Ahmad, Bilal, Friar, Emily P., Taylor, Emerald, Vohra, Muhammad Sufyan, Serpell, Christopher J., Garrett, Michelle D., Ee Loo, Jason Siau, Fong, Isabel Lim and Wong, Eng Hwa (2022) Anti-pancreatic lipase and anti-adipogenic effects of 5, 7, 3',4',5'-pentamethoxy and 6, 2',4'-trimethoxy flavone - An In vitro study. European Journal of Pharmacology, 938 . ISSN 0014-2999.

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Anti-pancreatic lipase and anti-adipogenic effects of 5,7,3’,4’,5’-pentamethoxy and 6,2’,4’-trimethoxy flavone - An In vitro study

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ARTICLE INFO

Keywords:
Polymethoxy flavones
Anti-Obesity
Pancreatic lipase
Anti-adipogenic

ABSTRACT

In this study, the anti-obesity effects of 5,7,3’,4’,5’-pentamethoxyflavone (PMF) and 6,2’,4’-trimethoxyflavone (TMF) were evaluated through two distinct mechanisms of action: inhibition of crude porcine pancreatic lipase (PL), and inhibition of adipogenesis in 3T3-L1 pre-adipocytes. Both flavones show dose dependent, competitive inhibition of PL activity. Molecular docking studies revealed binding of the flavones to the active site of PL. In 3T3-L1 adipocytes, both flavones reduced the accumulation of lipids and triglycerides. PMF and TMF also lowered the expression of adipogenic and lipogenic genes. They both reduced the expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ), CCAAT/enhancer-binding protein α and β (C/EBP α and β), sterol regulatory element-binding protein 1 (SREBP 1), fatty acid synthase (FASN), adipocyte binding protein 2 (aP2), and leptin gene. In addition, these flavones enhanced adiponectin mRNA expression, increased lipolysis and enhanced the expression of lipolytic genes: adipose triglycerides lipase (ATGL), hormone sensitive lipase (HSL) and monoglycerides lipase (MAGL) in mature 3T3-L1 adipocytes. Overall, PMF was seen to be a more potent inhibitor of both PL activity and adipogenesis versus TMF. These results suggest that PMF and TMF possess anti-obesity activities and can be further evaluated for their anti-obesity effects.

ARTICLE INFO

1. Introduction

The prevalence of obesity is increasing rapidly worldwide. It arises due to abnormal accumulation of body fats, and has numerous negative effects on health (Lin and Li, 2021). Obesity is known to be a principal cause of other disorders including cancer, hypertension, type-2 diabetes, asthma, dyslipidemia and atherosclerosis (Ahmad et al., 2020a; Chen et al., 2018; Je et al., 2021; Yamaguchi et al., 2017). PL is an enzyme important to the digestive tract, produced and secreted by the pancreas into the duodenum (Seyedan et al., 2015). PL hydrolyses fats (triglycerides) into monoglycerides and free fatty acids (FFAs). These monoglycerides and FFAs then enter enterocytes, where triglycerides are resynthesized and ultimately stored in mature adipocytes. This accumulation of triglycerides in mature adipocytes in white adipose tissue (WAT) is the primary cause of obesity (Ahmad et al., 2020b).

Mature adipocytes are lipid-laden cells formed from pre-adipocytes through a well-orchestrated process known as adipogenesis. The process of adipogenesis involves the increased expression of various adipogenic and lipogenic genes and transcriptional factors such as PPAR-γ, C/EBP-α and β, FASN, SREBF-1. This causes an increase both in number (hyperplasia) and size (hypertrophy) of adipocytes (Zhao et al., 2022) which ultimately leads to obesity. Correspondingly, inhibition of PL activity and adipogenesis in white adipocytes are among approaches being investigated to tackle obesity. Anti-obesity drugs have been approved and marketed, but so far, their adverse effects have outweighed their beneficial effects (Je et al., 2021; Park et al., 2019).

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https://doi.org/10.1016/j.ejphar.2022.175445
Received 28 July 2022; Received in revised form 29 November 2022; Accepted 30 November 2022
Available online 5 December 2022
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Flavones are a class of flavonoids found in fruits (specifically citrus fruits) and vegetables (Wang et al., 2017), and may possess various anti-disease effects such as anti-cancer, anti-microbial, estrogenic, anti-obesity, and anti-inflammatory properties. (Khalilpourfarshbafi et al., 2018; Singh et al., 2014). In this study, we have evaluated the anti-PL and anti-adipogenic effects of two flavones: 5,7,3′,4′,5-pentamethoxyflavone (PMF) and 6,2′,4′-trimethoxyflavone (TMF) (Fig. 1). Both flavones are found naturally in plants. PMF is found in Murraya paniculata (Cai et al., 2009; Kinoshita and Firman, 1997) and has been reported previously to have anti-cancer activity (Cai et al., 2009). TMF is found in Tripodanthus acutifolius and has been shown to possess anti-inflammatory and anti-arthritic activities (Serban et al., 2019; Ticona et al., 2021). Previously, a number of other flavones have been reported also to possess anti-obesity effects (Lee et al., 2015; Miyata et al., 2011; Okabe et al., 2014; Song et al., 2016). However, the anti-obesity effects of these two poly-methoxyflavones have not been evaluated. Hypothesizing that PMF and TMF may display anti-obesity effects, the aim of this study was to investigate the anti-PL and anti-adipogenic effects of PMF and TMF. In accordance with our hypothesis, both of the flavones (PMF and TMF) inhibited PL activities, and also adipogenesis in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Flavones

For this study, PMF was synthesized according to literature procedures (Lan et al., 2015), and TMF was purchased from Sigma Aldrich (CAS No. 720675-90-1) (Fig. 1). Both of the flavones had purity level ≥98%.

2.2. PL inhibition by PMF and TMF

The inhibitory effects of PMF and TMF against PL were determined following the methods described by Li et al. (2020) and Ong et al. (2016). Briefly, 5 mg/mL of PL (EC 3.1.1.3; Sigma Aldrich) was dissolved in Tris-HCl buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 10 mM MOPS) and thoroughly mixed for 15 min. The mixture was then centrifuged at 1000 RPM for 15 min and the supernatant used for further assays. The solution was incubated with a range of concentrations of flavones (20, 40, 60, 80, 100 μg/mL in DMSO) for 15 min at 37 °C. Following incubation, 5 μL of p-nitrophenyl butyrate (PNPB) (10 mM in acetonitrile) was added and the solution further incubated for 15 min at 37 °C, and the optical absorbance was read at 410 nm against a blank which was a denatured enzyme. Enzyme denaturation was achieved by boiling the enzyme solution in a water bath for 10 min. Orlistat, a known inhibitor of PL, was used as a positive control in the assays. The total amount of DMSO in the assay was maintained at below 1%. All assays were performed in triplicate, and the inhibitory activity of PMF and TMF or orlistat against PL was calculated according to the formula:

\[
\text{Inhibition Rate (\%I)} = \left(1 - \left(\frac{B - b}{A - a}\right)\right) \times 100
\]

where \(B\) is the activity of PL with inhibitor, \(b\) is the negative control with inhibitor, \(A\) is the activity of PL without inhibitor and \(a\) is the negative control without inhibitor.

2.3. Enzyme kinetics (mode of inhibition)

To evaluate the mode of inhibition of PMF and TMF against PL, two concentrations of PMF and TMF (100 and 200 μg/mL in DMSO) were incubated with PL for 15 min at 37 °C. After incubation, various concentrations (20, 40, 60, and 80 μM) of PL substrate (PNPB) were added to the mixtures and the optical absorbance at 410 nm was read continuously for 15 min at 30 s intervals in a microplate reader (BMG LABTECH SPECTROstar, Germany). Enzyme kinetics were studied using a double reciprocal plot (Lineweaver Burk Plot) which was obtained from plotting the velocity (V; μM/min) of the enzyme versus time in the presence and absence of flavones. The maximum rate of enzymatic reaction (V_{\text{max}}) and Michaelis Menton Constant (K_{m}) were also calculated by evaluating the Lineweaver Burk Plot (Ong et al., 2016; Rodliti et al., 2020).

2.4. Molecular docking

Molecular docking of the flavones in PL was performed by using the Glide docking program as implemented in the Schrodinger suite (Husson et al., 2020). The 3D structure of PL at 2.46 Å resolution (PDB ID: 1LBP) was retrieved from the RCSB Protein Data Bank (RCSB PDB: https://www.rcsb.org) (Egloff et al., 1995) and used in docking. The protein structure was prepared using the standard protein preparation flow: non-ligand molecules and crystallographic waters were removed, incomplete side chains were repaired, appropriate protonation states were assigned at pH 7.4, and the resulting structure was energy-minimized prior to docking. The 3D structures of the two ligands were constructed in Maestro before preparing for the docking using LigPrep. The ligands were then docked into the active site of the protein which was defined by the crystallographic bound ligand using induced-fit docking protocol.

2.5. Cell viability

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (ATCC, USA). 5 × 10^4 cells/well were seeded in 96 well plates and cell viability assays were performed by following the methods of Dunkhunthod et al. (2017) and Je et al. (2021) using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT). The 3T3-L1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS) and 1% penicillin-streptomycin (pen-strep) for 24 h. After 24 h, the media was removed and cells were washed with phosphate buffered saline (PBS). Cells were then treated with a range of flavone concentrations (0, 3, 6, 10, 15, 20, 25 μM) for 24, 48 or 72 h. The final concentration of DMEM in cell culture was 0.1%. After 24, 48 or 72 h of treatment, the media was exchanged with fresh media containing 10% sterile filtered MTT solution in DMEM and incubated for a further 4 h at 37 °C, 5% carbon dioxide (CO₂) in complete darkness. After 4 h, the media was removed and 100 μL of DMEM/well was added. The absorbance was read at 570 nm in a microplate reader (BMG LATECH SPECTROstar, Germany) after 15 min. The cells without treatment were considered as the control (100% viable) and all values were expressed in terms of percentage (%) relative to the control.

2.6. Cell culture and differentiation

3T3-L1 pre-adipocytes were seeded in 96 or 24 well plates at density of 4 × 10^3 or 2 × 10^3 cells/well respectively in DMEM supplemented with 10% BCS and 1% pen-strep. Two days post confluency (considered as Day 0; D0), the pre-adipocytes were induced to differentiate for 3 days. The differentiation induction cocktail comprised 1 μM dexamethasone, 500 μM BMX and 1.5 μg/mL insulin in DMEM supplemented with 10% fetal bovine serum (FBS). At day 3 (D3), the differentiation induction media was changed to differentiation
maintenance media which contained DMEM + 10% FBS + 1.5 μg/mL insulin and the cells were incubated for a further 2–3 days. The cells were kept in differentiation maintenance media until day 10 (D10), and the media was changed subsequently every 2–3 days. A range of concentrations of flavones was also added during the differentiation induction and differentiation maintenance media during the 10 days differentiation process. The cells receiving no treatment with flavones were taken as control and their differentiation rate was considered as 100%.

2.7. Oil Red O staining and lipids quantification

4 × 10^3 cells per well were seeded into 96 well plates and two days post seedling, they were induced to differentiate as described previously. At D10 the cells were harvested and stained with Oil Red O staining dye. Oil red O stock solution (0.5% w/v) in isopropanol was prepared and diluted to working solution with a ratio of 6:4 (60% of Oil Red O stock solution + 40% dH2O). The media was removed, and the cells were washed twice with ice-cold PBS. The cells were then fixed with a 10% formalin solution for 2 h. After fixation, the cells were washed again with PBS followed by washing with 60% isopropanol and allowed to dry at room temperature. Oil Red O working solution was added to the fixed cells and incubated further for 2 h. After incubation, the Oil Red O working solution was removed and the cells were washed twice with dH2O and allowed to dry. The stained lipid droplets were viewed at 40X magnification in an inverted fluorescence microscope (Nikon Eclipse, Japan) and photographed.

For the quantification of lipids, the stained lipid droplets were eluted with 100% isopropanol and incubated for 20 min at 37 °C. After incubation, optical absorbance was measured at 490 nm in a microplate reader. The absorbance is directly proportional to the amount of stained lipids. The untreated cells were taken as control and lipid quantification was calculated in terms of percentage relative to the control (Castillo et al., 2019; Je et al., 2021).

2.8. Triglyceride quantification

Triglyceride content in the control and treated groups of 3T3-L1 adipocytes was measured on D10 after differentiation using a commercial triglyceride assay kit (Elabscience, Cat. No. E-Bc-K238) following the manufacturer instructions. The harvested adipocytes were washed twice with ice-cold PBS followed by trypsinization and centrifugation. The supernatant was removed and the remaining pellet of cells was lysed in RIPA lysis buffer. The cells were then centrifuged at 13,000 g for 20 min. The supernatant was carefully removed and added to 96 well plates followed by addition of ready-made working solution and standards as instructed by the manufacturer. The mixture was incubated in the dark for 1 h and the absorbance was measured at 510 nm in a 96 well microplate reader. Water was used as a blank and the group of cells which received no treatment with flavones were taken as control. The absorbance of the supernatant is directly proportional to the quantity of triglycerides (Han et al., 2018).

2.9. Lipolysis (glycerol release)

The effect of the flavones on lipid breakdown in mature adipocytes was investigated at D10. The fully differentiated cells were stimulated with PMF or TMF, and glycerol release into the medium was measured using a glycerol assay kit (Catalog No. MAK117, Sigma Aldrich) according to the manufacturer instructions. Briefly, after stimulation, 10 μL of the glycerol standard and samples were pipetted into a 96 well plate, and then 100 μL of the master reaction mix (assay buffer 100 μL, enzyme mix 2 μL, ATP 1 μL and dye reagent 1 μL) was added to each standard and sample wells, and incubated for 30 min in the dark at 37 °C. The samples receiving no treatment with flavones served as control and the samples containing only water and master reaction mix were taken as a blank. All readings were taken in triplicate and the absorbance values were corrected by subtracting the absorbance of the blank from the absorbance of each sample. All values were expressed as percentage increase in the release of glycerol relative to the control.

2.10. RNA extraction, cDNA synthesis and RT-qPCR

To evaluate the effect of flavones on the expression of specific marker genes related to adipogenesis, lipogenesis, and lipolysis, RNA was extracted from the differentiated adipocytes at D10 (Treated and untreated Adipocytes). RNA extraction was achieved using the commercial RNA extraction kit from Promega (ReliaPrep™ RNA Cell Miniprep System,USA, Catalog No. Z6011) according to the manufacturer instructions. 1 μg of total RNA was reverse transcribed to first strand cDNA by using GoScript™ Reverse Transcription System from Promega USA (Catalog No. A5001). The reverse transcription was carried out at 25 °C for 5 min (annealing), followed by extension at 42 °C for 1 h and finally inactivation of reverse transcriptase at 70 °C for 15 min in a thermocycler (BIO-RAD T100™).

The expression levels of specific adipogenic, lipogenic, and lipolytic marker genes were investigated through RT-qPCR by using GoTaq™ Master Mix From Promega USA (catalog No. A6001). The targeted first strand cDNA after synthesis was amplified by using the respective sense and anti-sense primers for the specific marker genes (Table 1) in an RT-qPCR (Eppendorf realplex2 Master cycler, Germany). The reaction mix was prepared by mixing qPCR Master mix (10 μL), 500 nM of forward primer, 500 nM of reverse primer and finally 2 μL of 10 ng template cDNA. The total volume of the reaction mixture was adjusted to 20 μL with nuclease-free water. The standard thermal cycling conditions were activation (1 cycle) at 95 °C for 2 min followed by denaturation (40 cycles) at 95 °C for 15 s, and finally annealing and extension (40 cycles) at 60 °C for 1 min.

2.11. Statistical analysis

Graphpad prism statistical software (version 8.0) was used for the

Table 1

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-α</td>
<td>5′-GCTTGAGGAGGTGCAAG-3′</td>
</tr>
<tr>
<td>C/EBP-α</td>
<td>5′-GTGGTTGCGAAATGGTC-3′</td>
</tr>
<tr>
<td>C/EBP-β</td>
<td>5′-GGCGGACCTACTCTTT-3′</td>
</tr>
<tr>
<td>SREBF-1</td>
<td>5′-TTGTCGTTGGCCCTTGA-3′</td>
</tr>
<tr>
<td>aP2</td>
<td>5′-CCGGGATGAGAGGCTGTA-3′</td>
</tr>
<tr>
<td>FASN</td>
<td>5′-TTGGTTTTCGAGTATGAGT-3′</td>
</tr>
<tr>
<td>ATGL</td>
<td>5′-CCGGGAGCTGAGCCAC-3′</td>
</tr>
<tr>
<td>HSL</td>
<td>5′-CGTCTGCACTGCGAATT-3′</td>
</tr>
<tr>
<td>MAGL</td>
<td>5′-GGGAGCTGAGCCAC-3′</td>
</tr>
<tr>
<td>Adiponecin</td>
<td>5′-CCGGGAGCTGAGCCAC-3′</td>
</tr>
<tr>
<td>Leptin</td>
<td>5′-CCGGGAGCTGAGCCAC-3′</td>
</tr>
<tr>
<td>Beta Actin</td>
<td>5′-CCGGGAGCTGAGCCAC-3′</td>
</tr>
</tbody>
</table>
statistical analysis of the study. All results were expressed as mean ± standard deviation. A one way analysis of variance (ANOVA) followed by Tukey test was used for the statistical analysis of the data. Values of \( p < 0.05 \) were considered to indicate the statistical significance.

3. Results

3.1. Effects of PMF and TMF on the inhibition of PL

Both flavones (PMF and TMF) inhibited the activity of PL in a dose dependent manner (Fig. 2). The highest concentration of the flavones used in this study was 100 \( \mu \text{g/mL} \); at this concentration, PMF inhibited the activity of PL by 45.42 ± 1.07%, while TMF inhibited the PL activity by 36.04 ± 0.74% at the same concentration. Orlistat at 100 \( \mu \text{g/mL} \) inhibited the activity of PL by 35.06 ± 1.20%. According to the results obtained, the estimated IC\(_{50}\) values for PMF and TMF are above 100 \( \mu \text{g/mL} \). The inhibition of PL by PMF was significantly higher than TMF or orlistat (\( p < 0.05 \)) at 40–100 \( \mu \text{g/mL} \), while there was no significant difference between the inhibition of PL by TMF and Orlistat at these concentrations.

3.2. Mode of inhibition of PMF and TMF

After analysing the inhibitory effects of PMF and TMF on PL activity, next their mode of inhibition was determined. The kinetic analysis of PL activity was investigated by generating double reciprocal (Lineweaver Burk) plots for inhibition of PL by PMF or TMF and calculating the \( K_m \) and \( V_{\text{max}} \) in the presence and absence of each flavone. As shown in Fig. 3, the kinetic parameters calculated from double reciprocal trend lines indicated that these flavones display competitive inhibition (\( V_{\text{max}} \) remains the same and \( K_m \) varies). In this study, the \( V_{\text{max}} \) values in the presence and absence of flavones were almost equal (around 21 \( \mu \text{M/min} \)) but the \( K_m \) values increased with increasing flavone concentration (Table 2).

3.3. Molecular docking

To validate the mode of inhibition with results obtained in vitro, molecular docking was performed to investigate the interaction pattern, binding affinities and possible binding sites of the flavones with PL. The docking studies revealed that PMF and TMF can bind deeply within the active site of PL. Both PMF and TMF were modelled interacting with the amino acids of catalytic triad in the active site (Fig. 4). The active site of PL is composed of three amino acids (Ser152, His263 and Asp176). PMF interacted with Ser152, His263 and Phe 77 through three hydrogen bonds at 2.2, 2.3 and 1.7 Å (Fig. 4 C) which resulted in reasonable binding energy (−9.632 kcal/mol) of PMF with PL. The interaction pattern of TMF was similar to that of PMF by interacting with Ser152, His263, and Phe 77 and formed three hydrogen bonds at 2.6, 2.4 and 1.8 Å (Fig. 4 D). The binding energy of TMF with PL was −8.473 kcal/mol.

3.4. Effects of PMF and TMF on cell viability

3T3-L1 pre-adipocytes were cultured as described in the methodology section, and treated with 0, 3, 6, 10, 15, 20 and 25 \( \mu \text{M} \) of each

![Fig. 2. Inhibition of PL by PMF and TMF. \( P^* < 0.05 \), \( P^{**} < 0.001 \). All the values are represented as mean ± SD, (\( n = 3 \) biological repeats).](image-url)

![Fig. 3. Double reciprocal plots of PMF (A) and TMF (B), each at 100 and 200 \( \mu \text{g/mL} \). (\( n = 3 \) biological repeats).](image-url)

<table>
<thead>
<tr>
<th>Concentration (( \mu \text{g/mL} ))</th>
<th>Velocity of enzyme activity at different substrate concentrations (( \mu \text{M} ))</th>
<th>( V_{\text{max}} ) (( \mu \text{M/ min} ))</th>
<th>( K_m ) (( \mu \text{M} ))</th>
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<tbody>
<tr>
<td>20</td>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.191 ± 0.009</td>
<td>0.097 ± 0.002</td>
<td>20.83 ± 0.009</td>
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<td></td>
<td>±</td>
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<td>±</td>
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<tr>
<td></td>
<td>0.009 ± 0.002</td>
<td>0.001 ± 0.003</td>
<td>0.003 ± 0.003</td>
</tr>
<tr>
<td>40</td>
<td>PMF 100</td>
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<tr>
<td></td>
<td>0.375 ± 0.003</td>
<td>0.156 ± 0.002</td>
<td>21.23 ± 0.002</td>
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<tr>
<td></td>
<td>0.003 ± 0.002</td>
<td>0.002 ± 0.002</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>60</td>
<td>PMF 200</td>
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<td></td>
<td>0.44 ± 0.002</td>
<td>0.186 ± 0.001</td>
<td>21.32 ± 0.001</td>
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<tr>
<td></td>
<td>0.01 ± 0.002</td>
<td>0.01 ± 0.001</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>80</td>
<td>TMF 100</td>
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<td></td>
<td>0.248 ± 0.002</td>
<td>0.111 ± 0.002</td>
<td>19.84 ± 0.002</td>
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<td>±</td>
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<td>0.02 ± 0.002</td>
<td>0.007 ± 0.007</td>
<td>0.009 ± 0.009</td>
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<tr>
<td>200</td>
<td>TMF 200</td>
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<tr>
<td></td>
<td>0.36 ± 0.014</td>
<td>0.163 ± 0.012</td>
<td>20.68 ± 0.014</td>
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<td>±</td>
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<td>0.014 ± 0.004</td>
<td>0.001 ± 0.001</td>
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![Table 2. Kinetic analysis of inhibition of PL by PMF and TMF.](image-url)
flavone. PMF did not show any cytotoxicity relative to the control up to 25 μM after 24 h of incubation but after 48 and 72 h, it reduced cell viability at 25 μM by a small margin (Fig. 5A). TMF did not show any significant cytotoxicity at the same concentrations even after 72 h of incubation relative to the control (Fig. 5B). It was therefore decided to use non-toxic concentrations below 25 μM, and 15 μM was the highest concentration used for both PMF and TMF in subsequent assays.

3.5. Effect of flavones on lipid accumulation during adipogenesis

To investigate the anti-adipogenic effects of the flavones, 3T3-L1 pre-adipocytes were first differentiated into mature adipocytes in the presence and absence of flavones at 0, 3, 6, 10 and 15 μM for 10 days. Both PMF and TMF dose-dependently reduced adipogenesis and inhibited the accumulation of lipids as evidenced by Oil Red O staining. The intensity of the stained-lipid droplets (red colour) decreased with the increase in concentration of flavones - an indication of inhibition of adipogenesis and suppression of lipid accumulation. Moreover, in contrast to pre-adipocytes, the accumulation of lipids was much higher in the control (non-treated) mature adipocytes which also indicated that pre-adipocytes were fully differentiated into mature adipocytes and actively synthesized lipids. Consistent with the stained-lipid droplet observation, the accumulation of lipids by 16, 35, 45 and 60% at 3, 6, 10 and 15 μM respectively as compared to the control (untreated) cells (Fig. 6). Likewise, TMF also significantly decreased lipid accumulation by 8, 13, 23 and 32% at the same concentrations respectively (Fig. 7). There was no significant difference between lipid content at 3 μM of TMF and control.

3.6. Effects of flavones on triglyceride accumulation

3T3-L1 pre-adipocytes were seeded at 1.5 × 10⁴ cells per well in 24 well plates and induced to differentiate as described in the methodology section. At D10, the cells were harvested for the quantification of triglycerides. Consistent with the general lipid accumulation observed in mature adipocytes, PMF and TMF also inhibited the accumulation of triglycerides (Fig. 8). In concordance with the effects of these flavones on lipid accumulation, PMF significantly inhibited triglyceride accumulation to a greater degree than TMF with inhibition values being 16, 25, 37 and 49% at 3, 6, 10, and 15 μM respectively for PMF versus 10, 17, 23 and 30% at 3, 6, 10 and 15 μM respectively for TMF.

3.7. Effects of flavones on lipolysis

To investigate whether PMF and TMF have any effects on the release of free glycerol as a result of lipolysis, 3T3-L1 pre-adipocytes were differentiated for 10 days. The fully differentiated adipocytes were incubated with a range of concentrations of PMF or TMF (0, 3, 6, 10, 15 μM) for 24, 48 and 72 h. After incubation, the release of glycerol into the media was measured (Fig. 9). There was no significant effect of either PMF or TMF on glycerol release at 3 μM. However, a significant increase

Fig. 4. Molecular docking prediction of PMF with PL. (A) Whole enzyme structure and docked PMF and TMF. (B) Cartoon model of PMF and TMF and PL interaction. (C) 3-D structure of PMF with interacting amino acids of the PL active site. (D) 3-D structure of TMF with interacting amino acids of the PL active site. The binding energy of PMF with PL is −9.632 kcal/mol while that of TMF is −8.473 kcal/mol.
μin glycerol release was observed with 6 μM of PMF, but not TMF at the same concentration. At 10 μM, PMF enhanced the release of glycerol to 122, 126 and 139% after 24, 48 and 72 h of incubation respectively, whilst TMF enhanced glycerol release to 112, 119 and 123% at the same time points respectively. Both flavones showed highest lipolytic effects at 15 μM and after 72 h incubation. PMF increased glycerol release to 150% at 15 μM concentration and 72 h incubation. Likewise, TMF increased glycerol release to 126% at the same concentration and incubation period. In conclusion, both of the flavones effectively increase lipolysis at 10 and 15 μM concentrations when incubated with 3T3-L1 adipocytes for 48 and 72 h.

3.8. Effects of flavones on adipogenic gene expression

3T3-L1 pre-adipocytes were induced to differentiate in 24 well plates in the presence and absence of PMF and TMF. The two highest concentrations (10 and 15 μM) of each flavone were used for measuring the effects of these flavones on mRNA expression of the adipogenic genes PPARG-γ, C/EBP-α, C/EBP-β, and SREBF1 (Fig. 10). PMF and TMF both significantly reduced the expression of adipogenic genes compared to control cells. PMF downregulated PPARG-γ by 70 and 75% at 10 and 15 μM. Similarly, C/EBP-α was downregulated by 65 and 66%, C/EBP-β expression was reduced by 58 and 79%, and finally SREBF1 was downregulated by 78 and 81% at both PMF concentrations respectively. Likewise, TMF also downregulated these genes but to a lesser degree than PMF. PPARG-γ was reduced by 14 and 22%, C/EBP-α by 10 and 41%, C/EBP-β by 4 and 20% and SREBF1 by 33 and 59% at 10 and 15 μM respectively. The expression of C/EBP-β at 10 μM of TMF was not significantly lower than the control (untreated cells) (P < 0.05). Overall, PMF had strongest inhibitory effects on the expression of adipogenic genes. All the values were normalized to β-actin which served as an internal control.

3.9. Effects of flavones on lipogenic gene expression

After evaluating the effects of flavones on adipogenic genes, next their effects on expression of lipogenic genes was evaluated. Pre-adipocytes were differentiated into mature adipocytes in the presence and absence of PMF and TMF. The mRNA expression of lipogenic markers in adipocytes was evaluated by treating the cells throughout the differentiation process at 10 and 15 μM of PMF and TMF respectively. PMF downregulated the mRNA expression of FASN by 43 and 60% at 10 and 15 μM. Similarly, TMF also significantly reduced FASN mRNA expression but was less effective than PMF, downregulating FASN by 24 and 45% at 10 and 15 μM (Fig. 11). PMF and TMF also had consistent downregulatory effects on a second lipogenic gene (aP2) mRNA with PMF reducing its expression by 42 and 67%, and TMF by 10 and 36% at 10 and 15 μM respectively. At 10 μM, the effect of TMF on the inhibition of aP2 was not significantly lower than the untreated cells (Fig. 11B). In this case also, TMF was less effective than PMF. β-actin was used as an internal control.

3.10. Effects of PMF and TMF on Leptin and Adiponectin gene expression

Adipokines are produced by mature adipocytes. To gain a deeper understanding of the effects of PMF and TMF, the mRNA expression of two prominent adipokines (adiponectin and leptin) was evaluated both in treated and non-treated adipocytes. Consistent with previous results, both PMF and TMF significantly reduced the expression of leptin and enhanced the expression of adiponectin (Fig. 12). At more than 90%, the downregulatory effect of PMF on leptin was greater than its effects on other adipogenic and lipogenic genes. TMF also downregulated leptin, again, its effect was reduced versus PMF. TMF at 10 and 15 μM downregulated leptin by 34 and 51% which is almost half the effect of that of PMF. In contrast to leptin expression, adiponectin expression was increased in the treated cells. PMF and TMF both enhanced the expression of adiponectin. PMF increased the expression of adiponectin by 2.6- and 2.8-fold at 10 and 15 μM. Likewise, TMF also enhanced the adiponectin expression by 1.4 and 2.1-fold compared to the untreated control group. All values were normalized to that of β-actin (internal control).

3.11. Effects of flavones on lipolytic genes

To investigate the effects of PMF and TMF on the expression of lipolytic genes, 3T3-L1 adipocytes were incubated in the presence of PMF or TMF for 72 h and gene expression analysis of ATGL, HSL and MAGL was performed (Fig. 13). PMF enhanced the expression of ATGL by 1.7 and 2.1-fold at 10 and 15 μM while TMF only significantly enhanced its expression by 1.4 at 15 μM concentration. TMF had no effect on ATGL expression at 10 μM. Similarly, both flavones had no effect on HSL expression at 10 μM while at 15 μM, PMF enhanced significantly HSL expression 1.9-fold, and TMF 1.37-fold. Likewise, TMF had no significant effect on the expression of MAGL at 10 μM, and at 15 μM it significantly enhanced MAGL expression by a factor of 1.48. PMF significantly enhanced the expression of MAGL both at 10 and 15 μM by 1.4 and 1.6 fold.

4. Discussion

A main cause of obesity is the accumulation of lipids in the form of triglycerides in the mature adipocytes of WAT. Obesity is a great threat to human health worldwide due to its close association with metabolic syndromes (Chang and Kim, 2019) and is a potential risk factor for numerous other illnesses, and therefore treating obesity through reduction or prevention of triglyceride accumulation is a vital task. Flavones are known to possess various medicinal effects including anti-obesity. In pre-clinical studies, the potential role of flavones as anti-obesity agents is well established (Sudhakaran and Doseff, 2020).
Previously, various pre-clinical (in vitro and in vivo) studies have reported the anti-obesogenic effects of flavones (Gire et al., 2021; Peng et al., 2019; Su et al., 2020; Sun and Qu, 2019) which shows that flavones are a potential alternative for the management of obesity (Cercato et al., 2021).

In the present study, two flavones (PMF and TMF) were evaluated for the first time for their anti-obesity effects via two different mechanisms of action in vitro. In the first part, the inhibitory effects of PMF against the PL enzyme were examined and found to be higher than for TMF ($P < 0.05$). The presence of methoxy (-OCH$_3$) groups at 5 and 7 position of A ring has been shown to have significant effects on the activities of flavones (Kawaii et al., 2018), with the higher inhibitory effects of PMF against PL possibly due to the presence of these additional –OCH$_3$ groups, which would influence both the geometric and electronic properties of the molecule.

Next, the modes of inhibition of PMF and TMF against PL were investigated. Both PMF and TMF showed competitive inhibition, i.e., they competed with the substrate (PNPB) for the same site of attachment (active site), and therefore would be expected to interact with the active site of the enzyme to form an enzyme-inhibitor complex (EI complex).

The in vitro results were further confirmed through molecular docking studies which revealed that these flavones have the capacity to bind to the active site of PL. Three important amino acid residues of PL active site are Ser 152, Asp 176 and His 263 (Nguyen et al., 2020). The binding pattern of PMF and TMF was same in that both interacted with Ser 152, His 263 and Phe 77 and formed the same hydrogen bonding pattern, but the binding energy of TMF was weaker (–8.473 kcal/mol) versus PMF (–9.632 kcal/mol). Again, this could be due to the number and position of –OCH$_3$ groups attached to the flavone ring. In summary, the docking results obtained in this study were in accordance with the in vitro results.

Fig. 6. Effects of PMF on lipid accumulation during adipogenesis. (A) Lipid staining with and without PMF. (B) Lipid quantification with and without PMF. Data is represented as mean ± SD (n = 3 biological repeats). $P^{**} < 0.01$, $P^{***} < 0.0001$. PA: Pre-Adipocytes. C: Control.
in that the flavones showed a competitive mode of inhibition. Competitive inhibitors bind to the active site of the enzyme, decrease the amount of enzyme-substrate complex generated and ultimately result in diminishing the rate of catalysis (Pelley, 2012; Singh et al., 2017).

The anti-adipogenic, anti-lipogenic, and lipolytic effects of the two flavones were next investigated in 3T3-L1 adipocytes. As would be expected from the enzymatic studies, PMF showed higher inhibitory effects than TMF. The intracellular activities of the flavones are dependent on both the type of cells and the associated interactions between flavones and type of cell. Methylation of flavones increases their chemopreventive effects and metabolic stabilities (Walle, 2009). Flavones with multiple –OCH₃ groups have high membrane permeabilities due to their hydrophobic nature, which helps them to cross the phospholipid layer (Li et al., 2007), and thus may increase their intracellular bioactivities. So, the higher anti-adipogenic effects of PMF than TMF may be due to the presence of more –OCH₃ groups at both A and B rings. The lipid reduction effects of the flavones used in this study were higher than those reported by Choi et al. (2016) in the case of 7,8-dihydroxyflavone and apigenin (5, 7, 4′-trihydroxyflavone) (Kim et al., 2010, 2014). This could be due to the presence of –OCH₃ groups on PMF and TMF when compared to those reported by Choi et al. (2016) and Kim et al. (2010, 2014) that have only –OH substituent groups. Consistent with the data from the lipid assay, a similar pattern of inhibitory activity was observed in the case of triglyceride accumulation studies. Both PMF and TMF reduced the accumulation of triglycerides, and PMF was more a potent inhibitor of both lipid and triglyceride accumulation versus TMF.

It was evident from the lipid staining, and quantification of lipid and triglyceride accumulation that PMF and TMF have anti-adipogenic and anti-lipogenic properties, and following on from this, their inhibitory effects on the adipogenic and lipogenic genes PPAR-γ, C/EBP-α, C/EBP-β, SREBF-1, FASN, and aP2 specifically were investigated. The adipogenic genes such as PPAR-γ, C/EBP-α, C/EBP-β, and SREBF-1 play an

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**Fig. 7.** Effects of TMF on lipid accumulation during adipogenesis. (A) Lipid staining with and without TMF. (B) Lipid quantification with and without PMF. Data is represented as mean ± SD (n = 3 biological repeats). P** < 0.01, P*** < 0.001. PA: Pre-Adipocytes. C: Control.
important role in the positive regulation of adipogenesis and lipogenesis, and their collaboration significantly affects the expression of lipogenic genes and their respective enzymes such as FAS and ACC (Chayaratanasin et al., 2019). PPAR-γ is one of the crucial transcription factors required for the induction of differentiation in pre-adipocytes (Je et al., 2021). Likewise, C/EBP-α and -β are well-known positive regulators of adipogenesis. The expression of C/EBP-β is induced rapidly just after the induction of adipogenic stimuli (Guo et al., 2015) and then together with C/EBP-δ, it induces the expression of C/EBP-α and PPAR-γ (Ahmad et al., 2020b). Anti-obesity agents which suppress PPAR-γ expression should also suppress C/EBPs (Wang et al., 2016). In this study, the expression of PPAR-γ was reduced by 70 and 75% (PMF), and 14 and 22% (TMF), and on the same lines downregulation of C/EBP-α and C/EBP-β was also observed. In this scenario our results were consistent to those reported by He et al. (2015) and Kanda et al. (2012). SREBF-1 is another pro-adipogenic factor and promotes the differentiations of adipocytes and may induce the expression of PPAR-γ (Khaliqpourfarshabi et al., 2018). In this study, as shown in Fig. 10D the expression of SREBF-1 was also significantly reduced by PMF and TMF.

The results obtained in this study thus suggest that the flavones reduced the production of lipid and triglycerides in differentiating 3T3-L1 adipocytes by downregulating expression of PPAR-γ, C/EBP-α/β, and SREBF-1.

In previous studies, it has been reported that methoxylated flavones enhance the process of adipogenesis instead of inhibiting it. 3,5,7,4′-Tetramethoxyflavone (nobiletin) and 3,5,7,3′,4′-pentamethoxyflavone have been shown to enhance differentiation of 3T3-L1 adipocytes (Horikawa et al., 2012; Saito et al., 2007). The results obtained in this study therefore contrast with what was observed by Saito et al. (2007) and Horikawa et al. (2012). However, other studies both in vitro and in vivo have shown that polymethoxy flavones possess anti-adipogenic, anti-lipogenic, and lipolytic activities, and also cause thermogenesis and increase brown adipocyte development (Chen et al., 2018; Choi et al., 2020; Kobayashi et al., 2016; Okabe et al., 2014; Tsutsumi et al., 2014; Wang et al., 2015).

Adipogenesis and lipogenesis are interrelated activities, so the inhibitory effects of flavones on lipogenic gene expressions (FASN and aP2) was investigated. An increase in the activity and expression of lipogenic genes contributes to the development of obesity (Berndt et al., 2007; Choi et al., 2020). Consistent with the lipid and triglyceride results, the lipogenic genes were also downregulated by these flavones, which further confirmed the anti-lipogenic effects of PMF and TMF. In addition, the effects of flavones on adiponectin and leptin mRNA expression were also investigated. PMF and TMF reduced the mRNA expression of adiponectin and leptin.
expression of leptin which is used as an index of obesity (Je et al., 2021). In contrast to leptin, the expression of adiponectin reduces in obesogenic conditions (Je et al., 2021). In the present study, PMF and TMF enhanced the expression of adiponectin as compared to the control cells. The effect of PMF in both cases was more potent than TMF. Lipolysis in adipocytes is also one of the crucial points at which obesity can be tackled, so, lastly, the lipolytic effects of PMF and TMF were also investigated. Both flavones significantly enhanced lipolysis and increased glycerol release as shown in Fig. 9. Following glycerol release, the effects of PMF and TMF on lipolytic genes was also investigated. Expression of lipolytic genes and proteins (ATGL and HSL) is decreased in obesity (Jocken et al., 2007; Okabe et al., 2014), but in the current
study, both flavones significantly enhanced the expression of lipolytic genes (ATGL, HSL, MAGL). Similar results were also obtained by Okabe et al. (2014) in the case of 3,5,7,3′-pentamethoxyflavone and 5,7,4′-trimethoxyflavone.

5. Conclusion

In the present study, PMF and TMF significantly inhibited the activity of PL, inhibited both adipogenesis and lipogenesis in 3T3-L1 adipocytes and increased lipolysis in mature adipocytes. In the previous studies, it has been observed that –OCH3 group of flavonoids play a significant role in the determination of their effects in cells. This might be the reason that greater effects were seen with PMF versus TMF on inhibition of adipogenesis. Overall, the results obtained in the current study suggest that these flavones possess anti-obesity effects in vitro, and thus further research is needed to investigate these effects in animal models and then in the clinical setting.

References


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