In-silico and *In-vitro* antiplatelet activity from chloroform fraction of *Carica papaya L.* leaf extract

Cite as: AIP Conference Proceedings **2554**, 070003 (2023); https://doi.org/10.1063/5.0103700
Published Online: 25 January 2023

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In-silico and *In-Vitro* Antiplatelet Activity from Chloroform Fraction of *Carica papaya L.* Leaf Extract.

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Abstract. Ischemia and stroke are the top ten deadliest diseases in the world, where both of these diseases are related to blockage of blood vessels. Clopidogrel as an antiplatelet agent for the therapy for those diseases still has many side effects. The treatment with *Carica papaya* L. extract containing alkaloid compounds, traditionally has an anti-platelet effect. This research aimed to observe antiplatelet activity of chloroform extract of *Carica papaya* L. leaves by *in-silico* study into ADP (Adenosin Diphosphate) receptor and *in-vitro* study of platelet rich plasma induced by ADP which was observed with spectrophotometer UV/Vis method. Based on the results of *in-silico* study, alkaloid compounds such as dehydrocarpain I, dehydrocarpain II and emetin have docking score (-121.77 until -142.75 Kcal / mol) had lower binding energy than Clopidogrel (-111.09 Kcal / mol) toward ADP receptor. The result of *in-vitro* study, found that the addition of 100 ppm of chloroform fraction extract of *Carica papaya* L. leaves inhibited platelet aggregation about 2.55% ± 0.36, which was better than the standard drug, Clopidogrel (2.13% ± 0.34). Based on the results of *in-silico* and *in-vitro* study, the chloroform fraction extract of *Carica papaya* L. which contained alkaloid compounds, had activity to inhibit platelet aggregation, therefore they are potential to be developed as new drugs.

INTRODUCTION

Cardiovascular disease and stroke are among the 10 deadliest diseases in the world and both are in the top ranking in 2019 based on data from Health line Networks, Inc (1). Cardiovascular disease and stroke are the main causes of high rates of morbidity and mortality, which are currently claimed to reach 15 million deaths each year. The death rate from heart disease is estimated to continue to increase until it reaches 23.3 million in 2030. In the US, about 600,000 people died because of heart attacks each year based on data from the Centers for Disease Control and Prevention (CDC) (1). Cardiovascular disease and stroke in Indonesia have increased. Based on the results of the Indonesian Ministry of Health's Basic Health Research (Riskesdas) 2013, the prevalence of coronary heart disease in Indonesia reaches 0.5% and heart failure is 0.13% of the total population aged 18 years and over (2).

Many factors contribute to the increased incidence of death from cardiovascular disease, which one is the formation of a thrombus which triggers the main occurrence of myocardial infarction and stroke (3,4,5). Antiplatelet is one of the therapies that can be given to avoid the incidence of myocardial infarction, stroke and other complications associated with thrombus (3,4).

Platelet aggregation plays a major role in the pathogenesis of thromboembolic diseases. Several endogenous compounds such as thrombin, thromboxane A2, collagen, von Willebrand factor, and ADP stimulate platelet aggregation through different biochemical pathways (3,6). Clopidogrel is an option as a prophylactic for the incidence

The 8th International Conference and Workshop on Basic and Applied Science (ICOWOBAS) 2021
AIP Conf. Proc. 2554, 070003-1–070003-8; https://doi.org/10.1063/5.0103700
Published by AIP Publishing. 978-0-7354-4261-0/$30.00

of thromboembolism, in cardiovascular disease (3). Clopidogrel is an antiplatelet that binds to the ADP receptor or human P2Y12 receptor, thereby inhibiting platelet aggregation. (7,8,9). The problems that arise when Clopidogrel is used as a prophylactic therapy for ischemia are neutropenia and thrombotic thrombocytopenic purpura (8,10). In addition, Clopidogrel can inhibit the P450 cytochrome enzyme so that it affects the metabolism of several drugs such as phenytoin, tolbutamide, warfarin, Fluvastatin, and tamoxifen if given together (8).

The clopidogrel deficiency motive has led researchers to find better antiplatelet drugs derived from natural products. *Carica papaya* L. leaves are one of the traditional Indonesian ingredients that are often used for meat tenderizers (11). The alkaloid content in the leaves is strongly suspected to have anti-platelet activity, thereby preventing blood clots in the meat. The major phytochemicals in *Carica papaya* L. leaves extract is carpaine, pseudocarpaine, dehydrocarpain I, dehydrocarpain II, emetin which are alkaloid compounds. The presence of the N group in alkaloid compounds is strongly suspected to have an inhibitory interaction with the ADP receptor such as Clopidogrel, cilostazol, dipyridamole (4,6,8,12).

The focus of this research is examining *Carica papaya* L. leaves extract containing alkaloid compounds tested in-silico on ADP receptors and in-vitro tests on Platelet Rich Plasma (PRP) induced by ADP with clopidogrel as a positive control observed by spectrophotometry UV/ Vis.

METHOD

Papaya leaves (*Carica papaya* L.) were obtained from Materia Medika Batu Malang, Indonesia. The solvents used were ethanol 96%, chloroform and ethyl acetate (Emsure, Merck, Germany), NH_4OH 1 N (Emsure, Merck, Germany), H2SO4 (Emsure, Merck, Germany). Reagents used include: HCl 2 N (Emsure, Merck, Germany), Mayer reagent (Nitra Kimia Indonesia), Dragendorff (Emsure, Merck, Germany), and Wagner (Nitra Kimia Indonesia), Mg (Emsure, Merck, Germany), $FeCl_3$ (Emsure, Merck, Germany). Antiplatelet drugs Clopidogrel was used as positive control (Dexa Medica Indonesia) The reagent used for *in-vitro* antiplatelet activity was Adenosine Diphosphate (ADP) from Biotop Medical Netherlands.

Extraction Process

About 500 g of papaya leaves' powders were macerated with ethanol 96% for 24 hours three times. The filtrate was evaporated using a rotary evaporator (Rotavapor R-300, Switzerland) to obtain a thick ethanolic extract of papaya leaves (19). The ethanol extract was weighed 10 g then dissolved in 100 mL ethyl acetate and filtered. The residue was dissolved with 100 mL ethanol and 2 N HCl was added to pH 2, then partitioned with 100 mL chloroform and 30 mL distilled water, then the chloroform layer was separated. The ethanol layer was added with 1 N NH_4OH to pH 12 then partitioned again with 100 mL chloroform, followed by evaporating the filtrated to obtain chloroform extract, prepared for the determination of the total alkaloid

Phytochemical Screening

Phytochemical screening was carried out qualitatively to determine the presence of alkaloids, flavonoids, saponins and tannins. Alkaloid screening was carried out with Mayer, Dragendorff, and Wagner reagents. Flavonoid screening using concentrated Mg and HCl powder reagent. Saponin screening was carried out using 1N HCl reagent, while the tannin test was carried out using 10% $FeCl_3$ reagent.

In-silico Study

Molecular docking study was performed by MVD (Molecular Virtual Docker) Ver.5. The ADP receptor or human P2Y12 receptor was downloaded from Protein Data Bank (www.pdb.org) with PDB ID code 4NTJ (14). Validation process was performed by docking stimulation with its native ligand and the acceptance criteria RMSD $\leq$ 2 Å.

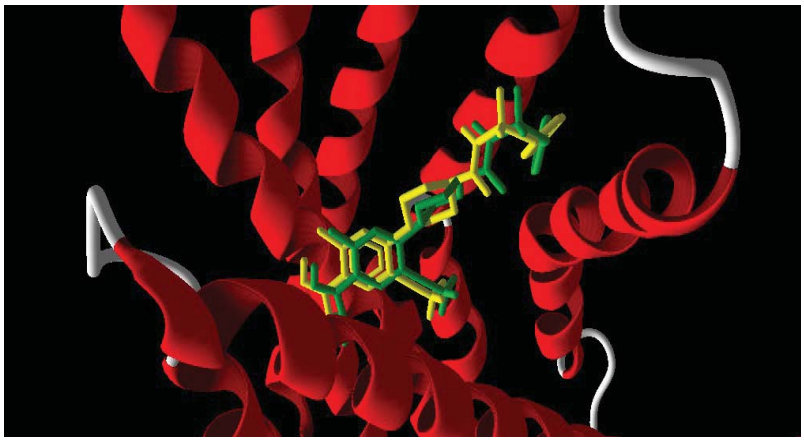


FIGURE 1. Validation its native ligand into active site ADP Receptor

Alkaloids contained in *Carica papaya* L. extract such as carpiane, pseudocarpaine, dehydrocarpain I, dehydrocarpain II, emetin were drawn using ChemBioDraw 3D. The geometrical optimation was calculated by MMFF94 calculation and save as mol. format afterward it were docked into active site ADP receptor or human P2Y12 receptor.

In-vitro Antiplatelet Study

The *in-vitro* antiplatelet activity test was carried out by observing the inhibition of platelet aggregation that occurs when blood plasma is treated along with induction with Adenosine Diphosphate (ADP). The platelet aggregate formed was assessed by comparing the plasma absorption before and after being given ADP using a spectrophotometer UV/Vis (Thermo Fisher Scientific, USA). The greater the decrease in plasma platelet absorption, the greater the aggregate formed. 1 mL Platelet Rich Plasma (PRP) was added with a test solution of 250 µl and then incubated at temperature 37°C in a water bath (Health, Korea) for 20 minutes. After incubation, PRP absorption was measured using a UV-Vis spectrophotometer using Platelet Poor Plasma (PPP) as a blank. The PRP absorbance which had been measured was then added with 20 µL of ADP and then incubated in a water bath at 37 ° C for 20 minutes and then the absorption was measured again (15-18).

The percentage of inhibition of platelet aggregation was calculated by the following formula (19):

$$\%\ \text{Aggregation inhibition} = \frac{(1-\text{B})}{\text{A}} x\ 100\% \quad (1)$$

B: absorbance after addition of ADP
A: absorbance before addition of ADP

The test materials in this study consisted of placebo control (distilled water), positive control, and total alkaloid extract of papaya leaves. The drug used in the positive control group was Clopidogrel 1 mg/ml (Dexa Medica Indonesia). The total alkaloid extract was suspended in distilled water with three concentrations of 0.50, 1.00 and 2.00 mg/mL. Calculation of IC_{50} values was used the IC_{50} Calculator (https://www.aatbio.com/tools/ic50-calculator).

RESULT AND DISCUSSION

Phytochemical Screening

500 grams of *Carica papaya* L. leaves powder was extracted by ethanol 96% producing the dark green extract about 42.6 grams (8.52%) ensure that the total alkaloid compounds of *Carica papaya* L. leaves extract separated from other metabolite compounds, we used fraction extraction method to process it. Table 1 showed the results of the phytochemical screening of the total alkaloid extract of papaya leaves. The chloroform fraction containing only alkaloids were being tested in vitro for antiplatelet activity.

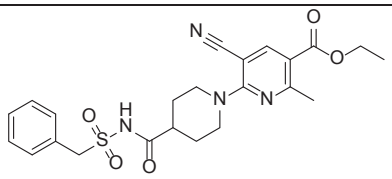
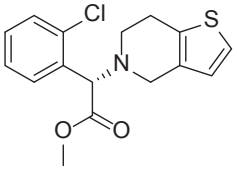
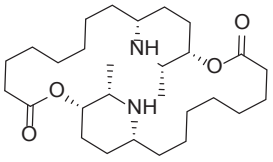
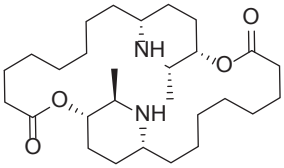
TABLE 1. Result of Phytochemical screening of *Carica papaya* L. extract

Phytochemical screening	*Carica papaya* L. extract	
	Ethanol Fraction	Chloroform Fraction
Alkaloid		
Mayer	+	+
Wagner	+	+
Drugendorf	+	+
Flavonoid	+	-
Saponin	+	-
Tanin/Fenol	+	-

In-silico Study

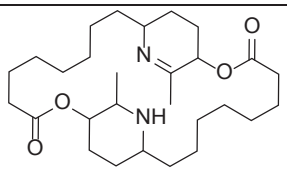
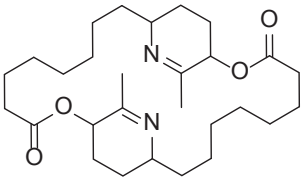
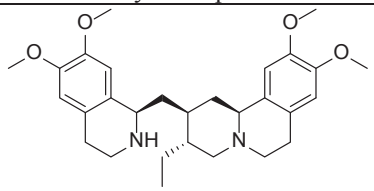
Based on literature review, the main alkaloids contained in the extract of *Carica papaya* L. are carpaine, pseudocarpaine, dehydrocarpain I, dehydrocarpain II, emetin (20-23). The five alkaloids were docked to the ADP receptor, which is one of the targets of the clopidogrel drug as an antiplatelet agent. Based on the result of *in-silico* studies dehydrocarpain I, dehydrocarpain II and emetin have docking score -121.77 until -142.75 Kcal / mol having lower binding energy than clopidogrel (-111.09 Kcal /mol) to the ADP receptor. dehydrocarpain I, dehydrocarpain II and emetin have the potential to bind to the ADP receptor more strongly than Clopidogrel. The bonds formed on the its native ligand were the hydrogen bond interactions on the amino acid Tyr 109; Asn 159; Gln 195; Arg 256 and its steric interactions with the amino acid Asn 191; Phe 252; Arg 256; Lys 280. The alkaloids contained in *Carica papaya* L. also had hydrogen bonding interactions on the amino acid Asn 159.

TABLE 2. Molecular Docking Result Clopidogrel and *Carica papaya* L' s alkaloid into active site ADP receptor

Compunds	Moldock score (kcal/mol)	Hydrogen bond interaction	Steric interaction
 Native ligand (AZJ_1201)	-162.74	Tyr 109 Asn 159 Gln 195 Arg 256	Asn 191 Phe 252 Arg 256 Lys 280
 Clopidogrel	-111.09	Asn 159	Asn 191 Cys 194
 Carpaine	-107.15	Asn 159 Lys 280	Gln 98 Ser 101 Tyr 105 Tyr 109 Asn 191 Lys 280
 Pseudocarpaine	-102.50	Asn 159 Lys 280	Val 102 Tyr 105 Asn 159 Val 190 Asn 191 Cys 194 Lys 280

Continued on next page

TABLE 2. Continued

Compunds	Moldock score (kcal/mol)	Hydrogen bond interaction	Steric interaction
 Dehydrocarpain I	-133.41	-	Gln 98 Val 102 Tyr 105 Tyr 109 Val 190 Asn 191 Cys 194
 Dehydrocarpain II	-121.77	Asn 159 Arg 256 Lys 280	Val 102 Tyr 105 Tyr 109 Asn 191 Cys 194 Lys 280
 Emetin	-142.75	Cys 194	Tyr 105 His 187 Phe 252

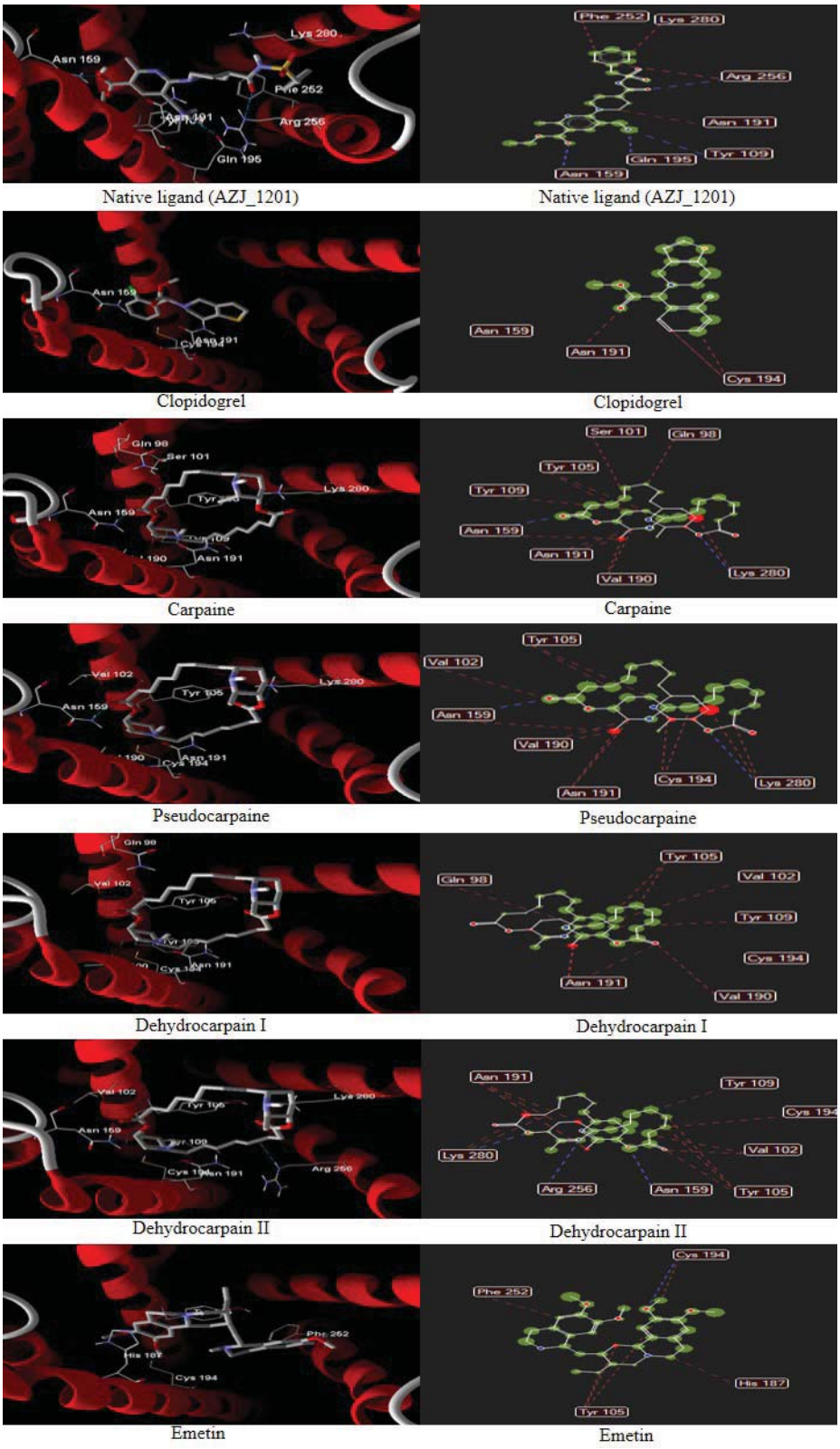


FIGURE 2. 3D and 2D molecular docking interaction its native ligand, clopidogrel and some alkaloid into active site ADP Receptor

In-vitro Antiplatelet Study

Biochemical mediators such as adenosine diphosphate (ADP), thromboxane A2, serotonin, platelet activation factor (PAF) and thrombin are inducers of platelet aggregation [8]. In this study, ADP was used to inhibitor the ADP receptor on Platelet Rich Plasma (PRP) to form platelet aggregation. the standard drug used for antagonist ADP receptor is clopidogrel, therefore clopidogrel is used in in vitro and in-silico study.

The addition of chloroform fraction of *Carica papaya* L. leaf extract aims to antagonist the ADP receptor so that it can inhibit the process of platelet aggregation. Native ligands and Clopidogrel contain the N atom *in-silico* binding to the ADP receptor on amino acid ASN 159 via hydrogen bonds. Alkaloids are compounds containing N atoms so that the chloroform fraction of *Carica papaya* L. leaf extract can inhibit platelet aggregation through ADP receptor antagonist.

Platelet rich plasma (PRP) was induced by ADP and was tested to Clopidogrel at 100 ppm. The result shows that it was able to inhibit platelet aggregation 2.13% ± 0.34 as shown in Table 3. The addition of *Carica papaya* L. chloroform fraction extract 100 ppm inhibited platelet aggregation by 2.55% ± 0.36 better than the standard Clopidogrel drug. The alkaloids content in *Carica papaya* L had activity in inhibiting platelet aggregation with a value of IC_{50} = 206.51 ppm ± 0.12 which needed to be investigated further.

TABLE 3. The Result of IC_{50} value from *Carica papaya* L Chloroform fraction extract

Compounds	% aggregation inhibition	IC_{50} (ppm)
Clopidogrel (100 ppm)	2.13 ±0.34	-
Carica papaya L Chloroform fraction extract (100 ppm)	2.55 ±0.36	
Carica papaya L Chloroform fraction extract (200 ppm)	2.63 ±0.12	206.51 ±0.12
Carica papaya L Chloroform fraction extract (400 ppm)	2.74 ±0.15	

CONCLUSION

Chloroform fraction extract of *Carica papaya* L. leaves which contained alkaloid compounds, are potential to be developed further as novel drug for the treatment of cardiovasdular diseases.

ACKNOWLEDGMENTS

We would like to thank Prof. Siwandono from Airlangga University for MVD (MOLECULAR VIRTUAL DOCKER) VER.5 licensed for obtained the result of *in-silico* study.

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