Pomegranate leaf attenuates lipid absorption in the small intestine in hyperlipidemic mice by inhibiting lipase activity

YU Xuan¹Δ, WANG Xin-Pei¹Δ, LEI Fan², JIANG Jing-Fei¹, LI Jun³, XING Dong-Ming¹*, DU Li-Jun¹*

¹ Laboratory of Molecular Pharmacology and Pharmaceutical Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China;
² School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, China;
³ State Key Laboratory of Innovative Drugs and Efficient Energy-saving Pharmaceutical Equipment, Jiangxi University of Chinese Medicine, Nancang 330006, China;

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[ABSTRACT] Pomegranate leaf (PGL) has a definite role in regulating lipid metabolism. However, pharmacokinetic results show the main active ingredient, ellagic acid, in PGL has lower oral bioavailability, suggesting that the lipid-lowering effect of PGL may act through inhibiting lipid absorption in the small intestine. Our results demonstrated that pomegranate leaf and its main active ingredients (i.e., ellagic acid, gallic acid, pyrogallic acid and tannic acid) were capable of inhibiting pancreatic lipase activity in vitro. In computational molecular docking, the four ingredients had good affinity for pancreatic lipase. Acute lipid overload experiments showed that a large dosage of PGL significantly reduced serum total cholesterol (TG) and triglycerides (TC) levels in addition to inhibiting intestinal lipase activity, which demonstrated that PGL could inhibit lipase activity and reduce the absorption of lipids. We also found that PGL could reverse the reduced tight-junction protein expression due to intestinal lipid overload, promote Occludin and Claudin4 expression in the small intestine, and enhance the intestinal mucosal barrier. In conclusion, we demonstrated that PGL can inhibit lipid absorption and reduce blood TG and TC by targeting pancreatic lipase, promoting tight-junction protein expression and thereby preventing intestinal mucosa damage from an overload of lipids in the intestine.

[KEY WORDS] Pomegranate leaf; Lipase activity; Hyperlipidemia; Ellagic acid; Pyrogallic acid

[CLC Number] R965

Introduction

Hyperlipidemia, a form of dyslipidemia, increases the risk of cardiovascular disease [1]. Common causes include diabetes mellitus and medication, but acquired hyperlipidemia can also be closely associated with excessive energy intake, which is called postprandial hyperlipidemia [2]. Whole fruit or the peels of pomegranates (Punica granatum L.) have been reported to have anti-hyperlipidemia, antioxidant [3-4], and antibacterial [5] effects and to provide protection for the gastro-intestinal system from barium chloride [6] and for the liver from injury due to alcohol and chemicals [7-8]; they have also been linked to the chemoprevention of tumorigenesis [10] and anticoagulant and antiplatelet [10] activity, among other roles. A recent study has shown that pomegranate fruits have neuroprotective effects against Alzheimer’s disease mediated by ellagitannin-gut-microbial metabolites [11]. The juice, peels, fruit, seeds, and leaves of pomegranates can benefit human health [12]. Phenolic compounds are the active ingredients in pomegranates [13].

Pomegranate peel has been used as a traditional Chinese medicine for centuries to treat acidosis, hemorrhage, diarrhea, helminthiasis, and microbial infections [14-15]. Additionally, Pomegranate leaves (PGL) can modulate lipid metabolism,
decrease levels of TC and TG in the serum of hyperlipidemia animals after a long-term, high-fat diet, and inhibit weight increases in mice \cite{16}. However, the pharmacokinetic results of pomegranate show that the major active ingredient, ellagic acid, has poor absorption \cite{17}, which implies that there may be an absorption target during pomegranate administration. In the presented study, we investigated the effects of PGL on intestinal lipase activity in acute hyperlipidemic mice to look for a potential active target of PGL in lipid metabolism.

**Materials and Methods**

**Animals**

Male ICR mice, weighing 20–22 g, were purchased from Vital River Laboratories (Beijing, China). The animals were housed in temperature- and humidity-controlled rooms, kept on a 12 h/12 h light/dark cycle and provided with unrestricted amounts of rodent chow and drinkable water. All experimental procedures were approved by the IACUC (Institutional Animal Care and Use Committee) of Tsinghua University (Approval ID: 15-DLJ1). The laboratory animal facility was accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International).

**Chemicals**

An extract of PGL (Pomegranate leaves) (*Punica granatum* L.), containing 8.3% ellagic acid (EA), 1.49% gallic acid (GA), 0.79% pyrogallic acid (PGA), and 18.27% tannic acid (TA), according to an HPLC assay, and ellagic acid (purity of 98%) were prepared by Dr. Xiang Lan at the Laboratory of Molecular Pharmacology and Pharmaceutical Sciences, Tsinghua University, Beijing, China. The chemical structures and CID numbers in PubChem at GeneBank for EA, GA, PGA and TA are shown in Fig. 1. Tannic acid was purchased from the Yuehai Chemical Plant (Zhejiang, Yuehai, China). Gallic acid was purchased from the China Pharmaceutical Company (Beijing, China). Pyrogallic acid was purchased from the Zunyi Second Chemical Plant (Guizhou, Zunyi, China). Triglycerides (TG), total cholesterol (TC), glucose and lipase assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Porcine pancreatic lipase (PPL, type I >100 U·mg⁻¹) and orlistat (>98%) were purchased from Sigma Aldrich (Shanghai, China).

**Fig. 1** Chemical structures of ellagic acid, gallic acid, pyrogallic acid and tannic acid in the extracts of pomegranate leaves

**The inhibition of lipase activity in vitro**

Lipase activity was determined according the instructions of the Lipase Activity Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The lipase activity index is shown as U/mg protein.

To determine lipase inhibitory activity, TA, GA, PGA, EA, and orlistat at different concentrations were pre-incubated with PPL for 10 min. After pre-incubation, a lipase substrate
was added and incubated for 10 min at 37 °C. All components to be detected were dissolved in dimethyl sulfoxide (DMSO) and then diluted with water.

**In vitro computer-based docking of lipase**

The computer-based docking assay was carried out according to the procedures of Feng et al. [18]. A human pancreatic lipase receptor structure was downloaded from the NCBI Protein Structure Data website (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=21535) [19]. The structures of ellagic acid, garcinol acid, pyrogallic acid and tannic acid were obtained from the PubChem database as .sdf files. Then, the files were transformed to .pdb files with Pymol version 0.99 (http://www.pymol.org/).

The structure files of the protein and the small molecules were analyzed with AutodockTools-1.5.6 (http://mgltools.scripps.edu/downloads) and were transformed to .pdbqt files. The calculation was conducted with Autodock Vina (http://autodock.scripps.edu/). The results were analyzed and visualized with Pymol. The affinity that most closely resembled the protein is shown as kcal·mol⁻¹.

**In vivo lipid absorption experiment**

The mice were randomly divided into ten groups (n = 10/group). The lipid emulsion was prepared with yolk powder and olive oil (1 g: 10 mL, 10/group). The lipid emulsion was prepared with yolk powder described [22-23]. For real-time PCR, all primer sequences were real-time PCR and a Western blot (WB) assay, as previously described.

**Statistical analysis**

All the values are expressed as means ± SD. The data were statistically analyzed using a one-way analysis of variance (ANOVA) with an F value determination. The F test was performed using Excel software 2016 (Microsoft, Redmond, WA, USA). The student’s t-test between two groups was performed after the F test. P values below 0.05 were considered to be statistically significant. GraphPad Prism 5 (http://www.graphpad.com/), GraphPad Software Inc., La Jolla, CA, USA) was used to determine the IC₅₀ (50% inhibitory concentration) to evaluate the inhibition of lipase activity in vitro.

**Results**

**Effects of PGL extracts on lipase activity in vitro**

As shown in Fig. 2, all four components of the PGL extracts and orlistat, the positive control, inhibited lipase activity to varying degrees. Among them, EA and GA had the same effects at a concentration of 120 μg·mL⁻¹ (Figs. 2A and B); PGA completely inhibited enzyme activity at a concentration of no greater than 14 μg·mL⁻¹ (Fig. 2C). TA inhibited lipase activity by only 25% at the same concentration (Fig. 2D). The concentration of orlistat had a logarithmic correlation with lipase activity (Fig. 2E). The linear equation of lipase activity was Y = 370.76 X + 2.559 9 (R² = 0.9558) (Fig. 2F).

To further analyze the inhibition abilities of the four isolated compounds of the PGL extracts, we used GraphPad Prism 5 to calculate the IC₅₀, which reflected the effectiveness of each component at inhibiting lipase activity. The results showed that GA had the lowest IC₅₀ at a concentration of 6.683 μg·mL⁻¹, which showed a stronger inhibition ability. The IC₅₀ values of GA, EA, and TA were 44.4, 55.05, and 239.4 μg·mL⁻¹, respectively. TA showed weaker inhibition ability than others. The IC₅₀ value of the positive control orlistat was 17.05 μg·mL⁻¹ (Table 1).
Fig. 2  Lipase activity after the in vitro administration of four ingredients in PGL. A: Ellagic acid (EA); B: Gallic acid (GA); C: Pyrogallic acid (PGA); D: Tannic acid (TA); E: Orlistat, used as a positive control; F: Linear standard equation of lipase activity for the determination of lipase activity. The Lipase concentration was 0.1 mg·mL⁻¹.

Table 1  IC₅₀ Values of orlistat, TA, GA, PGA and EA on lipase activity (μg·mL⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>Orlistat</th>
<th>TA</th>
<th>GA</th>
<th>PGA</th>
<th>EA</th>
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<tr>
<td><strong>Best-fit values</strong></td>
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<tr>
<td>LogIC₅₀</td>
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<td>2.379</td>
<td>1.648</td>
<td>0.825</td>
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<tr>
<td>HillSlope</td>
<td>−1.221</td>
<td>−1.837</td>
<td>−2.094</td>
<td>−2.809</td>
<td>−2.786</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>17.05</td>
<td>239.4</td>
<td>44.5</td>
<td>6.683</td>
<td>55.05</td>
</tr>
</tbody>
</table>

|          |          |        |        |         |         |
| **Std. Error** |          |        |        |         |         |
| LogIC₅₀  | 0.021 03 | 0.011 3| 0.024 37| 0.017 33| 0.046 69|
| HillSlope| 0.064 14 | 0.121 2| 0.205  | 0.273 7 | 0.763 3 |

|          |          |        |        |         |         |
| **95% Confidence Intervals** |          |        |        |         |         |
| LogIC₅₀  | 1.187 to 1.276 | 2.355 to 2.403 | 1.597 to 1.700 | 0.789 0 to 0.860 9 | 1.637 to 1.845 |
| HillSlope| −1.357 to −1.085 | −2.094 to −1.580 | −2.528 to −1.659 | −3.376 to −2.241 | −4.487 to −1.085 |
| IC₅₀     | 15.39 to 18.89 | 226.5 to 253.0 | 39.51 to 50.12 | 6.152 to 7.260 | 43.33 to 69.95 |

In vitro docking with the lipase

Through computer calculations, we can predict macro-molecular protein binding affinity for small molecules. As shown in Fig. 3, the compounds in PGL could be docked with human pancreatic lipase. The binding affinity was −12.4 (TA), −7.2 (EA), −5.7 (GA), and −4.9 (PGA) kcal·mol⁻¹, and TA showed a stronger affinity with the lipase than the other compounds (Fig. 3).
Effects of PGL on the mouse model of acute hyperlipidemia

To verify whether the PGL extracts could inhibit lipase activity in mice, we investigated the effects of PGL extracts on serum TG, TC, and glucose levels in normal mice. After oral administration of the lipid emulsion, serum TG and TC in the model group showed a notable increase compared to that of the control group (Figs. 4A and B). The large dosage of PGL (800 mg·kg⁻¹) and all four components significantly decreased serum TG levels in a mouse model of acute hyperlipidemia (Fig. 4A). The large dose used for the PGL and PGA groups yielded lower serum TC levels compared to those of the model groups (Fig. 4B). Orlistat showed an effect on serum lipids for both TC and TG. For overloading lipids, the serum glucose displayed no significant increase compared to the normal control. However, EA could decrease the serum glucose to a lower level than the levels in both the normal and model mice, suggesting that EA might potentially have an effect on glucose metabolism (Fig. 4D).
the model groups were much easier to break, which suggested that there was an impaired connection in the intestinal epithelium; the intestines in the PGL groups were more stable after the same single dose of an oral lipid emulsion. According to the literature, both Occludin and Claudin4 are connective proteins that facilitate the tight junctions of the small intestinal mucosa [24-25, 22]. To observe the barrier conditions during lipid overloading, we measured the Occludin and Claudin4 proteins using a Western blotting assay (Figs. 6A and B). The results showed that the mice in the model groups had lower levels of Occludin and Claudin4 proteins distinctly, which suggested the mucosa-barrier was disrupted during lipid overloading. PGL was able to enhance both the protein expressions of Occludin and Claudin4, which suggested PGL could protect the barrier from lipid damage. TA and GA upregulated Occludin and Claudin4 protein expression, whereas PGA and EA upregulated only Claudin4 protein expression. Orlistat up-regulated Occludin and Claudin4 expressions, suggesting that there might be a same mechanism between orlistat and PGL in the intestinal mucosa. The RT-PCR results were similar to the protein results for Occludin and Claudin4 (Fig. 6C).

Discussion

The digestion and absorption of lipids mainly happen in the small intestine, and phosphatidylcholine, which is produced by the liver, provides assistance during this process [26-28]. Lipid digestion is actually started in the stomach by gastric lipase, and lipids are then thoroughly digested by pancreatic lipase in the small intestine where triglycerides are transformed into fatty acids, mono- and di-glycerides [29]. The products of lipid digestion are then absorbed by the intestinal epithelia and released into the blood after being resynthesized from triglycerides [30].

In the present study, we found a direct target of PGL to explain why PGL extracts can decrease serum TC and TG in the acute hyperlipidemia mice. Three different doses of PGL and all four components showed an inhibition of lipase activity both in vivo and in vitro.

Molecular docking results also showed that four compounds had an affinity for pancreatic lipase. EA, GA and PGA mainly bound to the enriched α-helix of pancreatic lipase, whereas TA bound to the junction of the α-helix and β-folding. For affinity, TA was the strongest, followed by EA, GA, and PGA. However, PGA had the strongest inhibition of pancreatic lipase activity (IC50), followed by GA and EA. TA was weaker, which suggested the in vivo environment might be more complex.

To support the in vitro results, we employed an acute oral lipid-overloading model to observe the absorption of lipids in mice. The results showed that PGL can inhibit the intestinal absorption of lipids, especially after a large dose of PGL. The other four compounds were able to inhibit intestinal lipid absorption.
absorption, which significantly reduced serum TG. Because the substrate of pancreatic lipase is fat, it has been suggested that the decrease in serum TG by PGL and the four compounds was caused mainly by the inhibition of lipase activity in the intestine.

Large doses of PGL may inhibit intestinal lipase activity while reducing serum TG and TC. Therefore, we might confirm that intestinal lipase is one of the important targets for PGL in hyperlipidemia. In other words, inhibiting lipase activity and reducing fat absorption is the important role of PGL in lipid metabolism.

As shown by the results, the IC$_{50}$ of TA on lipase activity in vitro was 239.7 μg·mL$^{-1}$, which reflected lower activity. However, in the in vivo experiments, TA showed strong activity against lipase, which suggested that the action of TA on lipase strengthened in the more complex in vivo environment. During acute lipid overload, the blood glucose of mice showed little change. However, EA showed a certain degree of hypoglycemic effect, which suggested that EA might have potential medicinal value.

Intestinal absorption is an inseparable bond between intestinal epithelial cells that is mainly related to Claudin4 and Occludin expression in intestinal epithelial cells and reflects the tight junctions in the small intestinal barrier [24-25]. Intestinal lipid overload is able to induce lipid accumulation and increased lipid peroxidation, which results in DNA damage and disruptions to cell membrane integrity, as well as glucose imbalance [31-32]. A recent study has found that gut microbes could invade the small intestinal mucosa and cause mucosal barrier damage [33-34]. In the present experiment, we found that when lipid overload occurred, the expression of Claudin4 and Occludin in the small intestinal mucosa were distinctly downregulated, which implied that the barrier of the intestines was being interrupted. PGL could significantly upregulate the expression of Claudin4 and Occludin, which suggested that lipid overload was prevented in the intestinal mucosa. Additionally, the antioxidant effect of PGL could help protect the intestinal barrier against lipid overload [35-36]. PGL not only inhibited lipase activity but also enhanced the small intestinal mucosa barrier and ultimately inhibited fat absorption. TA and GA played a role in promoting protein expression, but it was not as obvious as PGL. Taken together, PGL had a more complex effect on the mucosa than the other four compounds.

Orlistat is a specific long-lasting gastrointestinal lipase inhibitor that can inhibit the hydrolysis of triglycerides into absorbable free fatty acids and single acylglycerols [37]. The primary function of orlistat is to prevent absorption of fats from dietary source by functioning as a lipase inhibitor, which ultimately decreases energy intake [38]. In the present study, we used orlistat as a positive control to compare it to PGL. As the results showed, orlistat inhibited lipase and decreased the blood levels of TG in mice, which up-regulated Occludin and Claudin4 expressions, suggesting that there appeared to be a same mechanism on the gut-mucosa between orlistat and PGL.

In conclusion, our research demonstrated that PGL could decrease serum TG and TC in acute hyperlipidemic mice and attenuate lipid absorption by inhibiting lipase activity in the small intestine of mice. Simultaneously, PGL could promote the expression of the tight-junction proteins Occludin and Claudin4, which enhanced the intestinal barrier for the prevention of lipid intake. TA, GA, PGA, and EA showed significant effects on inhibiting lipase activity, which suggested they were the most active ingredients of PGL. The results provided us with a new perspective on the anti-hyperlipidemia effects of PGL, which might contribute to the overall understanding and application of PGL.

Acknowledgement

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References


