The activity of Flavonoid Isolates from Papaya (Carica papaya L.) Seed as Pancreatic Lipase Inhibitor

Subandi1*, Pancasari Wiji Utami1, Tatas H.P. Brotosudarmo2
1Department of Chemistry, Universitas Negeri Malang (UM), Jl. Semarang 5 Malang
2MRCCP Universitas Machung Malang

*Corresponding author: subandi.fmipa@um.ac.id

Abstract. Excessive fat consumption can cause obesity, which is currently one of the global problems, because it can trigger various diseases such as coronary heart disease, diabetes, and so on. Absorption of fat into the body requires pancreatic lipase enzymes, which can be reduced by lipase inhibitors such as orlistat drugs. Previous research has shown that the extract of papaya seed, which contains many flavonoids, was able to inhibit pancreatic lipase. Therefore, the aims of this study are to isolate and identify the flavonoid compounds from papaya seeds that are active as pancreatic lipase inhibitors. Papaya seed powder was extracted using ethanol 70%, followed by isolation using TLC, identification by phytochemical test and UV and IR spectroscopy, while pancreatic lipase inhibitory activity has been done using titrimetric method. To confirm the Inhibitory activity of the predicted flavonoid against the enzyme, also had been done in silico study. The results had shown that there are two types of flavonoid in the ethanol extract of papaya seed, that are active as pancreatic lipase inhibitors. Papaya seed powder was extracted using ethanol 70%, followed by isolation using TLC, identification by phytochemical test and UV and IR spectroscopy, while pancreatic lipase inhibitory activity has been done using titrimetric method. To confirm the Inhibitory activity of the predicted flavonoid against the enzyme, also had been done in silico study. The results had shown that there are two types of flavonoid in the ethanol extract of papaya seed, that are active as pancreatic lipase inhibitors. The two flavonoid isolates have inhibition power of 132 and 12 times stronger than orlistat in the same mass. Result of UV-VIS and IR spectrum has shown that both isolates were suspected to have a similar structure to the three flavonoid compounds: epicatechin, catechin or epigallocatechin-3-gallate (EGCG). In silico study, also confirmed that each of the predicted flavonoids has higher binding affinity against pancreatic lipase, than orlistat.

Keywords: Papaya seed, Flavonoid, Orlistat, Pancreatic lipase inhibitor.

1. Introduction
Obesity is a global problem [1] and is an important risk factor for type 2 diabetes mellitus (T2DM), cardiovascular disease, cancer, and premature death [2-4]. One of the anti-obesity drug, that is currently widely used, is tetrahydrolipstatin, isolated from actinomycetes (Streptomyces toxytricini), who works as an inhibitor of pancreatic lipase and is available commercially under the name of "Orlistat" [5]. Nevertheless, the consumption of orlistat can cause some side effects such as hyperuricemia, diarrhea, nausea, nyositis, gastric irritation, steatorrhoea, oily spotting, flatulence, flatus with discharge, fecal incontinence and dry skin [6]. Therefore, it has been widely studied some herbal remedies that can replace orlistat, as pancreatic lipase inhibitor.

Pancreatic lipase is an enzyme that plays an important role in the absorption of fat into the body. This enzyme secreted by the pancreas into the intestine and hydrolyze 50-70% triglycerides into fatty acids [7,8]. On other hand the papaya seeds, which contain about 40% of water [9], generally are only...
used as potential seeds to be planted again, but most are still regarded as trash and thrown away. In the case of papaya, the seeds contained in the cavity of the fruit with a mass about 15% of the fruit mass\(^{[10]}\), contains antioxidants\(^{[11,12]}\), protein, carbohydrates, calcium, and beta-carotene\(^{[9]}\); fatty acids, benzylisothiocyanate, benzylglucosinolate, glucotropacolin, benzylthiourea, beta-sitosterol, caricin, and myrosin enzyme\(^{[13-15]}\).

Previous study has revealed the presence of saponins, tannins, flavonoids, alkaloids, carbohydrates, phenolic compounds and carotenoids in the ethanolic extract of Carica papaya seeds\(^{[16]}\). The presence of flavonoids in the papaya seed suggested that the seed allegedly also has inhibitory activity against pancreatic lipase and potentially as an anti-obesity drug. The aims of this study are to isolate and identify the flavonoid compounds from papaya seeds that are active as pancreatic lipase inhibitor. The activity of the predicted flavonoids against the enzyme, also had been analyzed using in silico study.

2. Material and Method

The research has conducted in the laboratory of Chemistry, FMIPA, State University of Malang and Ma Chung Research Center for Photosynthetic Pigments (MRCPP) Ma Chung University. This research consists of 6 stages: 1) preparation of papaya seed powder (drying and smoothing) 2) extraction using ethanol-water solvent with maceration method, 3) separation of flavonoid compound with Thin Layer Chromatography (TLC), 4) phytochemical test of flavonoid compound, 5) activity test of flavonoid isolate as pancreatic lipase inhibitor, and 6) UV-VIS and FT-IR spectroscopy analysis to predict structure of flavonoid isolate. Lipase enzyme used is pancreatic lipase from porcine (Sigma Aldrich), olive oil was used as a source of triglycerides (substrate), and Orlistat (Xenical\(^{®}\)) was used as a standard inhibitor.

2.1. General

Papaya seeds that had been collected were washed with water and dried by drying in the sun until the relative weight was constant. Then papaya seeds roasted and mashed with a blender. Further it was macerated using a water-ethanol solvent (3:7) for 24 hours, and concentrated with a rotary evaporator until a viscous extract was obtained. The separation of the compound was performed by qualitative thin layer chromatography (TLC) to select the best eluent, followed by preparative TLC to separate the isolates. At each stage of isolation, the flavonoid existence were detected by phytochemical tests using concentrated H\(_2\)SO\(_4\) and the power of pancreatic lipase inhibition were test in vitro using titrimetric method, with and without inhibitor. The type of flavonoids isolate were predicted based on their UV and FTIR spectrum, and based on the appropriate literature, then were confirmed by in silico analysis using predicted compounds.

2.2. Inhibition power Test of papaya seeds powder/ extract/ isolate or Orlistat against Pancreatic Lipase

Olive oil emulsion consisting of 2.5 mL of olive oil and gum arabic solution of 22.5 mL 10% (w / v) in water is homogenized for 10 minutes. Then added a solution consisting of 20 mL H\(_2\)O, 15 mL CaCl\(_2\) 0.075 M, and 10 mL of 3 M NaCl, then homogenized back. The mixture was diluted with phosphate buffer to pH 7.5. until 100 mL, homogenized and was used as substrate solution. Taken 25 mL of the substrate solution, then add 1 mg of porcine pancreatic lipase, and plus the sample (papaya seeds powder/ extract/ isolate), shaken for 30 seconds. The mixture was incubated at 37\(^{\circ}\)C for 25 minutes (optimal incubation time). Hydrolysis was stopped by heating into a water bath of 100 \(^{\circ}\)C for 10 minutes. The mixture then was titrated with NaOH 0.1N using pp as indicators until the pink colour arise. As a control substrate was used water as a substitute of olive oil.

Power inhibition test of inhibitor (Orlistat or papaya seed extract) was use the same procedure, unless has to add fine powder of 1 tablet orlistat (120 mg) or extract or isolat of of papaya seed to the solution/ mixture before incubation at 37 \(^{\circ}\)C. Lipase activity with or without inhibitor can be calculated by equation 1.
Lipase activity = \frac{(V_{sm} - V_{sa}) \times N \times NaOH \times 1000}{25} \text{ mikroMol/minute}

Description:
\[ V_{sm} = \text{Volume of NaOH required to titrate the oil substrate} \]
\[ V_{sa} = \text{volume of NaOH required to titrate 'watersubstrate' (as control)} \]
\[ NNaOH = \text{normality of NaOH used} \]
\[ 1000 = \text{conversion factor from mMol to µMol} \]
\[ 25 = \text{incubation time (minutes)} \]

Inhibition power of an inhibitor can be calculated by equation 2 below,

\[
\text{Inhibition power} = \frac{\text{lipase activity without inhibitor} - \text{lipase activity with inhibitor}}{\text{lipase activity without inhibitor}} \times 100\% \tag{2}
\]

while power inhibition relative to the Orlistat can be calculated by equation 3 below,

\[
\text{Inhibition power relative to Orlistat} = \frac{\text{Papaya seed extracted inhibitor power}}{\text{Orlistat inhibitor power}} \times 100\% \tag{3}
\]

3. Result and Discussion

3.1. Sample Preparation and Extraction

The result of papaya seed sample preparation to become viscous extract can be seen in Table 1.

| No | Step          | Sample Mass/ Vol | Process                               | Mass/ Vol Result          |
|----|---------------|------------------|---------------------------------------|---------------------------|
| 1  | Preparation   | 6000 g of fresh Papaya | Seed Taking and washing               | 851 g (wet seed)          |
| 2  | Drying        | 851 g of wet papaya seed | Sun drying and roasted                | 104.6 g (dry seed)        |
| 3  | Smoothing     | 104.6 g of dry papaya seed | Blending                              | 104.0 g (seed powder)     |
| 4  | Sieving       | 104.6 g of dry papaya seed | 50 mesh sieving                      | 92.8 g (fine seed powder) |
| 5  | Extraction    | 85 g of papaya powder | Solution to water-ethanol 650 mL and stiring for 1 x 24 jam | 600 mL (water-ethanol extracted) |
| 6  | Concentration | 600 mL of extract | Rotary evaporator                     | 2.1 mL (conc extracted)   |

According to Table 1, sample preparation has been done by preparing 6 kg or of papaya fruit or 851 gram of papaya seed and produce 104.6 gram of seed papaya “coffee”, and finally 2.1 mL concentrated extracted.

3.2. Flavonoid Isolation From Concentrated Extract of Papaya Seed

Qualitative TLC analysis was performed to obtain the appropriate eluent in separating the components in the extract. The corresponding eluent is a mixture of ethyl acetate, methanol, and water with a ratio
of 0.5: 1.4: 0.1; which can separate the components in concentrated papaya seeds extract into two stains (isolates). The first stain has a Rf value of 0.675 and the second stain has an Rf value of 0.875 (Figure 1a). Furthermore, separation is done by preparative TLC with the same eluent using plate size 15x15 cm. The result of separation of concentrated extract by preparative TLC resulted also two stains (Fig. 1b). Each stain on the preparative TLC plate is then scraped and dissolved in methanol, then filtered and accommodated in different vials. The resulting filtrate was then solvent evaporated at room temperature. The resulting precipitate is weighed, and partially used for UV-Vis and FT-IR spectrophotometry and partially used for inhibition test against pancreatic lipase.

![Figure 1](image)

**Figure 1.** Qualitative (a) and Preparative TLC (b) result under uv lamp λ 254 nM

### 3.3. Phytochemical Analysis

The secondary metabolites content present in papaya seed samples, either in the form of powder, viscous extract, or 2 types of isolate, were analyzed phytochemically using specific reagents (concentrated sulfuric acid). The results had shown that all the samples are positives contain flavonoids, which were indicated by the occurrence of pink color.

### 3.4. Pancreatic Lipase Inhibition Test

The inhibition test on pancreatic lipase has been done by titrimetric method. In this method the lipase activity is proportional to the amount of liberated fatty acid by the oil substrate, which is also proportional to the amount of titrant NaOH used. So the smaller the amount of titrant used is the smaller the lipase activity, which indicates the presence of inhibitor activity.

| No | Inhibitor type                        | Average of NaOH 0.1 titrant (mL) | Inhibition power (%) | Inhibition power relative to Orlistat (%) | Inhibition power relative to orlistat (at the same mass) |
|----|---------------------------------------|----------------------------------|----------------------|------------------------------------------|--------------------------------------------------------|
| 1  | No inhibitor                          | 1,5                              | -                    | -                                        | -                                                      |
| 2  | 1 Tablet of orlistat (120 mg)         | 0,2                              | 86,96%               | 100%                                     | 1 x                                                    |
| 3  | 0,928 g of Papaya seed powder         | 0,7                              | 52,17%               | 59,99%                                   | 0,07 x                                                 |
| 4  | Papaya seed extract (850 mg)          | 1,0                              | 34,78%               | 39,99%                                   | 0,06 x                                                 |
| 5  | Flavonoid isolate 1 (0,5 mg)          | 0,8                              | 47,83%               | 55,00%                                   | 132 x                                                  |
| 6  | Flavonoid isolate 2 (1,5 mg)          | 1,3                              | 13,04%               | 14,99%                                   | 12 x                                                   |
Based on Table 2, the addition of various samples from papaya seeds can decrease the activity of lipase, indicating that the papaya seed sample is active as a pancreatic lipase inhibitor. Even the inhibition power of isolate flavonoid isolate 1 and 2 of papaya seeds was 132 times and 12 times larger than Orlistat at the same weight. This data, suggests that both of these flavonoid isolates are pancreatic lipase inhibitors and potential as an anti-obesity drug to replace Orlistat.

3.5. Identification Result using UV-Vis dan FT-IR Spectrophotometry

To know the type of flavonoids isolates 1 and 2, had been carried out UV-Vis and FT-IR analysis, with the result as shown in Figure 2 and 3.

The spectra of UV-Vis from isolate 1 and isolate 2 showed 3 peaks at wavelengths of 204 nm, 223 nm, and 272 nm in different patterns. These results indicate that isolates 1 and isolate 2 are different compounds.

![Figure 2. UV-Vis Spectrum of flavonoid Isolate 1 (a) and flavonoid isolate 2 (b)](image)

### Table 3. Interpretation of UV-VIS Spectra

| Peak at \(\lambda\) (nM) | Transition of | The existence on the spectra of | Functional group of |
|-----------------|---------------|---------------------------------|---------------------|
|                  |               | Isolate 1 | Isolate 2 |                      |
| 204              | \(\pi \rightarrow \pi^*\) | \(\checkmark\) | \(\checkmark\) | \(-\text{C}=\text{O}\ \text{group}\) |
| 270              | \(n \rightarrow \pi^*\)    | \(\checkmark\) | \(\checkmark\) | \(-\text{Benzene ring}\) |

The result of FT-IR analysis can be seen in Figure 3, and the interpretation of the spectra on Table 4.
Based on FTIR data, the functional groups of the two isolates are relatively the same, so other data are still needed, such as the mass spectrum or NMR to distinguish the structure. According to previous research, however, there are 6 main subgroups of flavonoids that are active as pancreatic lipase inhibitors, namely flavonol (including quercetin, kaempferol, and myricetin), flavanones (eriodictyol, hesperetin, and naringenin), isoflavones (daidzein, genistein, and glycitein), flavones (apigenin and luteolin), flavan-3-ol, and anthocyanin (cyanidin, delphinidin, malvidine, pelargonidin, peonidine, and petunidine) [17]. Based on the phytochemical test, and UV-Vis / FT-IR data, and the previous study [17], can be predicted that isolates 1 and isolate 2 may have characteristics functional group similar to subgroup of flavan-3-ol, that are catechins, epicatechins, and/or epigallocatechin-3-gallate (EGCG) with have structures as shown in Figure 4.

To supported the prediction, had been done in silico studies of those compounds as ligands against enzyme of pancreatic lipase, as receptor.

3.6. In Silico Studies of Cathecin, Epicatechin and EGCG as Pancreatic Lipase Inhibitor

This in silico studies had used software and 3D molecular data as follow.
Software used:
1. PyMol (Python Molecular Viewer) software for preparing docking and visualizing binding positions of ligand compounds with receptor, namely pancreatic lipase enzyme.
2. PyRx 0.8 software for docking.
3. Discovery Studio software for visualizing the interaction of ligand compounds with receptors

Structures used:
1. The 3D structure of the Pancreatic Triacylglycerol Lipase P00591 (LIPP_PIG, with 1ETH code) receptor is obtained from the Protein Data Bank UniProt database (https://www.uniprot.org/) in the PDB file format. The structure used is chain A/C.
2. The 3D structure of the Orlistat ligand compound is obtained from DRUGBANK (https://www.drugbank.ca), Catechin, Epicatechin and EGCG were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) in Sybil Data Files format (sdf)

In this in silico analysis had been used docking techniques that consist of 3 steps, that are: 1) sterilization of 3D structure of receptor (porcin pancreatic lipase), 2) determine the strength of ligand-receptor interaction using molecular docking and 3) visualization position of ligand-receptor interaction.

3.6.1. Sterilization of the Pancreatic Lipase Enzyme Computationally.

The Computationally Sterilization of the pancreatic lipase enzyme aim is to eliminate the water content and all the ligands contained in the enzyme. This process was done using PyMol software (Python Molecular Viewer). Because in vitro analysis has used porcine pancreatic lipase, the same type of enzyme in 3D structure is used in silico analysis, as shown in the Figure 5.

Figure 5. The 3D Structure of porcine pancreatic lipase visualized by PyMol software Source: Uniprot (2019)

Meanwhile the 3D structure of Ligands, has been used 3 molecules taken from PubChem, namely orlistat (as inhibitor standart), cathecin, epicathecin and EGCG as can be seen on Figure 6.
3.6.2. Identification of Ligand Interactions with Pancreatic Lipase Enzymes using Molecular Docking. The molecular docking process is carried out to identify the activity of compounds that have potential as candidates for pancreatic lipase inhibitors. This process uses the Autodock Vina program on the PyRx 0.8 software. The results obtained are binding affinity values of ligands to receptor as can be seen on Table 3.

| Ligand   | Mode 0 | Mode 1 | Mode 2 | Average |
|----------|--------|--------|--------|---------|
| Orlistat | -5.8   | -5.7   | -5.6   | -5.7    |
| Catechin | -9.8   | -9.1   | -9.0   | -9.3    |
| Epicatechin | -9.6   | -9.6   | -8.9   | -9.37   |
| EGCG     | -8.3   | -8.3   | -8.3   | -8.3    |

As shown on Table 3, that catechin, epicatechin and EGCG has greater binding affinity to pancreatic lipases than orlistat. This indicates that the inhibitory power of the enzyme is also greater than orlistat, respectively. These data support the results of in vitro analysis, that each of the two flavonoid isolates has higher inhibitory power than orlistat. Nevertheless, the data is not sufficient yet, to determine whichever of flavonoid that corresponds to isolate 1 or isolate 2. According to inhibition power (Table 2) and binding affinities (Table 3), however, due to the greater inhibitory activity and binding affinity, can be predicted that the isolate 1 is catechin or epicatechin, while isolate 2 is EGCG.

3.6.3. Visualization of Interactions between Ligand-Pancreatic Lipase. The interaction position between ligand (flavonoid) and receptor (enzyme) can be obtained through the PyRx software. The result (Figure 7-10) has shown that the binding position of orlistat in pancreatic lipases are different from the binding position of catechin, epicatechin or EGCG, in the same enzyme. Because orlistat has been known as a competitive inhibitor, it can be concluded that the flavonoid isolates are non-competitive inhibitors of pancreatic lipases.
Nevertheless, because in vitro inhibitory activity of the flavonoids were much larger than orlistat, so they were predicted as very potential as anti-obesity drugs. Meanwhile, the structure prediction also in line with the previous study [18], that the flavonoids: cathecin, epichatechin or EGCG also found in
green tea and have activities in vivo to reduce lipid, including cholesterol, in the blood. The results of this study also reinforce the results of previous study, that “coffee” drinks from papaya seeds have greater inhibitory power than orlistat and organoleptically acceptable to consumers [19].

4. Conclusion
Water-ethanol extracted of papaya seed contain two types of flavonoid that are active as pancreatic lipase inhibitors. The two flavonoid isolates have inhibition power of 132 and 12 times stronger than orlistat has, at the same mass. UV-VIS and IR spectrum analyzed has showed that both isolates were suspected to have a similar structure to compounds of epicatechin, catechin or EGCG. In silico analysis also supported, that each of the predicted flavonoids has higher binding affinity to pancreatic lipase than orlistat.

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