In vitro and In silico Analysis of Pomegranate (*Punica granatum* L.) Fruit Powder as Pancreatic Lipase and α-Amylase Inhibitor

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Abstract. This study aims are to produce pomegranate powder, then to extracted with boiling water and to find out the phytochemical compounds, total phenolic compounds (TPC), total flavonoid compounds (TFC) and its inhibitory activity against pancreatic lipase by in vitro analysed. Besides of that, a compound that exist in pomegranate will also be in silico analysed by docking technique, for its binding with the α-amylase enzyme compared to acarbose. In vitro inhibition tests were conducted by titrimetric method, using olive oil as substrate, pancreatic lipase as enzymes, and orlistat as a standard inhibitor; meanwhile the in silico test was conducted by molecular docking techniques using human α-amylase as a receptor and acarbose and a compound in pomegranate (quercetin) as ligand. The result has shown that hot water extracts of pomegranate fruits powder (1.5 gr/150 ml) contained flavonoids, polyphenols, and alkaloids and TPC and TFC contents were 2.09 ppm and 2.058 ppm, respectively; had pancreatic lipase inhibition activity of 0.54 times compared to orlistat at the same mass (120 mg), and based on its molecular docking, quercetin, a compound in the pomegranate can bind to the α-amylase enzyme in a position that is relatively the same as acarbose, even with slightly larger affinity bindings.

1. Introduction

Obesity is a disease has become world’s problem that can cause many diseases like hypertension, heart disease, and diabetes [1]. According WHO (2019), in 2016, 13 % of the world’s adult population are obese, while in 2019 38.2 million children under 5 years old are overweight. Obesity can occur when someone too much consume fatty foods and carbohydrates and it’s not imbalance with physical activity or exercise [2]. Someone is said to be obese when they have mass index (body mass in kg divided height in m square) more than 30 kg/m² [3].

Obesity can be reduced in three ways, they are: exercise, diet control, and or consumption of anti-obesity drug that can inhibit pancreatic lipase activity like orlistat, or that can inhibit the breakdown of carbohydrate as acarbose [4], [5]; because both pancreatic lipase and α-amylase contribute to obesity, through their activity in the absorption of fat and carbohydrates into the body [6], [7]. However, these two drugs (orlistat and acarbose) have side effects like diarrhea, allergies, and abdominal pain [8], [9]. So, the alternative from natural ingredients with less side effect is needed.

One of the compounds that can inhibit pancreatic lipase enzyme is flavonoids, which can be found in several types of fruit, such as pomegranate. Pomegranate extract (juice), have anti-diabetic, anti-inflammatory, and antioxidant effects [10]. According to the finding of Viuda-Martoz [11], pomegranate fruit contain catechins and quercetins, while the peel of fruit contain luteolins and quercetins. Moreover,
our previous finding has proven that the ethanol extract of pomegranate fruit is able to inhibit pancreatic lipase activity, and the quercetin isolate from pomegranate has ability to inhibit pancreatic lipase activity 10 times stronger than orlistat at the same mass [12]. However, the activity of pomegranate powder brewing as a pancreatic lipase inhibitor is unknown. Whereas in powder form, pomegranate storage as herbal medicine will be easier. In addition, if the powder is produced by freeze drying method, it is hoped that its properties will not be inferior to fresh fruit. Therefore, the aim of this study were to produce pomegranate powder and steep it in hot water, to find out its component of phytochemical compound, to test its in vitro as pancreatic lipase inhibitor, and to know the potential of one of the components in it (quercetin) as an alpha amylase inhibitor by in silico analysis.

2. Materials and Method

2.1 Material and apparatus
In this study has been used red pomegranate fruit that has a sweet taste, and for the other important materials are porcine pancreatic enzyme (Sigma Aldrich), gum Arabic 10 %, CaCl₂, H₂O, NaCl, Na₂HPO₄, NaH₂PO₄, phenolphthalein as indicator, NaOH, HCl, Bouchard reagent, Orlistat (Xenical), H₂SO₄, FeCl₃, Folin-Ciocalteau, NaNO₂, methanol, oxalic acid, Na₂CO₃, AlCl₃. For the apparatus are freeze drying instrument, mortar and pestle, micro pipette, volumetric flask, micro-burette, pH meter, spectrophotometer UV/VIS, incubator 37°C.

2.2. Method

2.2.1. Preparation of Sample. Arils and seeds of pomegranate fruit were dried by freezer for 24 hours, then put in freeze drying instrument for 24 hours during 3 days. The dried of arils and seeds were grinded by mortar and pestle using dry ice to make powder. The powder was brewed with 150 mL hot water (85 °C) for further analyzed.

2.2.2. Phytochemicals Test. The brewing of pomegranate powder was tested include, alkaloids test (Bouchard reagent), flavonoids test (concentrate H₂SO₄), polyphenols & tannins test (AlCl₃, 1 %), and saponins test (foam formation and HCL 2N).

2.2.3. The analysis of total phenolics content (TPC) and total flavonoids content (TFC) were conducted using AlCl₃ as reagents (for TFC) and Folin-Ciocalteau reagent (for TPC). These two of test are colorimetric method, and were conducted spectrophotometrically using standard cure of gallic acid and quercetin, respectively. The measurement of absorbance were accomplished at 415 nm (TFC) and 765 nm (TPC). The calculation of phenolics and flavonoids content can be done using equation (1)

\[
TPC/TFC = \frac{(\text{Abs of sample} - b)}{a} \quad \text{equation (1)}
\]

where: \(a\) = Intercept dan \(b\) = slope of standard curve

2.2.4. Inhibition test to pancreatic lipase has been done by in vitro analysis. Used titrimetric method that refer to the method of Subandi et al [7].

2.2.5. In silico Analysis. In silico analysis has been done by molecular docking technique using human salivary \(\alpha\)-Amylase (code PDB: 1XV8) as receptor and acarbose and quercetin (standard inhibitor, each) in pomegranate fruit as a ligand. Both ligand and receptor must be in 3D structure. First the receptor was sterilized by PyMol software, the docking technique by PyRx 0.8 software, to found out binding affinity value of interaction between ligand and receptor, and by Discovery studio software, to find out and visualized the amino acid residues, bond distances, and type of interactions.
3. Result and Discussion

3.1. Result of preparation pomegranate powder

There are three types of pomegranate: red, white, and purple. Red pomegranate has a sweet taste and contains antioxidants that are beneficial for health. In this study, the arils and seeds of pomegranate are dried by freeze drying method, which was thought to be able to maintain bioactive compounds in the fruit. While the dried fruit was ground by mortar and pestle. The making process of dried fruit is summarized in Table 1.

Table 1. The result of preparation of pomegranate powder

| No. | Procedure                                      | Starting material (grams) | Result (grams) |
|-----|-----------------------------------------------|---------------------------|----------------|
| 1.  | Separate of arils and seeds from whole pomegranate fruit | 513.000                   | 107.436        |
| 2.  | Drying with Freez dryer for 3x24 hours         | 107.436                   | 43.800         |

From Table 1, the yield of pomegranate fruit powder is just 8.53%.

3.2. Phytochemicals test against the brewing of pomegranate fruit powder

Qualitative phytochemicals test or phytochemicals screening is intended to find out phytochemical compounds in the brewing of pomegranate fruit powder. In this study, 1.5 grams of powder is brewing in 150 mL hot water and stirred for 5 minutes. Using temperature and brewing time, assumed that polyphenols and flavonoids compounds can be extracted well [13]. The result is summarized in Table 2.

Table 2. The phytochemicals test of brewing pomegranate fruit powder

| Bioactive compound        | Positive control | Negative control (water) | The brewing of powder |
|---------------------------|------------------|--------------------------|-----------------------|
| Alkaloids                 | +++ (quinine)    | ---                      | +                     |
| Flavonoids                | +++ (quercetin)  | ---                      | +                     |
| Polyphenols & tannins     | +++ (gallic acid)| ---                      | +                     |
| Saponins                  | +++ (cinnamon)   | ---                      | ---                   |

Based on the phytochemical tests on brewing of pomegranate powder showed the presence of alkaloids, flavonoids, and polyphenols & tannins.

Identify the alkaloids by adding a few drops of Bouchard reagent into the brewing powder, the result shows a blackish brown sediment as in the positive control, quinine. The sediment is formed due to the K⁺ ion is binding with the lone pair of the element N in the alkaloids structure [14], by this reaction.

\[ \text{I_2} + \text{I}^- \rightarrow \text{I}_3^- \] (Brown)

\[ \text{K}^+ + \text{I}_3^- \rightarrow \text{Potassium-Alkaloid} \] (Precipitate, Brown)

Figure 1. The reaction of alkaloid and ion K⁺ from potassium iodide
Flavonoids identification carried out by adding concentrate H$_2$SO$_4$ into the brewing samples showed colour changes in the sample become red cause by the formation of chalcone [15], [16].

![Flavonoids and Chalcone](image)

**Figure 2. The reaction of flavonoid with sulfuric acid**

The polyphenol & tannins identification of the brewing powder using iron (III) chloride. The result showed blue-green colour, shows the reaction between Fe (III) ions with polyphenols and / or tannins [17].

![Polyphenol and Iron Chloride Reaction](image)

**Figure 3. The reaction of polyphenols with iron (III) chloride**

The saponins identification was conducted by shaking of the brewing of the powder for 10 seconds, and then be added HCl 2 N, in this process, the foam that was originally formed then disappears, indicates the absence of saponins [16].

**3.3. Determination of TPC and TFC.**
In this study, the phenolics and flavonoids content in the brewing of pomegranate powder can be seen in the Table 3.

| Sample       | TPC (ppm) | TFC (ppm) |
|--------------|-----------|-----------|
| 1.5 g/150 mL | 2.090     | 2.058     |

Based on the TPC and TFC on Table 3, the brewing powder of pomegranate fruit contains polyphenols and flavonoids which are quite high, this indicates its potential as an anti-diabetes, because consumption foods high of flavonoids compound can reduce the risk of diabetes [18], and in the other hand, its high polyphenol content indicates high anti-oxidant properties, which are also required in the maintenance of cell function, including the function of pancreatic beta cells in producing insulin.

**3.4. Inhibition result against pancreatic lipase**

In this titrimetric method, inhibition analysis of pancreatic lipase has been done by determining the activity of pancreatic lipase without inhibitors, first, then use orlistat as standard inhibitor, and then just use steeping pomegranate powder as an inhibitor, the results can be seen in the Table 4. Determination of the powder dosage of 1.5 grams of powder in 150 ml of hot water, refers to the volume of tea cup and the results of previous studies [12].
Table 4. Inhibition power the brewing of pomegranate powder against pancreatic lipase, relative to orlistat

| No | Experiment                                      | Average volume of NaOH as titrant (mL) | Inhibition power against pancreatic lipase (%) | Inhibition power against Orlistat (%) | Relative inhibition power against Orlistat at the same mass (120 mg) |
|----|-------------------------------------------------|---------------------------------------|-----------------------------------------------|--------------------------------------|----------------------------------------------------------|
| 1  | Without inhibitor                               | 3.11                                  | -                                             | -                                    | -                                                        |
| 2  | With Orlistat as inhibitor (120 mg)             | 2.15                                  | 70.69                                         | 100                                  | 1 times                                                  |
| 3  | With 20 mL of brewing powder of pomegranate fruit as inhibitor (1.5 gr/150 ml) | 2.25                                  | 63.22                                         | 89.43                                | 0.54 times                                               |

According to Table 4, 20 mL of pomegranate powder brewing still can inhibit pancreatic lipase, by 63.22% compared to 1 tablet of orlistat (120 mg), so if we drink 1 cup (150 ml) of steeping pomegranate powder (1.5 gram/150 ml), by calculation, the efficacy will be equivalent to 4 tablets of orlistat.

3.5. In silico test of quercetin binding to α-Amylase enzyme

The result of in silico test with docking technique turn out two data, namely the position of ligand binding (quercetin and acarbose) against receptor (α-amylase) and binding affinity, respectively. From the docking result, the binding position of quercetin against α-amylase was not too much different than binding position of acarbose, a commercial anti-obesity drug as an α-amylase inhibitor, as shown in Figures 4 and 5.

Figure 4. Visualization of acarbose position binding to α-Amylase using PyMol software.

Figure 5. Visualization of quercetin position binding to α-Amylase using PyMol Software.
The similarity of binding position these ligands against α-amylase enzyme as a receptor can be seen more clearly on Table 5.

**Table 5.** Bond distance, type of interaction, and amino acid residues that bind to active site of α-amylase enzyme

| Ligand   | Amino acid residue | Distance (Å) | Type of interaction                  |
|----------|--------------------|--------------|--------------------------------------|
| Acarbose | ARG B:267          | 2.32         | Unfavorable Donor-Donor              |
|          |                    | 3.07         | Conventional Hydrogen Bond           |
|          |                    | 2.47         | Conventional Hydrogen Bond           |
|          | ASP B:317          | 2.89         | Unfavorable Acceptor-                |
|          |                    |              | Acceptor                             |
|          | PHE B:348          | 4.43         | Pi-Alkyl                             |
|          | ALA A:310          | 3.73         | Carbon Hydrogen Bond                 |
|          | ARG A:267          | 3.16         | Conventional Hydrogen Bond           |
|          | ASP A:317          | 1.97         | Conventional Hydrogen Bond           |
|          | ARG 346            | 3.14         | Conventional Hydrogen Bond           |
|          | GLY B:351          | 2.36         | Conventional Hydrogen Bond           |
|          | ALA B:310          | 2.72         | Conventional Hydrogen Bond           |
| Quercetin| ARG A:346          | 2.35         | Unfavorable Donor-Donor              |
|          | ARG A:267          | 2.19         | Unfavorable Donor-Donor              |
|          | HIS A:305          | 3.21         | Conventional Hydrogen Bond           |
|          | PHE A:348          | 4.45         | Pi-Pi Stacked                        |
|          |                    | 4.07         | Pi-Pi Stacked                        |
|          | ASN A:350          | 3.26         | Conventional Hydrogen Bond           |

The similarity binding position of quercetin and acarbose against α-amylase as can be seen on Table 5 show same residues of PHE-348; ARG-267 dan ARG-346 that bind in active site of enzyme, therefore, they are predicted having similar activity and inhibition mechanism to enzyme, that is competitive inhibitor. The next of docking result as binding affinity between ligand and receptor are mentioned on Table 6.

**Table 6.** Binding affinity values of Acarbose and Quercetin to α-Amylase Enzyme

| Ligand | Average of Binding Affinity (Kcal/mol) |
|--------|----------------------------------------|
| Acarbose         | -9.6                                   |
| Quercetin        | -9.4                                   |

As shown in the Table 6, the average value of binding affinity quercetin is slightly smaller than acarbose. This data is in line with the data of Sun, et al., [19] that quercetin compound contained in pomegranate rind also potential as an α-Amylase enzyme inhibitor. Furthermore, others in silico study in various bioactive compounds found in pomegranate fruit against α-Amylase and α-Glucosidase enzyme, also proven that curcumin, 16-hydroxy-cleroda-3,13-dine-16,15-olide (16-H), docosanol, tetracosanol, antroquinonol, berberine, catechin, quercetin, actinodaphnine, and rutin can bind to α-Amylase and α-Glucoside enzyme and were predicted to inhibited the enzymes [20]. This study, together with our previous studies [12], has assessed the data that pomegranate powder, is highly regarded as an anti-obesity brew drink, on the basis of its ability as an inhibitor of pancreatic lipase and α-amylase.
4. Conclusion
Hot water extracts of pomegranate fruits powder (1.5 gr/150 ml) contained flavonoids, polyphenols, and alkaloids and TPC and TFC contents were 2.090 ppm and 2.058 ppm, respectively; had pancreatic lipase inhibition activity of 0.54 times compared to orlistat has at the same mass (120 mg), and based on its molecular docking, quercetin, a compound that exist in the pomegranate, can bind to the α-amylase enzyme in a position that is relatively the same as acarbose, even with slightly greater affinity bindings. Those data suggested that pomegranate powder has potency as anti-obesity herb drug.

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