Active Fraction as Anti-obesity by in Vitro toward Pancreatic Lipase Activity

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Abstract. Fruit extract of *asamgelugur* (*Garcinia atroviridis*) can be used as an anti-obesity by *in vitro* against pancreatic lipase activity. The pancreatic lipase plays a role in degradation of lipids into fatty acids, as pancreatic lipase activity is hampered, the absorption of monoglycerides in the human body decreases. The purpose of the research was to identify and evaluate the *Garcinia atroviridis* fruit fraction as pancreatic lipase inhibitor. The extracted fruit with percolation method showed an inhibitory activity of 44.16% at 500 µg/mL. Xenical® as the positive control exhibited an inhibitory activity of 43.65% at 25 µg/mL. The best result from partitioning was using BuOH solvent with inhibitory activity of 56.42% at 100 µg/mL, whereas from Xenical® was 50.35% at 50 µg/mL. The best fraction from BuOH fractionation eluting with CH2Cl2:MeOH revealed an IC50 value of <10 µg/mL, or was better than Xenical®. Therefore, E fraction from BuOH solvent of *garcinia atroviridis* extract is potential as a pancreatic lipase inhibitor.

**Keywords:** *Garcinia atroviridis*, BuOH, in vitro, pancreatic lipase.

1. Introduction

Obesity has become a global health issue affecting people from all over the world, both in developed and developing countries like Indonesia. Obesity is defined as the energy balancing disorder as the intake calories is more than those are burned and needed. Several factors responsible to the condition are surroundings, genetic, psychological, drug effects and physical activities. The intake food should undergo metabolism processes as it becomes energy. However, the food exceeds the appropriate amount or contains high amount of lipid. The condition gets worse without sufficient physical exercise and the surplus energy is stored as excessive fat in the body. Obesity cause problems such as heart coronary disease, type 2 diabetes and high blood pressure [1]. The World Health Organization (WHO) estimated that in 2016 more than 1.9 billion adults were overweight (39% of the population) and over 650 million (13% of the population) were people with obesity [2].
50 – 70 % of the fat total amount from the intake food is hydrolyzed by the pancreatic lipase. This lipase separates the lipid into monoglyceride [3]. The enzyme prevents ester bond within lipid which was formed by glycerol and long-chain fatty acid (LCFA). In human body, there are three pancreatic lipase sources which actively hydrolyzed the lipid before the absorption process. Lipid hydrolysis begins with lingual lipase in oral activity, yet it has low activity and tends to hydrolyze the short-chain fatty acid (SCFA). SCFA blends easier with water so it was absorbed directly in the stomach to circulate through the portal vein to liver and soon to be oxidized into energy. In the stomach, lipid is hydrolyzed by gastric lipase which reacts actively to short-chain fatty acid and medium-chain fatty acid. Pancreatic lipase in the small intestine finalizes the lipid hydrolysis reaction and actively responds to short-chain and medium-chain fatty acid. Also, this lipase works on the lipid surface. As the enzyme works more actively, more amount of lipid and oil is hydrolyzed as well as more amount of monoglycerides is produced. Consequently, the excess monoglycerides is absorbed in the small intestine which later is stored as the excessive accumulation of fat reserves in the adipose tissue. This condition is called obesity which may lead to body organs dysfunction.

One way to setback weight is to consume anti-obesity medication or slimming agent. Some of the synthetic drugs of slimming agent work differently; for instance remove the appetite (amphetamine, fenfluramine and sibutramine), hinder the lipid absorption (orlistat) and increase the energy output (ephedrine, caffeine and thyroxine). Meanwhile, most of the available synthetic anti-obesity drugs is harmful to health [4]. As a result, herbal slimming agent is more preferable as it is considered less harmful and more affordable. In Indonesia, traditional herbal medication is long considered as the main reference for health as well as skin care. Pancreatic lipase inhibitor has been the alternative method to fight obesity by reducing excessive monoglyceride and free fatty acid. Orlistat is the firstly found lipase inhibitor and lipstatin derivative isolated from Streptomyces toxytricini [5]. A study from Indonesian herbal plants also proved its potency as pancreatic lipase inhibitor. The aqueous extract by in Vitro and ethanol of *garcinia atroviridis* can be the inhibitor for pancreatic lipase activity [6]. This is in accordance that *garcinia atroviridis* is highly potential as the anti-obesity since its extract effectively reduces cholesterol, triglyceride and total phospholipid in membrane and serum [7]. According to Iswantini, et.alresearch [6] in phytochemistry test, dry fruit of *garcinia atroviridis* only contains alkaloid and saponin. Saponin in *garcinia atroviridis* allegedly contains in glycoside which can only be extracted with water, while alkaloid would do with ethanol. This research reported that pancreatic lipase inhibitor activity on its optimum condition, pH 8 and 40 °C has the highest inhibition as much as 86.3% with 150 µg/mL concentration. Succeeding, *garcinia atroviridis* contains hydroxycitric acid (1,2-dihydroxy-propane-1,2,3-tricarboxylate acid) which would competitively hinder ATP-citrate lyase enzyme work. The enzyme particularly alters the citrate acid to acetyl coenzyme A (Acetyl-CoA). Hydroxycitric acid (HCA) is one of hydroxy carboxylic acid with two hydroxyl groups and three carboxylic groups. Rasyla et.al. 2017 [8] stated that *garcinia atroviridis* has antioxidant and is able to reduce weight.

2. Experimental Section

*Extraction and Partition* [9]

The applied extraction method was percolation method by PT Indofarma. The generated extract was scaled and partitioned using polar, semi polar and nonpolar solvents. As much as 50 gr concentrated extract was partitioned with *n*-Hexane and 90% MeOH–H₂O (1:1). Hexane layer was detached from 90% MeOH–H₂O layer. Next, 90% MeOH–H₂O layer was re-partitioned with CH₂Cl₂ and 50% MeOH–H₂O (1:1). CH₂Cl₂ layer was re-detached, and 50% MeOH–H₂O layer was re-partitioned with BuOH–H₂O
(1:1). The following process was separating BuOH, while the remaining MeOH-H2O was detached into MeOH and H2O. Three solvents were concentrated into yields of n-Hexane, CH2Cl2 and BuOH generated.

**Fractionation [9]**

The active layers generated from the liquids partition were being sterilized by open column chromatography silent phase silica gel 60 (0.040–0.063 mm) and motion phase CH2Cl2 and MeOH (65:35 to 40:60). Fraction result was checked with densitometry and stain-detected with λ 254 and 366 nm UV light. Fractions with similar retention factor (Rf) value were grouped together. The following active fractions were identified by its inhibition value.

**Pancreatic Lipase Inhibition Assays [10]**

Lipase inhibition assay method was completed to measure lipase inhibitory activity. The total test volume was 200 μL with p-Nitrophenibutirate (PNPB) as substrate. Working solvent PNPB was being prepared with 8.403 μL PNPB solution in a bottle with up to 10 ml volume of acetonitrile. Regular drug solution was prepared with dissolving one capsule filling Orlistat in 12 mL DMSO (dimethyl sulfoxide). Assay solution was prepared in 5–1000 µg/mL concentration diluted with buffer. The solution or standard (25 μL) was incubated with 50 μL enzyme solution, 100 μL PBS buffer solution dan 25 μL PNPB solution for 30 minutes in 37 °C temperature. Lipase activity was identified by measuring PNPB hydrolysis into p-nitrophenol in λ 400 nm using ELISA (Biotech) plate reader. Inhibition percentage (% inhibition) was calculated using the following formula:

\[
\% \text{ inhibition} = \frac{A_{\text{Blank}} - A_{\text{Extract}}}{A_{\text{Blank}}} \times 100
\]

### 3. Results and Discussions

**Yield of Extract and Partition**

The applied extraction method was percolation on simplicia from *garciniaatroviridis*. Simpliciapowder was meant to extend the samplings surface so that the generated extraction would be optimum. In this method, solvent was flowed periodically so that the solution with lower concentration would continuously substitute the previous solution. This method was to increase the degree of concentration difference since the solvent velocity could lessen the boundary layer and increase the concentration difference. Simplicia from dry *garciniaatroviridis* was extracted with deionized water for it was the very solvent which widely used to extract the traditional herbal medicinal plants and applied them as Indonesian traditional herbal medicine.

The percolation result was as much as 16.45% yield. Compounds separation process using two solvents of different phase resulted on the compounds dispersion into their solubility phase accordingly. Generated asamgelugur extraction was then partitioned with n-hexane and 90% MeOH-H2O 1:1 ratio. 90% MeOH-H2O was applied to dissolve both polar and nonpolar compounds. Table 1 showed result from the three solvents used in the partition process; the more polar the solvent is, the better its competence in binding the compounds of *garciniaatroviridis* extract. This fact is in accordance with the extraction process since water as polar solvent was applied.
Table 1 Weighted and Yield Partition of *garcinia atroviridis* extract

| Solvent   | Weighted | Yield  |
|-----------|----------|--------|
| *n*-hexane | 0.01 g   | 0.03%  |
| CH₂Cl₂     | 0.80 g   | 1.60%  |
| BuOH       | 4.12 g   | 8.24%  |

*n*-hexane solvent was expected to draw out nonpolar compounds within *garcinia atroviridis* crude extract. As much as 0.03% yield was produced after partition process with *n*-hexane solvent. This was due to water applying on *garcinia atroviridis* crude extract which resulted to less drawn nonpolar compounds. BuOH layer generated the most yield compare to all three solvents. However, this finding was not too strong to decide whether the more yield produced would be in line with the inhibition.

Fractionation

Generated BuOH extract was then eluted further using thin layer chromatography (TLC). TLC was a chromatography analytical method used to separate compounds in a mixture. TLC was effective in finding out amount of components in a mixture, compounds identity and purity. As [11] stated, TLC could be used to measure the number of components in a compound, identifying compound, observing the process of a reaction and monitoring the effectiveness of purification.

TLC assay in this study covered finding the appropriate mixture solvent that was able to separate the active compounds within BuOH extract. From the conducted experiments, an eluent was obtained to separate the active components within the extract as signified with the existence of detached ribbons chromatogram. Each ribbon generated from elution process of each eluent was observed under λ 254 and 366 nm UV light. Eluent used to separate BuOH extract active components was chloroform solvent mixture: methanol with 65:35 to 55:45 ratios. The eluent could separate components within the extract into 2 ribbons. As no ribbon existed on Fraction 1, 2 and 3, they were grouped on Fraction A (Table 2). Beam of blue and identical ribbons appeared on Fraction 4 to 8 which then were grouped into Fraction B. Fraction C was a merging of Fraction 9 to 12 as the TLC results indicated some similarities. No ribbon appeared on Fraction 13 to 20 as possibly because on this fraction the applied eluent had reached the washing phase. Thus, the separation process had reached final stage. This fraction considered as Fraction D. Last process was washing process with methanol 100% to entirely draw every remaining extract.

Table 2. *Garcinia atroviridis* BuOH fraction chromatogram

| Fraction | Merging Fraction | Number of Spot |
|----------|------------------|----------------|
| A        | 1,2,3            | 0              |
| B        | 4,5,6,7,8        | 1              |
| C        | 9,10,11,12       | 2              |
| D        | 13,14,15,16,17,18,19,20 | 2          |
| E        | Washing MeOH     | -              |

Rf value could be one of the evidences in compounds identifying. Compounds with similar Rf value indicated that the compounds possessed similar or identical characteristics [12].

Detecting and observing the BuOH extract compounds would be better operated in 366 nm wavelength compare to 254 nm wavelength. 254 nm wavelengths were used to detect flavonoid compound based on quenching. On the other hand, λ 366 nm was applied to detect flavonoid compound based on fluorescence which would appear in dark yellow, glowing green or blue. Next, Fraction A, B, C D and E would be lipase inhibition re-tested.
Pancreatic Lipase Inhibition Capacity

Pancreatic lipase in Vitro assay on *garcinia atroviridis* partition result referred to a method done by Chedda[10]. This method was completed to discover pancreatic lipase inhibitory activity in breaking down triglycerides into fatty acid which then being absorbed by mucosa duodenum. The inhibition assay used buffer (PBS with NaCl and Triton-X-100, on 7.2 optimum pH). Buffer was applied to retain the enzyme and substrate optimum condition. Commonly used substrate in pancreatic lipase test by in Vitro was the pure substrate such as triolein and trinilolein. Enzyme bound the substrate molecules in forming temporary enzyme-substrate complex. It then was dissolved into monoglyceride, glycerol and free fatty acid (Figure 1). Afterward, it would be transported into microvilli surface and absorbed by blood vessel [13].

![Figure 1. Lipid hydrolysis reactions](image)

Applied substrate was 2p-Nitrophenilbutirate (PNPB). The substrate used as it was rich of unsaturated fatty acid specifically oleic acid and linoleic acid. Meanwhile, test sampling was dissolved into DMSO (dimethyl sulfoxide). Pancreatic lipase enzyme activity measurement was completed on the optimum condition, incubated for 30 minutes in 37°C temperature. This condition was considered crucial for the enzyme could easily be broken without the very condition. Lipase enzyme operated by accelerating lipid hydrolysis into fatty acid and glycerol. Lipase activity was defined on 400 nm using ELISA (Biotech) plate reader. The applied positive control in this research was Xenical® which was a widely used commercial slimming product. Xenical® was chosen since its main ingredient was orlistat (tetrahydrolipstatin) which was the firstly discovered selective irreversible pancreatic lipase inhibitor. Along with that, according to [14] study, pancreatic lipase inhibition of orlistat was preceded by non-competitive inhibition mechanism. Blank solution (without additional extract) was applied as the negative control. The inhibition assay was performed using five identical concentration variants for every extract, i.e 5-1000 µg/mL. These concentrations variants were to observe the relations of extract concentration additional toward the generated inhibition potency. As a matter of fact, the relation of concentration value and inhibition potency value was unknown.

![Figure 2. Inhibition capacity of (♦) *garcinia atroviridis* crude extract, (■) n–hexane, (▲) dichloromethyl, (×) butanol, and (×) Xenical on pancreatic lipase by in vitro method](image)
Figure 2 showed the relation of five extract concentrations and its inhibition potency toward pancreatic lipase activity was not linear. The increase in concentration was not always in line with its inhibition potency rise. It was due to the applied extract contained with several compounds group allegedly responded differently to each other. In addition, the compounds group affected one another both synergistically as well as antagonistically in hindering pancreatic lipase activity on certain concentrations. Meanwhile, the standard deviation (SD) value was good enough. IC$_{50}$ value in five extracts showed that BuOH solvent has the lowest IC$_{50}$ as much as 46.9 µg/mL (Table 3). The value was higher compare to the positive control (Xenical®) IC$_{50}$ (30.34 µg/mL). This condition apparently due to orlistat lower solubility potency compare to the sample layer extract on buffer could lead to lower inhibition potency value of the positive control compare to the extract.

| Table 3. Pancreatic lipase inhibition capacity of *garcinia arovidis* extract |
| Extract | Concentration (µg/mL) | Inhibition capacity | IC$_{50}$ (µg/mL) | Yield (%) |
|---------|------------------------|---------------------|------------------|-----------|
| Crude extract | 50 | 39.98 | 640.62 | 16.45 |
| Xenical | 25 | 43.65 | 30.34 | - |
| *n*-hexane | 10 | 41.29 | 55.19 | 0.03 |
| CH$_2$Cl$_2$ | 100 | 41.33 | 501.68 | 1.60 |
| BuOH | 5 | 38.77 | 46.91 | 8.24 |

Positive control was tested under different concentration variations to the sample extract, i.e. 10-200 µg/mL. The inhibition test results were presented on Table 4. Table 4 showed that the positive control (Xenical®) could perform better pancreatic lipase inhibition. Yet, the applied method in this study was considered more effective as by using substrates and other less costly reactors had successfully presented the sample extract inhibition potency toward pancreatic lipase activity.

On the other hand, inhibition assay result of BuOH layer fractionalized results were grouped into 5 test fraction A, B, C, D and E. TLC test in Fraction A showed no indication of separation. Thus, it was in accordance as the inhibition assay result had IC$_{50}$ more than 100 µg/mL. Fraction E was the washing process result using MeOH 99.85% which presented the best IC50 value less than 10 µg/mL. Compare to [15] study result on 90 Palestinian medicinal herbal extracts, some of them had excellent potency as anti-obesity. There were Camellia sinensis, Ceratoniasilica, Sarcopoteriumspinulosum and Mentha spicata. Those plants operated by inhibiting or as pancreatic lipase enzyme inhibitor activity. The research results presented IC$_{50}$ values as follows; Camellia sinensis as much as 500 µg/mL, Ceratonia siliqua as much as 800 µg/mL, Sarcopoteriumspinulosum as much as 1200 µg/mL and Mentha spicata as much as 1200 µg/mL,[16], [17], [18]. Along with that, [19] research stated IC$_{50}$ pancreatic lipase enzyme activity to combination of *garcinia arovidis* ethanol extract with *kaempferiaangustifolia* rhizome in 1:1 ratio was 11.23 µg/mL. IC$_{50}$ Fraction E result was better as supported by appropriate standard deviation to facilitate its potency as pancreatic lipase inhibitor. In Figure 3, the relation between five extract variations with its potency inhibition to pancreatic lipase activity was adequately linear. This result was due to the separation process was sufficiently pure.

| Table 4. Pancreatic lipase IC$_{50}$ value on BuOH fraction |
|-----------------|-----------------|-----------------|-----------------|
| Fraction | Concentration (µg/mL) | Inhibition (%) | IC$_{50}$ (µg/mL) | Yield (%) |
| A | 10.0 | 33.52 | >100 | 0.13 |
| B | 10.0 | 38.82 | 34.55 | 35.45 |
| C | 10.0 | 44.49 | 31.59 | 45.66 |
| D | 10.0 | 32.40 | 85.87 | 2.59 |
| E | 10.0 | 52.89 | <10 | 2.24 |
| Xenical | 12.5 | 43.33 | 30.34 | - |

POSITIVE CONTROL TESTED UNDER DIFFERENT CONCENTRATION VARIATIONS TO THE SAMPLE EXTRACT, I.E. 10-200 µG/mL. THE INHIBITION TEST RESULTS WERE PRESENTED ON TABLE 4. TABLE 4 SHOWED THAT THE POSITIVE CONTROL (XENICAL®) COULD PERFORM BETTER PANCREATIC LIPASE INHIBITION. YET, THE APPLIED METHOD IN THIS STUDY WAS CONSIDERED MORE EFFECTIVE AS BY USING SUBSTRATES AND OTHER LESS COSTLY REACTORS HAD SUCCESSFULLY PRESENTED THE SAMPLE EXTRACT INHIBITION POTENCY TOWARD PANCREATIC LIPASE ACTIVITY.
Compounds group that actively functioned in pancreatic lipase activity inhibition was predicted under polar group since the best IC50 result was on Fraction E. Hydroxycitrate acid (1,2-dihydroxypropane-1,2,3-tricarboxylic acid) which commonly called as HCA was the main content of *garciniaatroviridis* other than saponin as the pancreatic lipase inhibitor. HCA content on Garcinia family could reach 20-30% based on dry weight and on *garciniaatroviridis* reached 45.17%. HCA could easily dissolve in water and alcohol which supported the theory. As [20] mentioned that HCA reduced carbohydrate conversion to fat, HCA content on *garciniaatroviridis* would competitively inhibited the enzyme ATP-citrate lyase activity which converted citric acid into Acetyl coenzyme A. In Krebs cycle, Acetyl-CoA would be transformed into malonyl-CoA which then being converted into fatty acid. It indicated that HCA could function as lipase activity inhibitor and ATP-citrate lyase. Hydroxycitrate acid on *garciniaatroviridis* had similar three carboxylate clusters with the citric acid known as the competitive inhibitor to ATP-citrate lyase activity [21]. HCA had higher affinity in reacting to ATP-citrate lyase compare to its other natural substrate; citric acid [22].

4. Conclusion

Identification process of the most active fraction potency from *garciniaatroviridis* extract activity to lipase enzyme by *in Vitro* was successfully completed. Separation using the best partition obtained on BuOH layer generated IC50 as much as 46.91 µg/mL. BuOH layer purification generated IC50 <10 µg/mL on the most active Fraction E. The IC50 most active fraction was better than the positive control Xenical®. The result evaluation showed that *garciniaatroviridis* had the best activity as pancreatic lipase enzyme inhibitor in polar group as well as the potency for anti-obesity.

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