# Inhibition of pancreatic elastase in silico and in vitro by Rubus rosifolius leaves extract and its constituents

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# Inhibition of pancreatic elastase *in silico* and *in vitro* by *Rubus rosifolius* leaves extract and its constituents

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## Abstract

**Objective:** Elastases are protease enzymes, which mainly hydrolyze proteins of the connective tissue, so they have a significant impact on human disease. *Rubus rosifolius* is one of the *Rubus* species found in Indonesian mountains, and it has potential as an elastase inhibitor. The objective of this research was to examine the *in vitro* elastase inhibitor activity of *R. rosifolius* leaves and to dock different ligands of its constituents against target protein of Porcine Pancreatic Elastase (PPE) receptor. **Method:** Dried leaves powder of *R. rosifolius* was extracted using Soxhlet apparatus with *n*-hexane, ethyl acetate, and methanol. The extract was evaporated, and *in vitro* elastase inhibitor activity was determined using PPE with the quercetin used as control positive. Selected nine constituents of *R. rosifolius* were evaluated on the docking behavior of elastase receptor using Protein–Ligand ANT System ( PLANTS ) computational software with PPE enzyme with Protein Data Bank (PDB) file 1BRU. **Result:** The methanol extract showed significantly inhibited elastase with IC50 186.13 µg/mL, but ethyl acetate extract showed weak activity, and *n*-hexane extract did not show any activity. Docking studies and binding free energy calculations and hydrogen bonding with some amino acids revealed that ellagic acid showed the least binding energy for the target enzyme. **Conclusion:** This research has opened new insights into understanding that constituents of *R. rosifolius* methanol extract are potential inhibitors against elastase, and suggested the active compound is ellagic acid.

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## Full Text

### Introduction

Elastases are proteolytic enzymes, a serine protease, that degrade a wide variety of connective tissue proteins in the lungs, arteries, skin, and ligaments such as elastin. Pancreatic elastase (PE) and neutrophil elastase (NE) represent two of these elastin-cleaving enzymes. Elastases predominate in the pathogenesis of emphysema, acute pancreatitis, rheumatoid arthritis, thrombosis, and stroke in the absence of suitable inhibitors. Elastase-1 is mainly expressed in skin, although it was first found as foreign material during phagocytosis.[1],[2],[3] The development of in vitro methods to test the inhibition of elastase enzyme also can be used in antiaging activity screening tests,[4],[5] and this research can also be used to develop a nutraceutical food. Some compounds are known to have acted as elastase inhibitor, that is, epigallocatechin gallate, catechin, procyanidin, and quercetin.[3],[6],[7],[8]

Indonesia is a tropical country and has many mountains with a lot of plants unexplored, including chemical and biological activities. Rubus rosifolius is one of the Rubus species found in Indonesian mountains. This fruit is economically important as fruit crops and is commercially sold as fresh fruit in the Tangkuban Perahu mountain tourism area at West Java. R. rosifolius fruit was reported to contain flavonoid, sterol, triterpenoid, and had antibacterial activity, antinociceptive, and antiproliferative. Leaves of R. rosifolius were reported to have 5,7-dihydroxy6,8,4'-trimethoxyflavonol, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, and tormentic acid, which had antiproliferative activity against ovary cancer cell.[9] The main isolated compound from the hexane extract of R. rosifolius herb was 28-methoxytormentic acid and was reported to have potential analgesic activity.[10] Others reported that essential oil of R. rosifolius contained β-caryophyllene, dihydroagarofuran, isokessane and β-kessane,[11] ellagic acid, euscaphic acid, pomolic acid, β-sitosterol d-glucoside-6'-acetate, trachelosperogenin A, and some had activity against the carcinogen-activating CYP1B1 enzyme.[12]

Some Rubus were reported to have potential activity as inhibitor enzyme such as elastase, collagenase, hyaluronidase, and tyrosinase.[13],[14],[15],[16],[17] So, the objective of this research was to examine the potential of R. rosifolius leaves as an elastase inhibitor and to dock different ligands of its constituents against target protein porcine pancreatic elastase (PPE) receptor. In this research, Suc-AAA-pNA (SANA) was used as a substrate; this method was established, and the hydrolysis mechanism also was known. After protease action, this substrate releases p-nitroaniline, which is detectable by a microplate reader at 410nm.[18]

The docking process was carried out on the reported compound of R. rosifolius. Nine constituents hypothesized to inhibit the work of PPE enzymes, were chosen. The nine constituents were tormentic acid, pomolic acid, euscaphic acid, ellagic acid, rosifoliol, β-caryophyllene, trimethoxyflavonol, pentamethoxyflavonol, and dihidroagarofuran. Molecular docking can be used to know the potential of compounds to be a drug candidate based on the affinity of binding to target proteins.[19] Binding affinity is a critical aspect to be considered in molecular and macromolecular interactions.[20] This binding affinity was illustrated by the value of the docking score. Lower binding affinities indicate that a compound requires less energy to engage in binding or interaction. In other words, the lower affinity value of binding increases the potential for binding to the target protein.[21],[22] One docking method that has been widely used is the method of docking using software PLANTS (Protein–Ligand ANT System). This method has been widely used in several in silico studies such as in silico tests of antimalarial, antiinflammatory, antioxidant, and antibacterial activity.[18],[23]

## Materials and Methods

### Chemicals and reagents

The chemical and reagents used in this study were buffer Trizma base (T1503; Sigma–Aldrich, St. Louis, MO), PPE (E1250; Sigma–Aldrich), substrate N-succinyl-Ala-Ala-Ala-pnitroanilide (SANA) (S4760; Sigma–Aldrich), quercetin (Sigma-Aldrich), and aquadest.

### Plant material

R. rosifolius leaves were collected from Mount Tangkuban Perahu, West Java. The specimen was authenticated by a botanist at Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia. Fresh samples were cleaned, air-dried, and grounded into a fine powder by laboratory mill.

### Extraction

The leaves were extracted using Soxhlet apparatus by using three different solvents, that is, n-hexane, ethyl acetate, and methanol. The organic solvents were evaporated using a rotary vacuum evaporator and then were dried using a vacuum oven.

### Elastase inhibitory assay

The assay was performed as described by Kraunsoe et al.[24] with slight modification. Elastase inhibitory assay using PPE with the substrate SANA. The product reaction, p-nitroaniline for 15 min at room temperature, was monitored by measuring the absorbance at 401nm using a microplate reader (VersaMax™ Microplate Reader (USA)). The reaction mixture contained 70mM Trizma–HCl buffer (pH 8.0), 16 mU PPE, and 0.29mM SANA, as shown in [Table 1]. It was preincubated for 15 min at 25°C, and the reaction was started by adding substrate. Blanks contained all the components except the enzyme. Quercetin was used as a positive control. The experiments were done in triplicate. The percentage of elastase inhibition was expressed as a percentage of inhibition of elastase activity.{Table 1}

### Hardware and software required Hardware

The hardware that was used to docking process is a laptop with AMD A6 processor, 4 GB RAM, Windows 10, 64-bit operating system.

### Software

The software used in drawing ligand structures is MarvinSketch. The docking process was done using PLANTS 1.1_mingwm.exe. Protein and ligand preparation using YASARA free software. The docking results are visualized using PyMOL.

### Ligand preparation

Chemical structures of two ligands as control positive were (1) gallic acid (CID370) and (2) quercetin (CID5280343). Chemical structures of the nine selected ligands, namely (1) tormentic acid (CID73193), (2) pomolic acid (CID382831), (3) euscaphic acid (CID471426), (4) ellagic acid (CID5281855), (5) rosifoliol (CID527256), (6) betacaryophyllene (CID5281515), (7) trimethoxyflavonol (CID14606539), (8) pentamethoxyflavonol (CID10453852), and (9) dihidroagarofuran (CID10775429). All structures were retrieved from PubMed compound databases (www.pubmed.com). All of the test ligands and 2-(2-hydroxy-cyclopentyl)pent-4-enal as native ligand were prepared to form their conformations using MarvinSketch and saved with file name ligand.mol2 and ref_ligand.mol2.

### Target protein preparation

The three-dimensional structures of the PPE enzyme with PDB ID: 1BRU (DOI: 10.2210/pdb1BRU/pdb) were obtained from www.rcsb.org was pre-processed. Protein and ligand are separated by YASARA software. Protein saved as protein.mol2 and the ligand save as ref_ligand.mol2.

### Molecular docking

The docking process was done using the modification of the standard procedure of molecular docking using PLANTS operating system.[23],[25] The docking protocol validation is performed by calculating the root mean square deviation (RMSD) values between the reference ligand (2-(2-hydroxy-cyclopentyl)pent-4-enal) of PDB ID: 1BRU with the conformation of the redocked ligand. Docking protocol is considered good and can be used for the further docking process, if it has a value less than 3 Å; the alignment closer to 0 is considered better. Binding site center and binding site radius of protein obtained in the process of redocking the reference ligand was input in the plantsconfig.file configuration. It was used as a validated docking protocol. Validated protocol (plantsconfig.file), ref_ligand.mol2, protein.mol2, and ligand.mol2 were prepared as input data. The docking process was done by typing commands on cmd worksheet. PLANTS 1.1_mingwm.exe software will run to calculate the value of the docking score according to the docking protocol that has been validated. The docking process obtained docking scores as output data that showed the energy of the ligand in binding to the target protein. The more negative the docking scores, the affinity of the ligand binding to the protein was stronger. Inhibition constant was determined by the Gibbs equation that was $\Delta G = RTLnKi$.[26]

## Results and Discussion

### Extraction and elastase inhibitory assay

The dried extracts were weighed, and the yield of each extract was calculated. The extraction rendement of hexane, ethyl acetate, and methanolic extracts of R. rosifolius was 2.11 %, 6.93%, and 6.23%, respectively. Elastase inhibitory assay showed that methanol extract gave the best activity in 100 $\mu$g/mL extract. The results are shown in [Table 2]. There are no previous reports available on the elastase inhibitor activity of this species. In vitro elastase activity was assayed using SANA as a substrate. This substrate was chosen because it is specific to the proteolytic activity of PPE, and it was used in similar works present in the literature.[7]{Table 2}

### Molecular docking evaluation

It has been reported in Rubus species that there are phenolic compounds such as ellagic acid (usually found as glycosylated glycosylation polymers), gallic acid, chlorogenic acid, and caffeic acid.[27] The docking score of compounds in R. rosifolius leaves extract can be seen in [Table 3]. The RMSD value was obtained from the redocking 1BRU PDB.ID to form the validated docking protocol, which was 3 Å; this value is eligible for the protocol to be used for further docking process.[28] This value ensures that the docked ligand was in the right position in the binding pockets of the receptor with a minimum shift to the reference ligand position. The alignment between the reference ligand and the conformation of the docked ligand of R. rosifolius inhibition active compound is shown in [Figure 1].{Table 3}{Figure 1}

The docking process of 11 compounds (as depicted in [Figure 2]) showed that two compounds could inhibit PPE enzyme with PDB ID: 1BRU [Table 3]. The gallic acid and quercetin as control positive and rosifoliol had lower docking scores than 2-(2-hydroxy-cyclopentyl)pent-4-enal in inhibiting elastase enzyme (1BRU). Ellagic acid had the lowest docking score in inhibiting the elastase enzyme (1BRU).{Figure 2}

The binding interactions of the most active docked conformation of the test ligands and the target proteins have been identified using PyMol. By checking one by one all amino acids within 4 Å of the active site of the target protein, the critical binding interactions were identified. The interaction of ligands with the binding pocket receptor is given in [Figure 3].{Figure 3}

The negative value of binding energy change (ΔG) reveals that the binding process is spontaneous; it can fit well in the binding pocket receptor forming the most stable drug receptor energetically.[29] The more negative of binding energy value of a compound is more feasible it is used as a drug.[30] The molecular docking results showed that control positive and two of test compounds in R. rosifolius have a docking score that was more negative than reference ligand (2-(2-hydroxy-cyclopentyl)pent-4-enal), which shows that the constituent in R. rosifolius potentially inhibits PPE enzyme. Ellagic acid and rosifoliol have the lowest docking score and inhibition constant; it was indicated that they have the best activity compared to other compounds in inhibiting PPE [Table 3]. Lower score docking and inhibition constant make the formed complex protein–ligand to be more stable and stronger. The interaction of ligand (gallic acid, quercetin, ellagic acid, and rosifoliol) with amino acids on 1BRU binding pocket proteins showed some hydrogen bonds [Table 4]. Some amino acids play a role in the formation of hydrogen bonds between the ligand and active amino acids of protein, that is, SER_190, ASN_192, SER_214, GLY_216, SER_195, CYS_191, HIS_57, and SER_217. Ellagic acid has the most affinity because it has the most number of hydrogen bonds. The SER_195 and HIS_57 are active amino acids that in accordance with the active amino acids form hydrogen bonds between proteins and reference ligand (2-(2-hydroxy-cyclopentyl)pent-4-enal) in PDB data, as depicted in [Figure 4].[31] Visualization results of ligand–protein interaction showed that the presence of the –OH and O groups in the ligand allows more hydrogen bonds to form. Besides hydrogen bonding, electronic bonding, hydrophobic, and van der Waals interaction have influenced the activity of ligand inhibiting the receptor.[32](Table 4) (Figure 4)

Some terpene constituents (such as β-caryophyllene, dihydroagarofuran, and tormentic acid) were isolated from n-hexane fraction. Trimethoxyflavonol and pentamethoxyflavonol were isolated from dichloromethane fraction.[9],[11] The docking test showed that all compounds have larger Ki, and the in vitro test showed that there is no inhibition activity from n-hexane extract. Pomolic acid and euscaphic acid were isolated from ethyl acetate extract.[12] Both of these compounds showed a large Ki even though ethyl acetate extract showed weak activity. Ellagic acid and gallic acid were the most predominant of the phenolic compound isolated from methanol extract.[12] Docking test and in vitro test showed that methanol extract has potential activity as an elastase inhibitor.

For the external preparation can be widely used in cosmetics to prevent skin aging and to maintain the skin in a youthful and healthy state; and also as a nutritional food. In these experiments, it was concluded that methanol extract showed potential activity as an inhibitor against elastase, and molecular docking to protein pancreatic porcine elastase (PPE) receptor (1BRU.pdb) indicated that the ellagic acid was suspected as one of the most active compounds. The result is also consistent with other studies that show that methanol is an efficient extraction medium for a broad spectrum of compounds.[33]

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There are no conflicts of interest.

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