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ANTIOXIDANT AND ANTITHROMBOTIC ACTIVITIES OF THE *DISTICHOCHLAMYS CITREA* LEAVES EXTRACT

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SUMMARY

Distichochlamys citrea (DC) is an endemic ginger species used in treating associated-heart diseases in traditional medicine in Vietnam. However, scientific evidence to support the local use of this plant was limited. The present study aimed to investigate the antioxidant and antithrombotic activities of *D. citrea* extracts for the first time. The antioxidant activity of DC extracts was assessed by scavenging DPPH radical and measuring their total phenolic content (TPC). The antithrombotic activity was evaluated by inhibiting platelet aggregation and prolonging blood coagulation. Volatile components elucidated by GC-MS were docked with typical platelet receptors, including COX-1 and P2Y12. Results showed that the methanol extract of *D. citrea* exhibited a stronger DPPH scavenging ability (IC_{50} = 0.33 ± 0.00 mg/mL) and a higher TPC (8.09 ± 0.21%) than other extracts ($p < 0.05$). On the other hand, the hexane extract of *D. citrea* (DC-HX) had a remarkable inhibiting impact on ADP-, collagen- and ristocetin–induced platelet aggregation in a dose-dependent manner (Pearson's correlation, $r > 0.90$, $p < 0.05$). In contrast, this extract did not lengthen the clotting time through any factors, such as PT (prothrombin time), and TT (thrombin time), except for APTT (activated partial thromboplastin time) at 4 mg/mL of the extract. GC-MS revealed that oxygenated hydrocarbons (54.45%) dominated the volatile profile of DC-HX, followed by sesquiterpens (37.18%) and diterpenes (6.66%). In the platelet aggregation process, several compounds in DC-HX were firmly bound to COX-1 and P2Y12, which might partly explain the significant antiaggregatory activity of this fraction. In conclusion, *Distichochlamys citrea* may be a potential source of active phytoconstituents for treating radicals- and cardiovascular-associated diseases.

Keywords: Antiaggregatory activity, anticoagulant activity, antioxidant activity, *Distichochlamys citrea*, molecular docking.

INTRODUCTION

In 2019, cardiovascular diseases (CVDs) killed 17.9 million people, representing approximately one-third of all deaths globally (WHO, 2021). More than 80% of all deaths related to cardiovascular disease are caused by the development of blood clots, often known as thrombosis (Mackman, 2008). Primary antithrombotic medications aim to inhibit platelet aggregation and blood coagulation, preventing thrombosis. Those drugs are relatively effective but also responsible for side effects such as bleeding and drug resistance, limiting their use (Mackman *et al.*, 2020). Therefore, finding more effective and safer antithrombotic agents in natural resources is necessary for people at high risk of CVDs.

In the literature, many studies showed that ginger (*Z. officinale*) exhibited a potential antithrombotic effect. In 1984, Srivastava and colleagues demonstrated the inhibiting impact of the aqueous extract of *Z. officinale* on ADP, collagen, epinephrine, and arachidonate-induced platelet aggregation (Srivastava, 1984). In 1997, a three-month study on those who have coronary artery disease indicated significant suppression of ADP and epinephrine-triggered platelet aggregation by a of 10 g of ginger powder each day (Bordia *et al.*, 1997). More recently, ginger was considered a potential antithrombotic agent by reducing platelet thromboxane-B_2 (TXB_2) production in rats administered 500 mg/kg orally for four weeks (Thomson *et al.*, 2002). Like the antiplatelet aggregation activity, ginger *Z. officinale* was also documented to have an inhibitory effect on blood coagulation. In 2016, an extract of *Z. officinale* rhizomes in water considerably inhibited the *in vitro* blood coagulation by increasing prothrombin time (Taj Eldin IM *et al.*, 2016). In 2017, Ajala and colleagues demonstrated that the ginger's methanol extract remarkably exhibited an *in vivo* prolongation of APTT, PT, and TT in rats (Ajala *et al.*, 2017). *Distichochlamys,* belonging to the Zingiberaceae family, is an endemic ginger genus in Vietnam (Newman, 1995). Four *Distichochlamys* species have been revealed, including *D. citrea*, *D. orlowii*, *D. benenica,* and *D. rubrostriata* (KaiLarsen and MarkNewman, 2001; Rehse and Kress, 2003; Quoc Binh Nguyen and Jana Leong-Škorničková, 2012). *D. citrea*, also called black ginger, has attracted the attention of domestic scientists for recent years. In 2015, Pham Viet Ty and colleagues from Hue University showed that the principal constituents contained in the essential oils of *D. citrea* rhizomes contained α-pinene, β-pinene, β-linalool, α-terpineol, 1,8-cineole, *cis*-geraniol, β-citral and α-citral. Recently, a few scientific reports on the bioactivities of *D. citrea* rhizomes extracts have been published, such as the antimicrobial effect (Van Hue *et al.*, 2022), antioxidant and anti-glucosidase activity (Van Chen *et al.*, 2022). However, to the best of our knowledge, the antithrombotic effect of this herb has yet to be proven.

The objective of the current study was to assess the antioxidant, antiaggregatory, and anticoagulant activities of DC extracts. GC-MS was used to analyze the volatile components in *D. citrea*. Molecular docking was utilized to partly explain the antiaggregatory effect of the active DC extract.

MATERIALS AND METHODS

Chemicals

DPPH (1,1- diphenyl-2-picrylhydrazil),

ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) ascorbic acid, trolox, aspirin, ticagrelor, ADP (adenosine diphosphate) collagen, ristocetin, heparin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. APTT (activated partial thromboplastin time), PT (prothrombin time), and TT (thrombin time) chemicals were provided by Dade Behring Marburg GmbH (Marburg, Germany). Solvents (n-hexane, ultrapure water), and membrane filters (PTFE, 0.22 µm) for GC-MS analysis were bought from Thermo Fisher.

Plant materials

Distichochlamys citrea was collected in Thua Thien Hue province, Vietnam, in June 2020. Dr. Nguyen Quoc Binh of the Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST) identified this plant. The Department of Life Sciences at the University of Science and Technology of Hanoi, VAST, received a voucher specimen of *D. citrea* with the number SH 1194.

Extraction

10 g of dried leaves of *D. citrea* were macerated at 40°C, under 1 h of sonication with 100 mL (2 times) of each solvent, such as hexane, ethyl acetate, ethanol, and methanol. The crude extracts were obtained after evaporation of solvents under reduced pressure and then stored at 4°C until analysis.

Antioxidant capacity

DPPH assay

The capacity of DC extracts to scavenge DPPH radical was performed following the previous method with slight modifications (Sridhar and Charles, 2019). The experiment was performed on a 96-well plate. In each well, 10 µL of the plant extract was incubated with 190 µL of DPPH (0.1 mM in methanol) for 15 minutes at 37°C. The mixture's absorbance was determined at 517 nm by a Microplate spectrophotometer (xMark, Bio-Rad). The positive control used was ascorbic acid. Following is the calculation of the inhibition percentage:

$$\% \text{ Inhibition} = 100 - (\frac{As}{Ac} \text{x} 100\%)$$

Where As is the sample's absorbance, Ac is the control's absorbance

The sample concentration that inhibited 50% of radicals was determined as the IC_{50} value.

Determination of total phenolic content

This experiment was carried out according to the method Folin-Ciocalteu described in a previous study (Ali *et al.*, 2018). 10 µL of DC extracts were incubated with 95 µL of Folin-Ciocalteu reagent and 95 µL of Na_2CO_3 6% at 40°C for 15 min in a 96-well plate. The absorbance was measured at 765 nm.

A standard curve of gallic acid was established at concentrations from 31.25 to 500 µg/mL. The total phenolic content (TPC) of DC extracts was calculated as grams of gallic acid equivalents per 100 grams of dried sample (g GAE/100 g sample): $TPC = (C_{GAE} \text{ x } 100) / C_o$

Where C_{GAE} is the concentration of gallic acid equivalent (µg/mL), and C_o is the concentration of DC extracts (µg/mL).

Antiaggregatory activity

The Ethics Committee of the School of Medicine and Pharmacy, Vietnam National University in Hanoi, approved this research

with the code: 02/2020/CN-HĐĐĐ. Healthy volunteers were free of antiplatelet or anticoagulant agents at least three weeks before the test. Anticoagulated blood samples were centrifuged at 500 rpm and 300 rpm for 10 min to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP), respectively. All the blood samples were used within three hours after collection from volunteers.

The experiment was investigated according to the previous protocol with slight modifications (Lordan *et al.*, 2020). 50 µL of DC-HX at final 1, 2, and 4 mg/mL concentrations and 450 µL of PRP were gently mixed for 3 min at 37°C. Then, 5 µL of ADP (10 µM), 1 µL of collagen (2 µM), or 5 µL of ristocetin (1.25 µM) were added to the mixture to induce the platelet aggregation. The negative control was 0.1% DMSO, while the positive control was ticagrelor (0.002 mg/mL) for ADP and aspirin (0.1 mg/mL) for the two remaining agonists. The highest aggregation percentage of samples and controls was measured by a Chrono-Log 530 VS aggregator (USA).

Anticoagulant activity

This test was done following the method of Dhahri and colleagues (Dhahri *et al.*, 2020). Briefly, 50 µL of the DC-HX extract at 1, 2, and 4 mg/mL in 0.1% DMSO and 450 µL of PPP were incubated in glass cuvettes for 5 min at 37°C. After adding APTT, PT, and TT reagents, the clotting time of the samples was determined using a coagulation analyzer (ACL TOP500, USA). The negative control was 0.1% DMSO while the positive control was heparin 0.2 IU/mL for APTT and TT or 2 IU/mL for PT parameter. All experiments were run in triplicate.

GC-MS analysis

The volatile constituents in the DC extract were analyzed following Madhumita and colleagues' method with slight modifications (Madhumita *et al.*, 2019). 1 µL of the DC-HX extract (at 1 mg/mL in n-hexane) was injected into a GC–MS machine equipped with a Thermo Scientific GC (TRACE™ 1300) and an MS (DSQ II). All runs were performed using a capillary column TG-5MS (30 m × 0.25 mm × 0.25 µm) (Thermo Fischer Scientific). A gradient oven temperature was carried out with the program as follows: the initial temperature of 50°C was kept for 2 minutes before being progressively increased to 180°C at the rate of 5°C/min and remained at 180°C for 3 minutes. Then, the temperature grew to 280°C at 4°C/min and remained at 280°C for 5 minutes. The gas carrier was helium at a 1.2 mL/min flow rate. The mass scan was set for acquisition from 40 to 800 (*m/z*). All volatile compounds' retention indices (KRI) were compared with reference values of 13 standard n-alkanes (C_8–C_{20}). The raw files were converted into cdf files by Xcalibur software (Thermo Scientific); then processed by Automated Mass Spectral Deconvolution and Identification System (AMDIS) software. The structure and percentage by mass of volatile components in the DC extract were determined using the NIST mass spectral library (NIST27, WILEY7) and essential oil components by GC/MS Version 4 by Robert Adams (Adams, 2007).

Molecular docking

24 compounds in the DC-HX extract were docked against COX-1 and P2Y12 (PDB-IDs of 3N8Z and 4NTJ, respectively) by using Autodock Vina 4 software. Aspirin and ticagrelor were used as the positive control.

The targets' structure was obtained from Protein Data Bank, and after ligands and solvent removal, the proteins were prepared using Autodock Tools version 1.5.7 by adding hydrogen and assigning Gasteiger charges. The structure of small molecules was extracted from the PubChem database in 2D sdf format, converted to pdb format by MarvinSketch version 22.3, and prepared to Autodock format (pdbqt) by Raccoon plugin (Forli *et al.*, 2016) with hydrogen addition, Gasteiger charge calculation, and torsion assignment. The docking protocols were validated by redocking the co-crystallized ligands through their ability to recover the docking pose and essential interactions of the co-crystallized ligands. Through docking procedures, the binding affinity of small molecules in the DC extract and positive controls were evaluated by the best pose's binding energy (BE) (kcal/mol). Discovery Studio Visualizer software obtained the interaction between ligands and protein targets.

Statistical analysis

GraphPad Prism 9.1 software was used to analyze the data of this study statistically. Data were determined as mean ± SD. Group means were compared using an independent t-test and one-way ANOVA. Differences were significant when $p < 0.05$.

RESULTS AND DISCUSSION

Antioxidant activity

DC extracts exerted an antioxidative activity against DPPH radical in a dose-dependent manner from 62.5 to 1000 µg/mL (Pearson coefficient $r > 0.987$, $p < 0.01$) (Figure 1). DC-ME was shown to be the most effective extract as it had a lower IC_{50} than other extracts ($p < 0.05$). However, all DC extracts significantly exhibited a weaker antioxidant activity than the positive controls ($p < 0.001$) (Table 1).

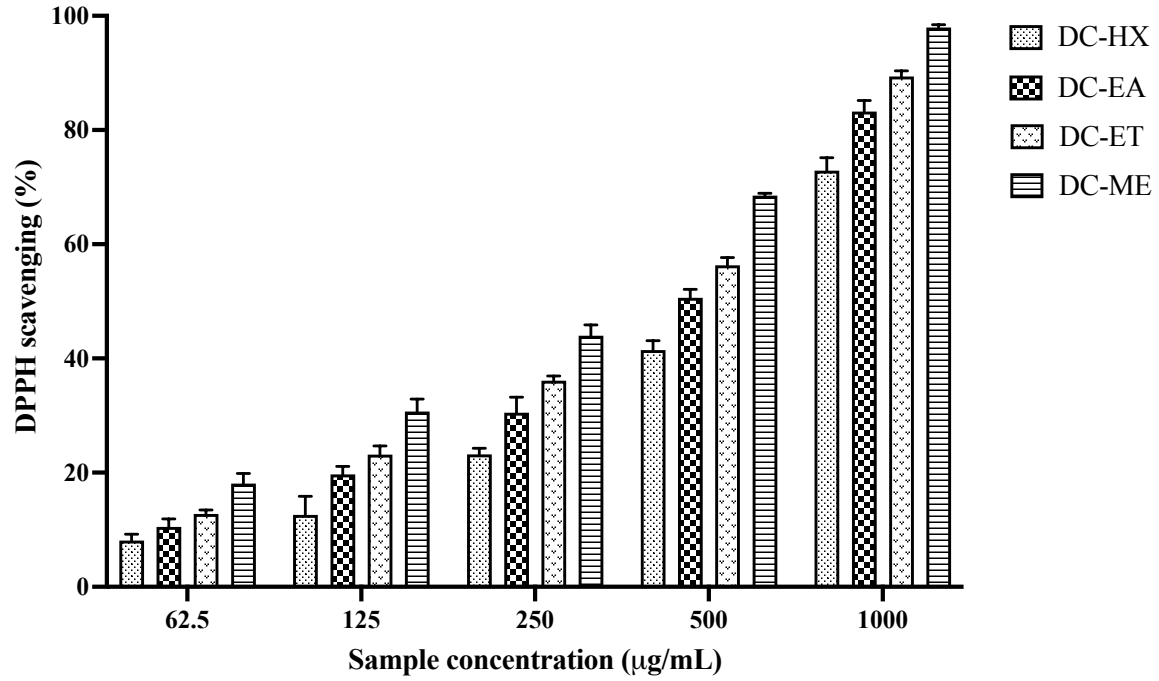

Figure 1. DPPH scavenging capacity of the DC extracts.

Table 1. Antioxidant activity of DC extracts.

Sample	DPPH (IC_{50}, mg/mL)	TPC (%)
DC-HX	0.58 ± 0.05^{a}	3.90 ± 0.50^{a}
DC-EA	0.48 ± 0.01^{b}	6.29 ± 0.09^{b}
DC-ET	0.40 ± 0.03^{c}	7.33 ± 0.26^{b}
DC-ME	0.33 ± 0.00^{d}	8.09 ± 0.21^{c}
Ascorbic acid	0.008 ± 0.001^{e}	

Various superscript letters in each column refer to significant differences among samples ($p < 0.05$)

According to the literature, the DPPH scavenging activity of ginger *Zingiber officinale* fractions was demonstrated with IC_{50} values ranging from 2.81 to 5.57 mg/mL (Yeh *et al*., 2014). In contrast, the essential oil of *Z. officinale* rhizomes seemed to be less effective when scavenging ABTS radical (IC_{50} value of110.14 mg/mL).

The TPC values of DC extracts ranged from 3.90 to 8.09%, much higher than the TPC value (less than 1%) of *Z. officinale* in Turkey using different drying methods *(Ghafoor et al.*, 2020). In another study, the average TPC value of 200 ginger extracts was around 1.7% (Johnson *et al.*, 2022), which was also smaller than the content of phenolic compounds in the *D. citrea* extracts. This result might explain the more vital scavenging ability of *D. citrea* compared to other ginger extracts.

Antiplatelet aggregation activity

In the following antithrombotic effect, only the result of DC-HX was described. The results showed that DC-HX displayed a robust inhibitory effect on ADP, collagen, and ristocetin-induced platelet aggregation for the first time. In addition, the antiaggregatory impact of DC-HX was obtained in a dose-dependent manner (Pearson's correlation, $r > 0.87$, $p < 0.05$) (Figure 2). Moreover, DC-HX at all tested concentrations significantly reduced the maximum percentage of platelet aggregation triggered by all three agonists ($p < 0.05$ compared to the negative control). There is no considerable difference between the three agonists regarding the magnitude of the reduction. However, the inhibitory effect of the DC-HX was much weaker than the positive control ($p < 0.05$).

`In the literature, the n-hexane extract of *Z. officinale* lowered platelet thromboxane generation and inhibited platelet aggregation. Notably, gingerol, a volatile compound isolated from *Z. officinale* displayed remarkable antiplatelet activity with different agonists such as collagen, arachidonic acid, or thrombin (Srivastava, 1986; Guh et *al.*, 1995). Therefore, the n-hexane extract of *D. citrea* was tested for the antithrombotic effect in the current study. The results demonstrated that DC-HX expressed an excellent inhibitory effect on the platelet aggregation triggered by all agonist tested including ADP, collagen, and ristocetin.

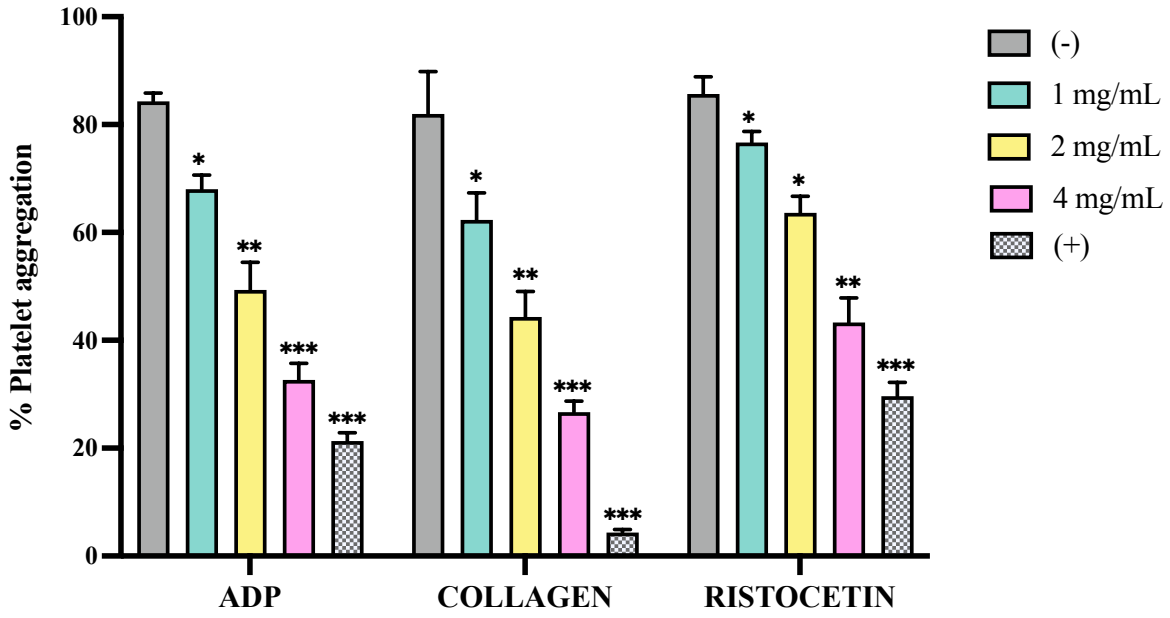

Figure 2. Antiaggregatory effect of DC-HX . (-) and (+): the negative and positive control, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ in comparison with the negative control.

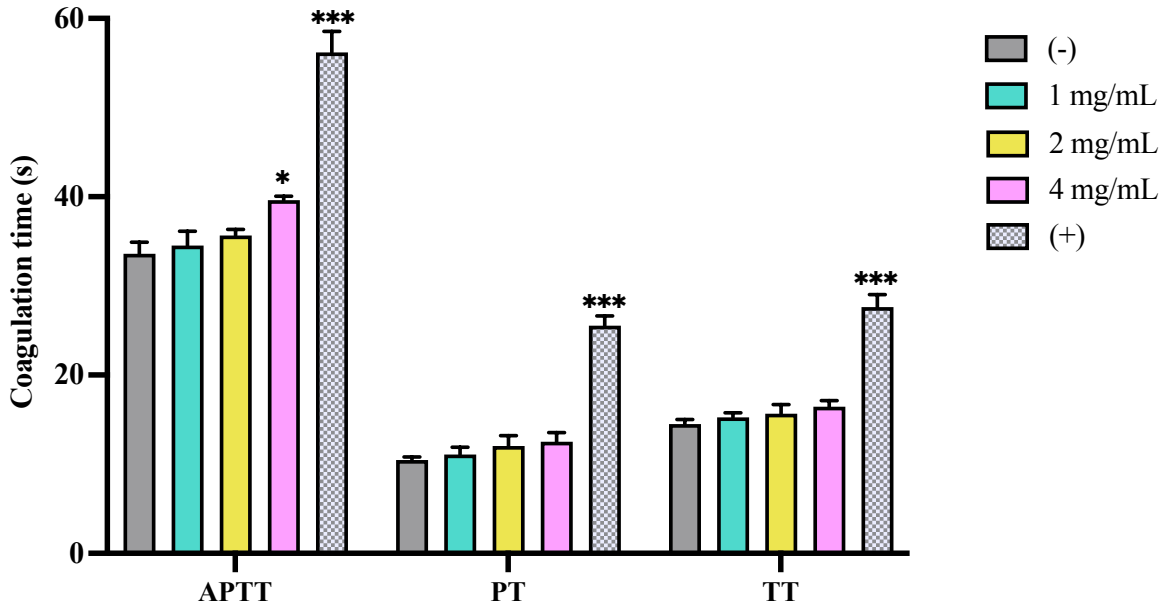

Figure 3. Coagulation time (s) of DC-HX. (-) and (+): the negative and positive control, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ in comparison with the negative control.

Anticoagulant activity

In this experiment, the anticoagulant activity of DC-HX was investigated through three pathways. The APTT test measures the coagulation time by the intrinsic pathway; the PT test determines the clotting time by the extrinsic pathway; and the TT test evaluates the clotting time by the common pathway where fibrinogen is converted to fibrin. The results in figure 3 showed that DC-HX did not prolong the clotting time through any parameters at three tested doses ($p > 0.05$), except for APTT at 4 mg/mL of the extract ($p < 0.05$ in comparison to the negative control).

In the current work, the non-polar fraction of Vietnamese black ginger extracted by n-hexane could only prolong clotting time through the APTT parameter at 4 mg/mL. This fact indicated that n-hexane is probably a suitable solvent to extract antiplatelet agents from *D. citrea* leaves, but it might not be an appropriate solvent for extracting anticoagulant agents from this plant.

Volatile components analysis

The volatile components in DC-HX were determined by analyzing the GC-MS chromatogram as presented in figure 4.

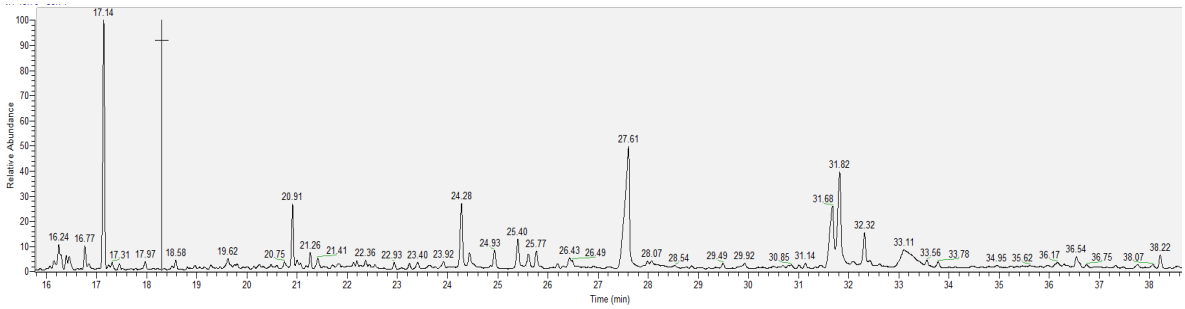

Figure 4. GC-MS profile of DC-HX

A total of 24 compounds in DC-HX, which accounted for 98.29%, were identified. The oxygenated hydrocarbons dominated with 54.45%, followed by sesquiterpenes (37.18%) and diterpene (6.66%). The main constituents in DC-HX included β-sesquiphellandrene (31.32%), n-hexadecanoic acid (17.33%), *trans*-13-octadecenoic acid (8.73%), 4-(1,5-dimethylhex-4-enyl)cyclohex-2-enone (7.25%), (9*Z*,12*Z*)-octadecadienoic acid (6.71%) and neophytadiene (6.66%).

Table 2. Volatile components in DC-HX.

ID	RT (min)	KRI	RI	Hit (%)	Chemical name	Formula	%
1	16.24	1494	1487	48.3	Aristolochene	$C_{15}H_{24}$	2.31
2	16.45	1503	1495	42.2	β-Bisabolene	$C_{15}H_{24}$	0.92
3	16.77	1517	1509	47.9	α-Bisabolene	$C_{15}H_{24}$	2.63
4	17.14	1533	1524	49.4	β-Sesquiphellandrene	$C_{15}H_{24}$	31.32
5	17.30	1539	1532	70.8	(7a*R*)-5,6,7,7a-Tetrahydro-4,4,7a-	$C_{11}H_{16}O_2$	0.48

					trimethyl-2(4H)-benzofuranone		
6	17.97	1568	1568	78.3	Dodecanoic acid	$C_{12}H_{20}O_2$	0.66
7	18.58	1594	1581	66.9	Caryophyllene oxide	$C_{15}H_{24}O$	0.93
8	20.91	1694	1698	84.9	4-(1,5-Dimethylhex-4-enyl)cyclohex-2-enone	$C_{14}H_{22}O$	7.25
9	21.41	1716	1722	43.2	ar-Curcumen-15-al	$C_{15}H_{20}O$	0.96
10	22.36	1757	1768	81.3	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.6
11	22.93	1781	1794	7.46	β-Bisabolenol	$C_{15}H_{24}O$	0.74
12	23.23	1794	1799	13.5	Isosericenin	$C_{16}H_{20}O_3$	0.58
13	23.39	1801	1814	83.3	Tris(2-chloroisopropyl)phosphate	$C_9H_{18}Cl_3O_4P$	0.52
14	24.28	1839	1837	52.6	Neophytadiene	$C_{20}H_{38}$	6.66
15	24.44	1846	1844	88.1	6,10,14-Trimethylpentadecan-2-one	$C_{18}H_{36}O$	1.22
16	25.40	1887	1889	28.9	*trans*-Phytyl acetate	$C_{22}H_{42}O_2$	2.86
17	25.77	1903	1909	5.31	Dihydro-columellarin	$C_{15}H_{22}O_2$	0.93
18	27.61	1982	1968	83.3	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	17.33
19	31.68	2156	2133	23.7	(9*Z*,12*Z*)-octadecadienoic acid	$C_{18}H_{32}O_2$	6.71
20	31.82	2163	2164	14.4	*trans*-13-Octadecenoic acid	$C_{18}H_{34}O_2$	8.73
21	32.32	2184	2172	29.8	Octadecanoic acid	$C_{18}H_{36}O_2$	2.05
22	33.78	2247	2224	59.6	3-Hydroxy-6-methylestra-1,3,5(10),6-tetren-17-one	$C_{19}H_{22}O_2$	0.42
23	36.54	2365	2383	47.2	(*E*)-Labda-8(17),12-diene-15,16-dial	$C_{20}H_{30}O_2$	1.48
24	38.22	2437	2464	83.5	1,3,5-Triphenyl-cyclohexane	$C_{24}H_{24}$	1.71
Diterpene (1 compound: 14)							**6.66**
Sesquiterpenes (4 compounds: 1-4)							**37.8**
Oxygenated hydrocarbons (18 compounds: 5-13, 15-23)							**54.5**
Total							**98.9**

RT: retention time, KRI: calculated Retention Index, RI: theory Retention Index reported by library.

GC-MS result showed that β-sesquiphellandrene was the most abundant component present in the leaves extract of *D. citrea*. A previous study also indicated that β-sesquiphellandrene was among the key constituents, with content ranging from 6.53 to 54.95% of *D. citrea* leave's essential oil from central Vietnam (Pham Viet Ty *et al.*, 2017). Similarly, β-sesquiphellandrene was also present as the main component in many traditional plants such as *Clerodendrum serratum (*5.3%) (Noreen *et al.*, 2018), *Curcuma longa* (7.37%) (D'Auria and Racioppi, 2019), *Zingiber officinale* (8.13%) (Yeh *et al.*, 2014), *Teucrium marum* (11.27%) (Ricci *et al.*, 2005) or *Tripleurospermum disciforme* (17.85%) (Chehregani *et al.*, 2010). The present study indicated that the content of β-sesquiphellandrene contained in *D. citrea* (31.32%) seemed higher than in other plants mentioned above. Earlier studies concluded that ginger oil's antioxidative impact can be linked to several volatile constituents, such as β-sesquiphellandrene and β-bisabolene (Höferl *et al.*, 2015). β-sesquiphellandrene was also one of the primary sesquiterpenes contributing to the antioxidant activity of *Curcuma* species (Zhao *et al.*, 2010). Moreover, the ability to scavenge free radicals of DC-HX might come from other significant phytoconstituents, such as n-hexadecanoic acid (Kalpana Devi V *et al.*, 2012) or trans-13-octadecenoic acid (Selvaraj *et al.*, 2021), but also from minor compounds with intense antioxidant activity, such as β-bisabolene (Kazemi and Rostami, 2015), caryophyllene oxide (Karakaya *et al.*, 2020) or dodecanoic acid (lauric acid) (Henry et *al.*, 2002).

Three fatty acids, including n-hexadecanoic acid (17.33%), *trans*-13-octadecenoic acid (8.73%), 9*Z*,12*Z*-octadecadienoic acid (6.71%) and the only diterpenes neophytadiene (6.66%) also existed with high contents in DC-HX. These compounds were reported in the *Distichochlamys* species for the first time. They also exhibited various pharmacological effects such as the anti-inflammatory property (Aparna *et al.*, 2012) and the anticancer potential (Bharath *et al.*, 2021) for n-hexadecanoic acid; the antiviral activity of trans-13-octadecenoic acid (Selvaraj *et al.*, 2021); the antimicrobial activity (Ceyhan-Güvensen and Keskin, 2016) and the anti-inflammatory effect (Bhardwaj *et al.*, 2020) for neophytadiene.

Taken together, the black ginger of Vietnam is rich in many compounds of great therapeutic value. However, none of those components were previously documented to have the antiaggregatory effect. Therefore, they were docked with the primary platelet receptors to assess their possible contribution to DC-HX antiaggregatory activity.

Molecular docking

COX-1 is a crutial factor that affect the generation of TxA2, a platelet aggregation simulator, and thus displays a thrombotic effect (Li and Diamond, 2014). P2Y12 receptor plays a key role in the activation and aggregation of platelets and has becomes an essential target for antithrombotic drugs (Gachet, 2012). Aspirin and clopidogrel work together to block the P2Y12 receptor and the COX-1 enzyme to reduce platelet activation and thrombosis (Jeffrey, 2012).

A total of 24 compounds in DC-HX exhibited a wide range of binding affinity when docked against COX-1 and P2Y12.

Table 3. Binding energy (BE) of 24 compounds to COX-1 and P2Y12.

No	Chemical name	COX-1 (kcal/mol)	P2Y12 (kcal/mol)
1	Aristolochene	-7.2	-7.5
2	**β-Bisabolene**	**-8.1**	-7.6
3	**α-Bisabolene**	**-8.2**	-7.4
4	**Sesquiphellandrene<beta->**	**-8.0**	-7.7
5	(7a*R*)-5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	-7.7	-7.4
6	Dodecanoic acid	-5.8	-5.3
7	Caryophyllene oxide	-7.5	-8.1
8	4-(1,5-Dimethylhex-4-enyl)cyclohex-2-enone	-7.7	-7.2
9	Curcumen-15-al<ar->	-7.8	-7.6
10	Tetradecanoic acid	-6.4	-5.8
11	Bisabolenol<beta->	-6.4	-7.1
12	Isosericenin	-6.4	-7.2
13	2-Propanol, 1-chloro-, phosphate (3:1)	-4.6	-4.8
14	Neophytadiene	-6.1	-7
15	2-Pentadecanone, 6,10,14-trimethyl-	-6.5	-6.6
16	Phytol acetate<E->	-5.6	-7
17	**Columellarin<dihydro->**	-7.2	**-9.0**
18	n-Hexadecanoic acid	-5	-6.1
19	9*Z*,12*Z*-Octadecadienoic acid	-5.8	-6.1
20	*trans*-13-Octadecenoic acid	-6.5	-6.1
21	Octadecanoic acid	-5.9	-5.9
22	**3-Hydroxy-6-methylestra-1,3,5(10),6-tetren-17-one**	-6.9	**-9.7**
23	(*E*)-Labda-8(17),12-diene-15,16-dial	-7.6	-8.8
24	**1,3,5-Triphenyl-cyclohexane**	4.3	**-9.6**
25	Flurbiprofen (FLP)	-9.6	
26	Aspirin	-6.9	
27	Ethyl 6-{4-[(benzylsulfonyl)carbamoyl] piperidin-1-yl}-5-cyano-2-methylpyridine-3-carboxylate (AZJ)		-9.9
28	Ticagrelor		-8.9

For COX-1, the BE values of all docked compounds are higher than the co-crystallized ligand (FLP, BE = -9.6 kcal/mol). However, several constituents had lower BE than aspirin used as the positive control in the experiment. The one with the strongest binding affinity to the receptor is α-bisabolene (BE = -8.2 kcal/mol), followed by β-bisabolene (BE = -8.1 kcal/mol), found as a minor compound in DC-HX. Interestingly, the most abundant molecule in the black ginger leaves extract (β-sesquiphellandrene, 31.32%) also possessed a strong affinity to COX-1 with BE of -8.0 kcal/mol. These compounds might contribute to the promising antiplatelet aggregation of DC-HX. The complex of both FLP and aspirin with the protein is stabilized by hydrogen bonding between the oxygen atom of the carboxylic group and the hydrogen atom of a residue in the protein (ArgA:120 and SerA:530 respectively) or by the alkyl - alkyl and alkyl - π interaction. Meanwhile, molecules in the plant extract are bound to the receptor mainly by the later interactions and/or by Van der Waals force (Figure 5). This somehow explains the weaker binding affinity of these molecules than that of co-crystallized ligands.

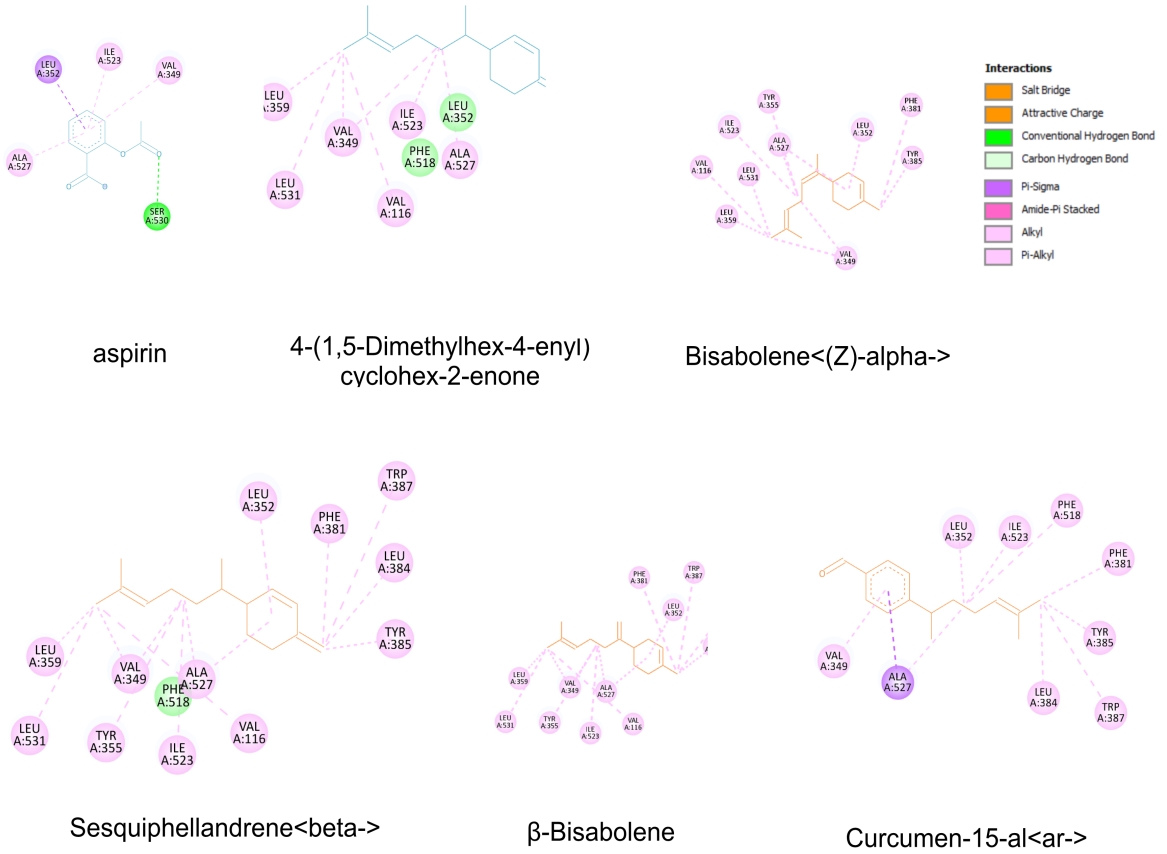

Figure 5. Interactions of α-bisabolene, β-bisabolene, β-sesquiphellandrene, curcumen-15-al<ar-> and 4-(1,5-dimethylhex-4-enyl)cyclohex-2-enone to COX-1.

For P2Y12, the co-crystallized ligand (AZJ) and positive control (ticagrelor) have BE of -9.9 and -8.9 kcal/mol, respectively. Two compounds in DC-HX had comparable BE to AZJ and lower BE than ticagrelor when docked with P2Y12, including 1,3,5-triphenyl-cyclohexane, (-9.6 kcal/mol) and 3-hydroxy-6-methylestra-1,3,5(10),6-tetraen-17-one, (-9.7 kcal/mol). Dihydrocolumellarin, another minor component in DC-HX, bound to P2Y12 as strong as ticagrelor (BE = -9.0 kcal/mol). Apart from hydrogen bonds, π – π stacking, especially the stacking between TyrA:105 of the receptor and an aromatic ring of the ligand (dihydrocolumellarin, 3-hydroxy-6-methylestra-1,3,5(10),6-tetraen-17-one and 1,3,5-triphenyl-cyclohexane), performs a crucial function in strengthening the binding of ligands and P2Y12 (Figure 6).

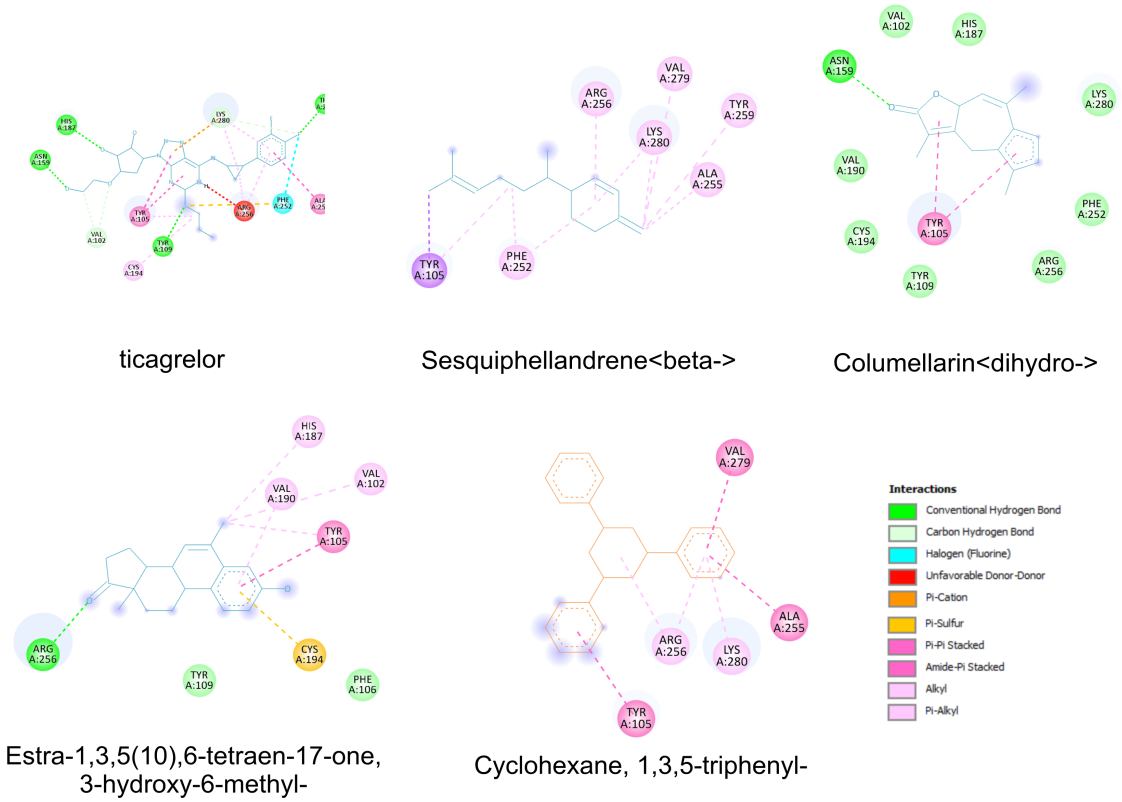

Figure 6. Interactions of β-sesquiphellandrene, dihydrocolumellarin, 3-hydroxy-6-methylestra-1,3,5(10),6-tetraen-17-one and 1,3,5-triphenyl-cyclohexane to P2Y12.

CONCLUSION

In this study, different DC extracts were investigated for their antioxidant capacity. DC-ME exhibited a more vital scavenging ability against DPPH and had a higher total phenolic content than the other extracts. DC-HX showed promising antithrombotic activity by inhibiting platelet aggregation induced by ADP, collagen, and ristocetin, but it only prolonged the clotting time by the APTT parameter. The findings of this research might partly explain the local use of *D. citrea* in Vietnam to treat heart-associated

diseases. Further studies should be investigated to find potential antiplatelet agents in this plant.

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