Canna edulis Ker Gawl, the only species from the genus *Canna* of the family Cannaceae, is widely distributed in South America, Thailand, China, Taiwan, and Vietnam. Traditionally, both the aerial part and rhizome of this plant have been used for the treatment of various ailments, including rheumatism, pain, fever, inflammation, hepatitis, and heart-related diseases [7]. The phytochemical investigation of *C. edulis* rhizome resulted in the identification of flavonoids and phenolic compounds with antioxidantactivity [8, 9]. Lignin from *C. edulis* rhizome was proved to have inhibitory effects on α-D-glucosidase, α-amylase, and trypsin [10 - 12]. Recently, we have demonstrated the potent antiplatelet and anticoagulant effects and isolated antithrombotic compounds such as epimedokoreanone A and nepetoidin B from the *C. edulis* rhizome [6]. It should be noted that the scientific data on phytoconstituents and pharmacological activities of *C. edulis* is very limited and mainly focused on the rhizome part. No studies regarding the potential of aerial *C. edulis* in the treatment and prevention of cardiovascular-related diseases have been documented. This study reports for the first time the potent inhibitory effect on human platelet aggregation of the aerial part of *C. edulis*.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Methanol, *n*-hexane and ethyl acetate were purchased from Xilong Scientific Co., Ltd., China. Dimethyl sulfoxide, aspirin and ticagrelor were obtained from Sigma-Aldrich, USA. Adenosine diphosphate and collagen were provided by Chronolog Corp., USA.

2.2. Plant materials and extraction

The aerial part of *C. edulis* Ker Gawl was collected in Thai Nguyen province, Viet Nam and identified by Dr. Thi Thanh Huong Le, Thai Nguyen University of Science, Thai Nguyen, Viet Nam. A voucher specimen (CE.A.TN) was deposited at the Department of Life Sciences, University of Science and Technology of Ha Noi. The aerial part of *C. edulis* was cleaned, air-dried and ground to fine powder. Then the powder (1.1 kg) was macerated three times with methanol at room temperature. The crude was filtered and evaporated under reduced pressure conditions at 50 ^{o}C to yield the methanol extract (CE.A.Me). The crude extract was then resuspended in water and subsequently partitioned with *n*-hexane and ethyl acetate to obtain the *n*-hexane residue (CE.A.H, 26.2 g), ethyl acetate residue (CE.A.EA, 4.5 g) and an aqueous layer (CE.A.W, 55.1 g). Extraction percentage yield was calculated as below:

$$\%\ \mathbf{yield} = \frac{\mathbf{mass\ of\ dried\ extract}}{\mathbf{mass\ of\ dried\ aerial\ parts\ before\ extraction}} \times \mathbf{100}(\%) \qquad (1)$$

2.3. Blood sample collection and plasma preparation

This study was approved by the Research Ethics Committee, School of Medicine and Pharmacy, Vietnam National University, Ha Noi, Viet Nam (document number 02/2020/CN-HĐĐĐ). Healthy, non-smoking volunteers (aged 18 - 35 years) with no medications taken for at least 3 weeks before blood sampling were recruited. After overnight-fasting, venous blood was collected from participants and transferred to 3.2 % sodium citrate (9:1, v/v) tubes. A complete

blood count was checked before experiments to ensure that the studied blood samples had normal blood cell counts. The citrate tubes were centrifuged at 500 rpm for 10 min at room temperature to obtain platelet-rich plasma (PRP), or at 3000 rpm for 10 min at room temperature to obtain platelet-poor plasma (PPP). Platelet count in PRP was adjusted to 3×10^8 platelets/mL using PPP. Plasma samples were used within 3 h after blood collection.

2.4. ADP- and collagen- induced antiplatelet aggregation assay

Canna edulis extracts were dissolved in DMSO and then diluted into different concentrations of 40, 20 and 10 mg/mL. To assess the influence of the extracts on platelet aggregation function, a turbidimetric method was applied [13]. Briefly, PRP (450 µL) was incubated with the tested sample at different concentrations (50 µL) for 3 min at 37 ^{o}C. PPP was used as the blank sample to establish the baseline light transmission. To initiate the aggregation process, ADP (10 µM) or collagen (2 µg/mL) was added to the PRP sample. 0.1 % DMSO was used as the negative control. 0.002 mg/mL ticagrelor and 1 mg/mL aspirin were used as the positive control for ADP and collagen-induced platelet aggregation, respectively. After 6 min, three parameters were collected: maximum percentage platelet aggregation, maximum aggregation slope, and area under platelet aggregation curve (AUC). The inhibitory percentage (%I) of each sample was determined as follows:

$$\%\,\mathbf{I} = \frac{\mathbf{X} - \mathbf{Y}}{\boldsymbol{X}} \times \mathbf{100}(\%) \qquad (2)$$

X and Y were maximum percentage aggregation of the negative control and tested samples, respectively. X and Y, measured as the change in light transmission through the PRP suspension, were recorded by the device. Maximum slope and AUC were also recorded by the device. I% indicates the maximal inhibitory effect on the platelet aggregation. The maximum slope of the platelet aggregation curve represents the aggregation velocity per min caused by the tested sample. Low aggregation slope values caused by the tested sample suggest that the tested sample inhibits the aggregation velocity. The determination of AUC is based on the height of the aggregation curve and its slope, thus AUC depends on both maximum platelet aggregation and aggregation velocity. AUC represents the overall platelet aggregation. Low AUC values caused by the tested sample suggest that the tested sample inhibits the overall platelet aggregation [6].

2.5. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The independent sample t-test and one-way ANOVA were performed using SPSS 23.0 software to determine the statistical differences between the group means. Pearson's correlation coefficient (r) was calculated to express the relationships between variables. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

The extraction of the aerial part of *C. edulis* yielded the total methanol extract (CE.A.Me, 11.5 %) and 3 fractions: CE.A.H, 2.3 %; CE.A.EA, 0.4 %; CE.A.W, 5.0 %.

3.1. Inhibitory effect of aerial *C. edulis* extracts on ADP-induced platelet aggregation

3.1.1. Percentage inhibition of platelet aggregation

In general, the total extract and all 3 fractions showed inhibitory effects on ADP-induced platelet aggregation at all tested concentrations. Moreover, the antiplatelet effects of all extracts were dose-dependent (Pearson correlation coefficient, $r > 0.86$, $p < 0.05$ for all extracts). At 4 mg/mL, the *n*-hexane extract CE.A.H showed a similar antiplatelet effect as 0.002 mg/mL ticagrelor (mean %I, 78.7 % vs 73.4 %, $p > 0.05$), while the other extracts exerted significantly higher percentage inhibition of platelet aggregation compared to the positive control ($p < 0.05$). In addition, the ethyl acetate fraction showed the strongest antiplatelet effect among extracts; its percentage inhibition at 4 mg/mL was 99.2 % ± 1.0 % ($p < 0.05$). Except for CE.A.W, other extracts at 1 mg/mL had significantly lower %I compared to ticagrelor ($p < 0.05$) (Figure 1, Table 1).

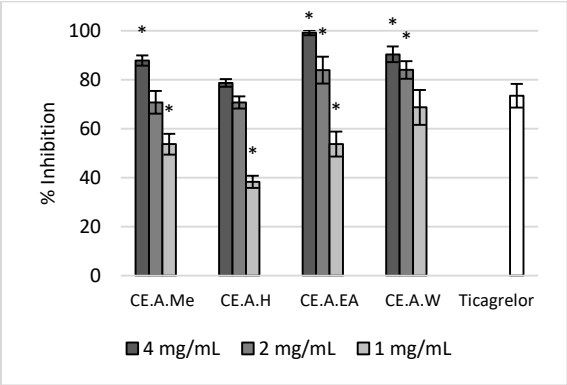

Figure 1. Percentage inhibition of aerial *C. edulis* extracts on ADP-induced platelet aggregation. CE.A.Me: methanol extract from aerial *C. edulis*, CE.A.H: n-hexane fraction from aerial *C. edulis*, CE.A.EA: ethyl acetate fraction from aerial *C. edulis*, CE.A.W: aqueous fraction extract from aerial *C. edulis*. *: $p < 0.05$ compared to 0.002 mg/mL ticagrelor.

3.1.2. Inhibitory effects of aerial C. edulis extracts on aggregation velocity and overall platelet aggregation

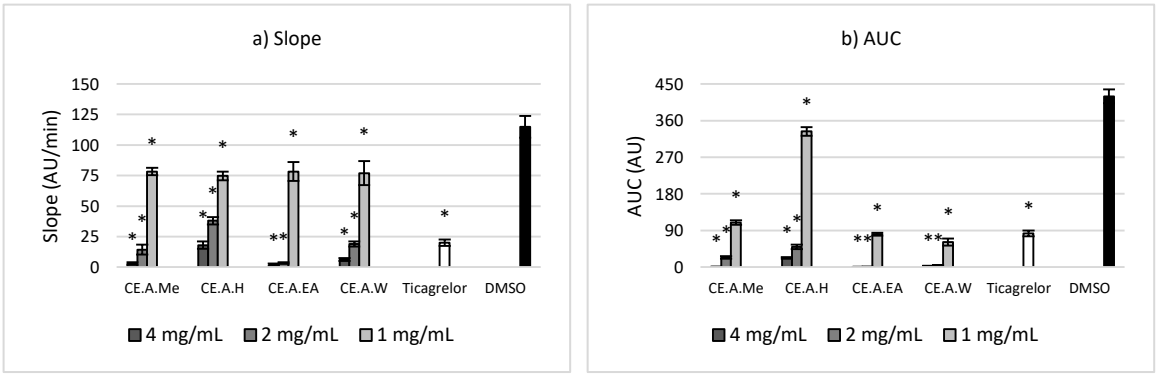

Figure 2. Slope and AUC of aerial *C. edulis* extract on ADP-induced aggregation. CE.A.Me: methanol extract from aerial *C. edulis*, CE.A.H: n-hexane extract from aerial *C. edulis*, CE.A.EA: ethyl acetate extract from aerial *C. edulis*, CE.A.W: aqueous extract extract from aerial *C. edulis*. *: $p < 0.05$ compared to 0.1 % DMSO.

When the platelet aggregation was induced by ADP, all extracts showed significantly lower slope and AUC compared to the negative control ($p < 0.05$). These two parameters of extracts were dose-dependent. The ethyl acetate fraction at 4 and 2 mg/mL, and the methanol extract at 4 mg/mL had similar slope and AUC ($p > 0.05$), and these values were the lowest compared to other extracts and the positive control ($p < 0.05$). At 4 mg/mL, the water fraction had significantly lower slope and AUC than the positive control ($p < 0.05$). The *n*-hexane fraction at a concentration of 4 mg/mL had a similar slope ($p > 0.05$), but lower AUC than the positive control ($p < 0.05$) (Figure 2, Table 2).

3.2. Inhibitory effect of aerial *C. edulis* extracts on collagen-induced platelet aggregation

3.2.1. Percentage inhibition of platelet aggregation

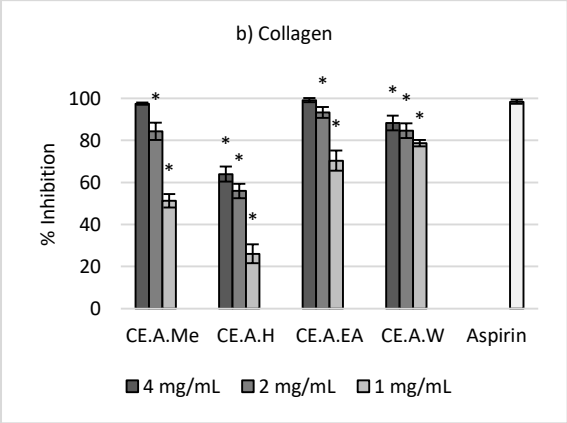

Figure 3. Percentage inhibition of aerial *C. edulis* extracts on collagen-induced plateletaggregation. CE.A.Me: methanol extract from aerial *C. edulis*, CE.A.H: n-hexane fraction from aerial *C. edulis*, CE.A.EA: ethyl acetate fraction from aerial *C. edulis*, CE.A.W: aqueous extract fraction from aerial *C. edulis*. *: $p < 0.05$ compared to 0.1 mg/mL aspirin.

In the case of collagen agonist, the results also showed that all extracts exhibited potent inhibitory effects on platelet aggregation, and their effects were dose-dependent (Pearson correlation coefficient, $r > 0.86$, $p < 0.05$ for all extracts). At the highest tested concentration of 4 mg/mL, the ethyl acetate fraction almost completely inhibited the collagen-induced platelet aggregation, and its percentage inhibition was similar to the positive control (mean %I, 99.2 % vs 98.4 %, $p > 0.05$). The inhibitory effect of the total methanol extract was also potent (at 4 mg/mL, mean %I, 97.5 %), however it was significantly lower compared to CE.A.EA at the same tested dose ($p < 0.05$). Both *n*-hexane and water fractions also showed good antiplatelet aggregation activity (at 4 mg/mL, mean %I, 64.0 % and 88.3 % for CA.E.H and CA.E.W, respectively), but their effects were significantly lower than both CE.A.EA and CE.A.Me at the highest tested concentration and 1 mg/mL aspirin at all tested doses ($p < 0.05$) (Figure 3, Table 3).

3.2.2. Inhibitory effects of aerial C. edulis extracts on aggregation velocity and overall platelet aggregation

Similar to the case of ADP agonist, all extracts also showed the ability to reduce slope and

AUC when the tested concentration increased (Pearson correlation coefficient, $r < -0.75$, $p < 0.05$). In addition, all extracts exhibited significantly lower slope and AUC than 0.1 % DMSO ($p < 0.05$). At 4 mg/mL, both the ethyl acetate and water fractions exerted a lower slope compared to 1 mg/mL aspirin ($p < 0.05$), while the AUC of these two extracts were similar to the one of the positive control ($p > 0.05$). The slope and AUC of both methanol and *n*-hexane extracts CE.A.Me and CE.A.H at all tested concentrations were significantly higher than the positive control ($p < 0.05$) (Figure 4, Table 4).

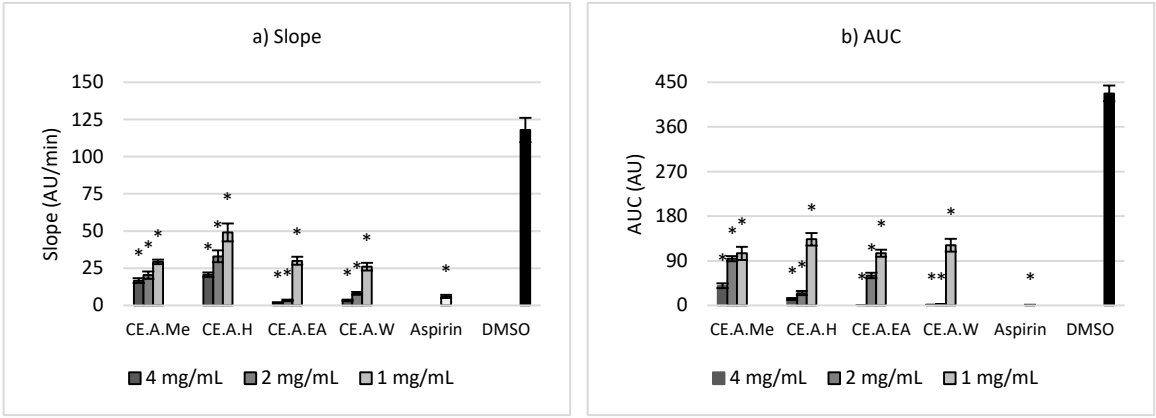

Figure 4. Slope and AUC of aerial *C. edulis* extracts on collagen-induced platelet aggregation. CE.A.Me: methanol extract from aerial *C. edulis*, CE.A.H: n-hexane extract from aerial *C. edulis*, CE.A.EA: ethyl acetate extract from aerial *C. edulis*, CE.A.W: aqueous extract extract from aerial *C. edulis*. *: $p < 0.05$ compared to 0.1 % DMSO.

3.3. Discussion

Medicinal plants are valuable natural resources for the development of functional food and pharmaceutical industries. This study proved for the first time the potent antiplatelet effect of the aerial *C. edulis* using the light transmission aggregometry, which is the gold standard for platelet function testing and evaluation of antiplatelet therapies [14]. Upon stimulation of an agonist (ADP, collagen, ristocetin, epinephrine, etc.), platelets in the PRP sample adhere, and the difference in the light transmittance between the PRP and PPP is measured to assess the aggregation ability of the tested sample. Maximum percentage aggregation, AUC and slopes are three parameters measured to investigate the antiplatelet effects of the samples. Maximum percentage aggregation represents the degree of aggregation, while slope and AUC express aggregation velocity and the overall aggregation, respectively. Based on the maximum percentage aggregation of the negative control and the tested samples, the percentage inhibition of platelet aggregation (%I) of different samples is calculated. This parameter along with the aggregation slope are important for antiplatelet studies. However, AUC is the best measure of overall platelet activity, and it is used mainly for diagnostic and clinical decision-making purposes [15].

In this study, the total methanol extract from aerial *C. edulis* showed a good antiaggregatory activity, with high percentage inhibition of platelet aggregation (%I at 4 mg/mL, 87.83 % ± 2.08 % and 97.50 % ± 0.50 % for ADP and collagen, respectively). It also significantly reduced slope and AUC compared to the negative control regardless of agonists used ($p < 0.05$). Furthermore, at 4 mg/mL, the slope and AUC of CE.A.Me were significantly

lower than the positive control in the case of ADP, indicating its potent inhibitory effect on both aggregation velocity and overall platelet aggregation. The three fractions of aerial *C. edulis* also had remarkable antiplatelet effects, in which the ethyl acetate fraction CE.A.EA showed the strongest antiplatelet activity ($p < 0.05$), followed by CE.A.W and CE.A.H. CE.A.EA almost completely blocked the aggregation process induced by both ADP and collagen, with %I of 99.17 % ± 1.04 %. Moreover, the slope and AUC parameters of CE.A.EA were significantly lower compared to the positive controls ($p < 0.05$), indicating its significant inhibitory effect on not only the maximum platelet aggregation, but also the aggregation velocity and the overall platelet aggregation. Despite the activity of CE.A.W and CE.A.H being not as high as CE.A.EA, their inhibitory effects on platelet aggregation were still good. At 4 mg/mL, CE.A.W exhibited a remarkable inhibitory effect on ADP-induced platelet aggregation, with significantly higher percentage inhibition compared to ticagrelor ($p < 0.05$). The water fraction also showed significantly lower slope and AUC compared to the positive control for ADP ($p < 0.05$). Additionally, CE.A.W also demonstrated a significantly reduced slope in collagen-induced platelet aggregation compared to aspirin. This indicates its impact on both the rate and extent of platelet aggregation for both ADP and collagen agonists. CE.A.H also exhibited a notable antiaggregatory effect on ADP-induced platelet aggregation, with significantly higher inhibitory percentage compared to ticagrelor, and a significantly lower AUC than the positive control ($p < 0.05$). The results obtained from the present study suggest that the aerial part of *C. edulis*, particularly the ethyl acetate fraction, is a potential natural source of antiplatelet compounds. Research into the biological effects and mechanisms of action of this plant may help develop alternative therapies for the treatment and prevention of thrombosis and heart diseases.

ADP and collagen are two of the frequently used agonists in platelet aggregometry testing. Upon stimulation, ADP is released from platelet granules and triggers the P_2Y receptors. This leads to a cascade of events, and finally results in platelet activation and aggregation [16]. Collagen induces platelet activation by interacting with glycoprotein VI (GPVI), leading to the synthesis of thromboxane A_2, granule release, platelet shape changes and clot amplification [17]. The observed strong effects of aerial *C. edulis* extracts for both ADP and collagen agonists suggest different mechanisms of action of aerial *C. edulis*. Active extracts and bioactive molecules of aerial *C. edulis* might interfere with P_2Y receptors, inhibit the GPVI receptor-mediated signialing pathway or thromboxan A2 synthesis. Several *Canna* species such as *C. edulis* rhizome, aerial *C. generalis* and *C. Indica* were also reported to possess antiplatelet effects. Moreover, the ethyl acetate fraction from these *Canna* plants showed the most potent antiaggregatory effect among extracts, which is similar to the present study [5, 6, 18]. Futher *in vitro* and *in vivo* experiments are needed to clarify modes of action of this plant.

Medicinal plants have long been used for the treatment and prevention of numerous diseases, including cardiovascular diseases. Various studies have been conducted regarding the antithrombotic potential of medicinal herbs and their relying mechanisms [19 - 21]. For example, three citrus plants (yuzu, hallabong and orange) grown in Korea were reported for their antiaggregatory effects, in which the ethyl acetate extract from these plants also exerted the highest inhibitory activity compared to other extracts [19]. The aqueous extract of *Zingiber officinale* rhizome was shown to inhibit platelet aggregation caused by ADP, collagen, epinephrine and arachidonic acid [20]. In addition, gingerol and shogaol, two pure compounds isolated from *Z. officinale* were proven to suppress the aggregation process [21]. Ginsenosides from the root of *Panax ginseng* were found to be the active antithrombotic constituents; they expressed antiplatelet effects through decreased calcium influx, thromboxane A_2 formation and MAPK downregulation [22, 23].

The consumption of plant-based diets is beneficial for the prevention of cardiovascular events. Polyphenol-rich fruits and vegetables are demonstrated to possess inhibitory effect on several biomarkers related to cardiovascular diseases, such as platelet aggregation, platelet hyperactivity and oxidative stress [24]. The rhizome part of *C. edulis* has been found to be a rich source of phenols, flavonoids, lignins and proanthocynidins [8, 12]. This study provides scientific evidence for the antithrombotic potential of aerial *C. edulis*, supporting the traditional use of *C. edulis* for the treatment of heart-related diseases [7]. Aerial *C. edulis* could be a potential source for the development of supplementary products for the management of cardiovascular events. However, more studies are required to investigate the chemical profile, as well as to determine bioactive components of this plant.

4. CONCLUSIONS

This is the first study to demonstrate the potent inhibitory effect of the aerial part of *C. edulis* on platelet aggregation induced by both ADP and collagen. This new finding provides scientific evidence supporting the traditional use of this plant for the treatment of heart-related diseases. Among tested extracts, the ethyl acetate fraction showed the highest inhibitory effect on platelet aggregation regardless of agonists used. The results also suggest that active fractions and bioactive compounds in aerial *C. edulis* may interfere with ADP receptors or the thromboxane pathway involved in the platelet aggregation process. The aerial part of *C. edulis*is a potential source for searching for new antiplatelet agents to develop safer and more effective alternative therapies for the treatment and prevention of cardiovascular disorders. Further studies are required to identify the bioactive phytoconstituents of *C. edulis* and clarify the mechanisms of antiplatelet action.

Acknowledgements. This study was funded by the Vietnam Academy of Science and Technology with the grant number: THTEXS.04/22-24.

CRediT authorship contribution statement. Thi Van Anh Nguyen: Methodology, Investigation, Funding acquisition, Formal analysis, Supervision, Writting paper. Duc Huy Bui: Formal analysis, Writting paper. Hong Luyen Le: Formal analysis, Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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