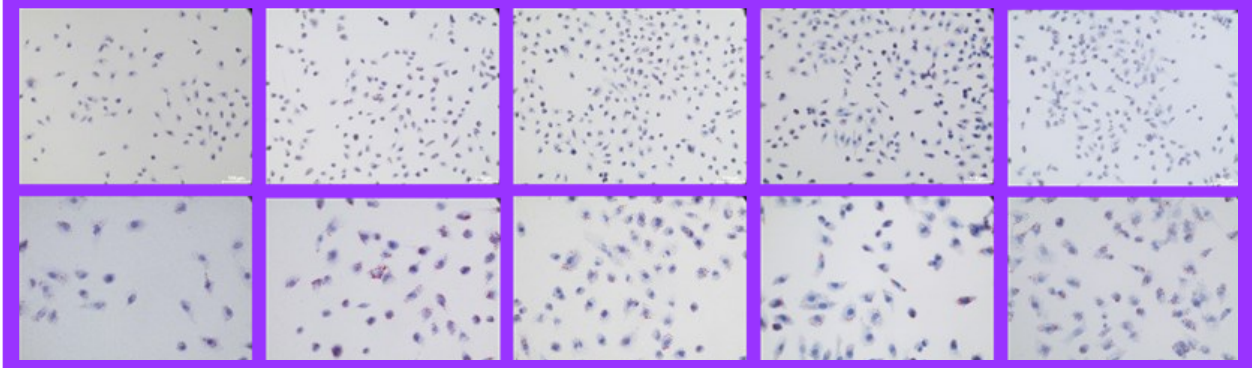

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Total flavonoids of *Abelmoschus manihot* ameliorate lipid deposition in HK-2 cells by inhibiting fatty acid uptake mediated by CD36

Xiaofang Wang[1], Chenquan Tang[2], Yi Jiang[2], Yi Xue[2], and Enchao Zhou[1]

[1]No. 1 Clinical Medical College, Nanjing University of Chinese Medicine (Jiangsu Province Hospital of Chinese Medicine), Nanjing 210000, China; [2]Department of Nephrology, Suzhou Hospital of Integrated Traditional Chinese and Western Medicine, Suzhou, Jiangsu 215100, China.

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Abstract

This study aimed to explore the mechanism by which total flavones of *Abelmoschl manihot* reduce intracellular lipid deposition in HK-2 cells (Human renal cortex proximal tubule epithelial cells) and thus reduce cell apoptosis. Palmitic acid was employed to induce intracellular lipid deposition. The cell proliferation activity was detected by the CCK-8 assay. The cell death status was evaluated by flow cytometry. The intracellular lipid deposition was observed by oil red O and BODIPY probe staining. The expression level of CD36 in HK-2 cells was determined by western blot. The results indicated that total flavones of *A. manihot* could inhibit the expression of CD36 in HK-2 cells in a dose-dependent manner and reduce lipid deposition. Consequently, total flavones of *A. manihot* protect HK-2 cells by reducing intracellular lipid deposition by inhibiting CD36-mediated fatty acid uptake.

Introduction

Lipid deposition constitutes a risk factor for chronic kidney disease and exerts detrimental effects on renal function. Lipid accumulation is associated with a high-fat diet, overexpression of fatty acid transporter proteins (such as CD36), and a decreased mitochondrial β-oxidation rate (Herman-Edelstein et al., 2014). Excessive fatty acids can damage podocytes, proximal tubular epithelial cells, and interstitial tissues through various mechanisms, including augmenting the production of reactive oxygen species (ROS) and lipid peroxides and promoting mitochondrial damage and tissue inflammation (Sun et al., 2020). Studies have demonstrated that disorders of lipid metabolism are closely linked to the development of chronic kidney disease, particularly in patients with diabetic kidney disease.

In the kidneys of diabetic kidney disease patients and db/db mice, lipids predominantly accumulate in the proximal tubular region. They are accompanied by up-regulated expression of adipose differentiation-related protein and sterol regulatory element-binding protein-1. These changes are positively correlated with tubule-interstitial injury scores and inflammation. Additionally, the content of adipose differentiation-related protein in urine is significantly increased in diabetic kidney disease patients. It is positively correlated with abnormal lipid metabolism, serum creatinine, urinary N-acetyl-β-D-glycosaminidase, albumin excretion (albumin/creatinine ratio), and tumor necrosis factor-α expression (Yang et al., 2018). These studies suggest lipid accumulation is a key factor in diabetic kidney disease. It not only impacts the metabolism of kidney cells but is also related to inflammation, oxidative stress, mitochondrial dysfunction, and cell death. Intervention in lipid metabolism may represent a new therapeutic target for diabetic kidney disease.

Abelmoschus manihot (L.) Medik is a traditional Chinese medicine that contains flavonoids as its main active ingredients. Studies have shown that *A. manihot* can ameliorate diabetic kidney disease through multiple pathways, such as activating the autophagy process, regulating mitochondrial function, and inhibiting inflammatory responses (Ge et al., 2016; Li et al., 2016). Multiple studies have explored the therapeutic effect of *A. manihot* on diabetic kidney disease through animal models (such as rats) (Li et al., 2018). The results indicate that *A. manihot* extract can significantly improve renal injury and dysfunction associated with diabetic kidney disease. Some clinical trials also support the effect of *A. manihot* in treating diabetic kidney disease, especially in reducing proteinuria and improving renal function (Zhao et al., 2022). Studies also suggest that *A. manihot* extract can reduce blood lipid levels, but its underlying mechanism remains unclear and warrants further investigation. Therefore, the purpose of this study is to determine the protective effect of total flavonoids of *A. manihot* on the kidneys and explore its potential mechanism using a palmitic acid-induced renal tubular cell lipid deposition model. We evaluated the improvement of intracellular lipid deposition and the protective effect on apoptosis by total flavones of *A. manihot* in the model.

Materials and Methods

Cell culture

HK-2 cells (Human renal cortex proximal tubule epithelial cells) were purchased from Icellbioscience (China) and maintained in DMEM F12 1:1 (Servicebio, China) supplemented with 10%fetal bovine serum (Bdbio, China) and 1%penicillin-streptomycin at 37°C in a humidified atmosphere containing 5%CO_2. Based on the cell growth status, subculture is carried out once every 2 to 3 days. Cells with good growth conditions were taken for the experiment.

Drug intervention

Upon adding palmitic acid (57-10-3, Aladdin, China) to a sodium hydroxide solution and subjecting it to full saponification in a water bath at 75°C for approximately 30 min, a colorless, transparent, and clear saponified sodium palmitate solution is ultimately obtained. While the solution is still hot, the prepared d-BSA solution (ST025, Beyotime, China) was added to phosphate-buffered saline (PBS, Servicebio, China) at 55°C and centrifuged at 8,000 rpm in a high-speed centrifuge for 20 min). When used, dilute it to the required concentration with a complete culture medium. Total flavones of *A. manihot* are provided by the Pharmacy Depart-ment of Suzhou Hospital of Integrated Traditional Chinese and Western Medicine.

Analysis using ultra-performance liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS)

The quantitative analysis of six constituents present in the total flavonoids of *A. manihot* was accomplish-ed by application of the LC-MS/MS technique. The reference substances, specifically hyperoside (No.: 111521 - 202310, purity: 94.7%), isoquercitrin (No.: 111809 - 201804, purity: 97.2%), and quercetin (No.: 100081 - 201610, purity: 99.1%), were obtained from the National Institutes for Food and Drug Control. Quercetin-3'-O-glucoside (No.: 478243, purity: 98.48%) was sourced from MedChemExpress. Hibifolin (No.: B2418319, purity: 98%) was procured from Shanghai Aladdin Biochemical Technology Co., Ltd. Myricetin (No.: OK20241019G, purity: 98%) was acquired from Beijing Vokai Biotechnology Co., Ltd.

Box 1: Oil red O staining

Principle

Oil red O staining exhibits specificity in coloring fat a vivid red. Using microscopic examination, the dimensions and dispersion pattern of lipid droplets within cells can be distinctly visualized, thereby serving as a valuable tool for evaluating the abnormal accumulation of lipids within cells.

Requirements

DMEM F12 1:1(G4610, Servicebio, China); 10%Fetal bovine serum (F801-050Hi, Bdbio, China); Hematoxylin staining solution (G1004, Servicebio, China); HK-2 cells; Isopropanol (80109218, Sinopharm, China); Oil red O dye (G1015, Servicebio, China); 4%Paraformaldehyde (G1101, Servicebio, China)

Procedure

Step 1: After various treatments on the cells in the 24-well plate, the cell culture medium was aspirated.

Step 2: The cells were briefly washed with PBS, followed by the fixation with 4% paraformaldehyde fixative at room temperature and rinsing with PBS.

Step 3: A small amount of 60% isopropanol was employed to differentiate the cells.

Step 4: The cells were stained with the working solution of oil red O at room temperature in the dark for 30 min.

Step 5: The staining solution was removed, and 60% isopropanol was added for rapid differentiation for 3-5 sec.

Step 6: The cells were rinsed with pure water, and a hematoxylin staining solution was added to stain the cell nuclei.

Step 7: The staining solution was discarded, and PBS was added to cover the cells before observation under an optical microscope

Reference

Zhong et al., 2024

Chromatographic parameters were set as follows: The chromatographic column adopted was the Acquity UPLC® HSS T3 (with dimensions of 2.1 mm x 100 mm and a particle size of 1.8 µm). Mobile phase A consisted of an aqueous solution of 0.1% formic acid, while mobile phase B was acetonitrile, and a gradient elution procedure was employed. Regarding the mass spectrometry conditions, the electrospray ionization source (ESI) was utilized, and multiple reaction monitoring (MRM) was carried out in the negative ion mode (Chen et al., 2021). For hyperoside, the specific ion transitions were m/z 463.15 → 300.15 and m/z 463.15 → 271.20; for isoquercitrin, m/z 463.15 → 300.15 and m/z 463.15 → 271.20; for quercetin, m/z 301.05 → 151.15 and m/z 301.05 → 179.20; for quercetin-3'-O-glucoside, m/z 463.05 → 301.15 and m/z 463.05 → 151.15; for hibifolin, m/z 493.05 → 317.20 and m/z 463.05 → 167.20; for myricetin, m/z 317.05 → 151.15 and m/z 317.05 → 179.15.

The total flavones sample was diluted to a volume of 50 mL using a 50%aqueous acetonitrile solution. After thorough mixing, it was subjected to centrifugation. Subsequently, 0.1 mL of the supernatant was taken and diluted to 50 mL with the same 50% aqueous acetonitrile solution. This was then filtered through a 0.22 µm microporous membrane. The resulting filtrate served as the test solution for MRM analysis. The peaks in the MS spectra were recorded, and the contents of the total flavones sample were determined by correlating with the concentrations of the respective standard substances.

Cell viability

CCK-8 experiment was performed to assess the impact of total flavones on the viability of HK-2 cells. The cell proliferation experiment was carried out by using the CCK-8 assay kit (Medicalbio, CCK-08). A 96-well plate was prepared with 100 µL of cell suspension (5,000 cells/well) and incubated in a cell culture incubator at 37°C with 5%CO_2 for 24 hours. Then, the cells in the 96-well plate were exposed to various treatments. After 24 hours, the culture medium was aspirated, and 100 µL of 10%CCK-8 solution was added to the corresponding experimental wells, followed by an incubator for 1 hour. The optical density was measured using an enzyme labeling instrument set at a wavelength of 450 nm (Wang et al., 2024).

Flow cytometry

The HK-2 cells were subjected to flow cytometry using the annexin V- ITC/propidium iodide apoptosis assay kit (Multisciences, China). The cells in the 6-well plate were subjected to various treatments. Then, the cells were dyed using annexin V-FITC/propidium iodide as per the supplier's instructions. Then, the cells were resuspended in 500 µL 1x binding buffer solution and then incubated at 4°C without light for 15 min with 10 µL annexin V-FITC and 10 µL propidium iodide. Finally, the flow cytometer (Beckman, DxFlex) was employed to identify the apoptosis rate (Zeng et al., 2024).

BODIPY probe staining

HK-2 cells were cultured on sterile glass slides and then treated with various drugs. Firstly, the medium was removed. Then, the cells were rinsed once with 1x PBS. Next, fixation was carried out with 4% paraformaldehyde at room temperature for 30 min. After a single rinse with 1x PBS again, BODIPY (C2055, Beyotime, China) working solution (working concentration 10 µg/mL) was prepared. Subsequently, 100 µL of the working solution was added, and staining was performed in the dark within a 37°C incubator for 30 min. After that, the working solution was aspirated. The cells were rinsed three times with 1x PBS. Subsequently, nuclear staining was conducted with DAPI (C1005, Beyotime, China) for 3 to 5 min. Finally, the glass slide was removed, sealed with an anti-fluorescence quenching agent, and observed under a fluorescence microscope (Jiang, 2020).

Western blotting analysis

The cells were incubated in a 6-well culture plate at 1x 10^6/mL for 24 hours and incubated for another 24 hours after exposure to various treatments. Then, the culture medium was discarded, and the cells were washed twice with PBS. The cells were lysed using RIPA and then collected. Thereafter, the cells were subjected to ultrasonic treatment for 60 sec (15 sec/time, in total- 4 times). The cells were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected to determine the concentration of the target protein. An equal amount of protein from every specimen was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride films. The membranes were incubated with 5% non-fat milk and TBS buffer (TBST) involving 0.1% Tween 20 for 60 min, and afterward CD36 rabbit polyclonal antibody (P16671, Biodragon, China, 1:1000) were added. The samples were incubated overnight at 4°C. After incubation with the primary antibodies, the films were washed 3 times with TBST and incubated with horseradish peroxidase coupled with secondary antibodies. The membranes were viewed using ECL (Medicalbio, PT01001) luminescence. The blots were quantitatively analyzed using the Image J (Huang et al., 2022).

Statistical analysis

The entire data were analyzed using Graphpad Prism 10.1.2. The data are expressed as means ± SD. One-way analyses of variance were employed to evaluate the differences between the groups. A p-value <0.05 was considered significant.

Results

LC-MS/MS analysis of total flavones

Using LC-MS/MS, 6 flavones were successfully identified from the *A. manihot*. The names with amounts within the brackets were hyperoside (4.9 mg/mL), quercetin-3-O-glucoside (2.4 mg/mL), isoquercitrin (5.1 mg/mL), hibifolin (4.6 mg/mL), quercetin (1.0 mg/mL), and myricetin (1.1 mg/mL) (Figure 1).

Effects of total flavones on palmitic acid-induced apoptosis of HK-2 cells

Apoptosis of renal cells is one of the significant etiologies of kidney injury. HK-2 cells were exposed to palmitic acid at different concentrations. The results revealed that as the concentration of palmitic acid ascended, cell viability decreased in a dose-dependent manner, suggesting that palmitic acid-induce injury to HK-2 cells. HK-2 cells were treated with total flavones of *A. manihot* at various concentrations. As the concentration increased, there was no statistical significance in the alteration of HK-2 cell viability, indicating that within a certain concentration range (<1600 mg/mL), the total flavones of *A. manihot* did not cause apparent damage to HK-2 cells. Finally, HK-2 cells were treated with total flavones of *A. manihot* to assess the impact on palmitic acid-induced cell death. The results of CCK-8 assay and flow cytometry demonstrated that total flavones of *A. manihot* attenuated palmitic acid-induced cell death (Figure 2).

Effects of total flavones on palmitic acid-induced lipid deposition in HK-2 cells

Lipid deposition in renal tubular cells constitutes one of the crucial pathological mechanisms of chronic kidney disease. Staining with oil red O and BODIPY probes indicates that treating HK-2 cells with 300 μM palmitic acid for 24 hours can induce significant intracellular lipid deposition. Total flavones of *A. manihot* attenuated palmitic acid-induced lipid deposition in HK-2 cells in a dose-dependent manner. In conclusion, total flavones of *A. manihot* ameliorate palmitic acid-induced apoptosis by inhibiting palmitic acid-induced intracellular lipid deposition (Figure 3).

Effects of total flavones on palmitic acid-induced lipid deposition in HK-2 cell

CD36 is a protein associated with fatty acid transport on the cell surface. The predominant fatty acid type it takes up is long-chain fatty acids, such as palmitic acid. The results of the western blot assay indicated that palmitic acid-induced an elevation in the expression of CD36 in HK-2 cells. Total flavones of *A. manihot* inhibited the intracellular lipid deposition resulting from the high expression of CD36 in palmitic acid-induced HK-2 cells in a dose-dependent manner (Figure 4).

Discussion

This study demonstrates that total flavones of *A. manihot* can safeguard HK-2 cells by diminishing intracellular lipid deposition. The underlying mechanism might be associated with the capacity of total flavones of *A. manihot* to restrain the expression of CD36 on the cell surface and decrease fatty acid uptake. Renal tubular epithelial cells are highly metabolically active and require a substantial amount of energy to maintain

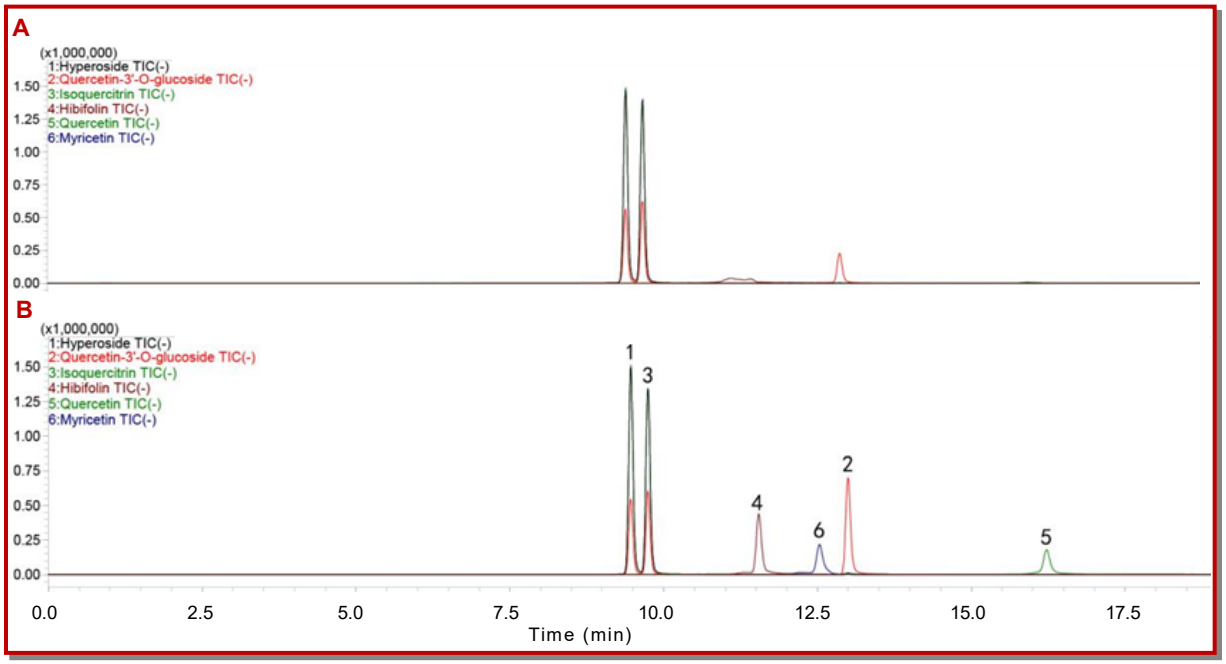

Figure 1: LC-MS/MS data showing peaks of total flavones of *A. manihot* (A); Mixed standards (B)

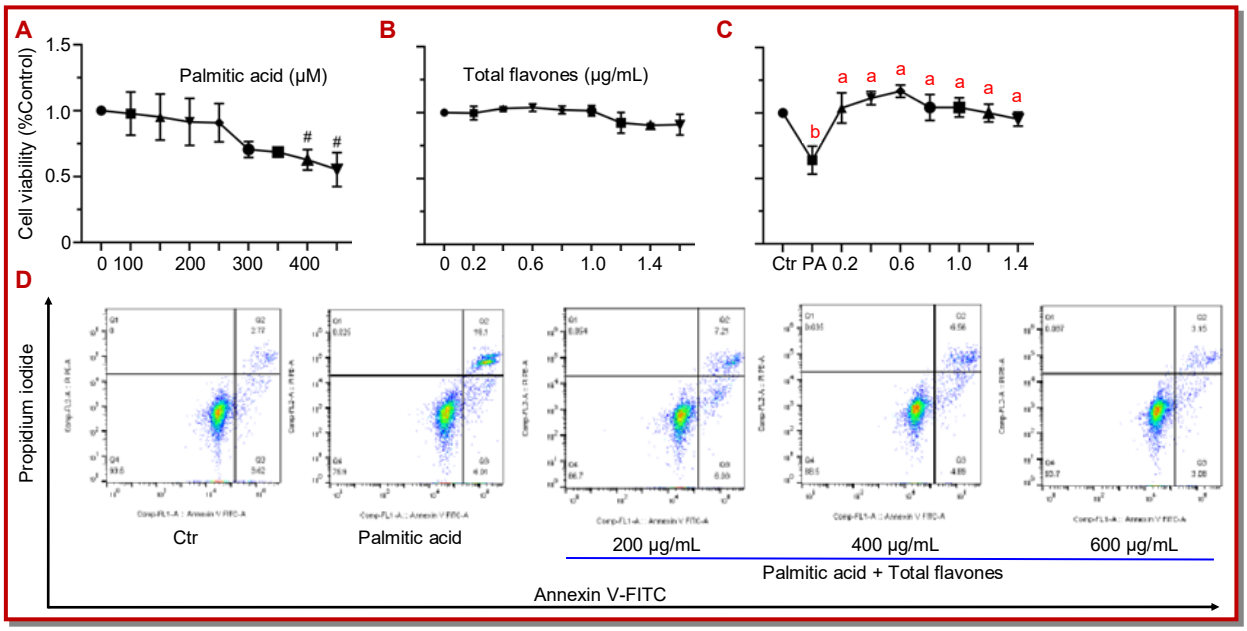

Figure 2: Total flavones of *A. manihot* improved the palmitic acid-induced cell damage *in vitro*. The impact of varying concentrations of palmitic acid (PA) and total flavones on the viability of HK-2 cells was investigated. Cells in 96-well plates were individually subjected to different levels of palmitic acid and total flavones for 24 hours. Subsequently, CCK-8 analysis was conducted to assess cell viability (A,B). Palmitic acid at a concentration of 300 μM was employed to induce damage in HK-2 cells. Simultaneously, different levels of total flavones were supplemented to the cells, which were then incubated for 24 hours. CCK-8 analysis was utilized to assess cell viability (C). Meanwhile, flow cytometry was used to evaluate the effect of total flavones of concentrations on cell apoptosis by labeling annexin V-analyst/propidium iodide (D). Data are means ± SD. [a]$p<0.01$ compared to the PA group. [b]$p<0.05$ compared to the control group.

normal functions. They primarily obtain energy by taking up long-chain fatty acids and generating ATP (Bhargava and Schnellmann, 2017). The control mechanism of fatty acid homeostasis in renal tubular epithelial cells is important (Stadler et al., 2015). In diabetic nephropathy, the free fatty acids in the renal tubular lumen increase (Weinberg, 2006), and the reabsorption of fatty acids by renal tubular epithelial cells is augmented, resulting in intracellular fatty acid overload. After fatty acid overload, it can induce dysregulation of lipid oxidation, manifested as incomplete fatty acid β-oxidation and fatty acid peroxidation. Accelerated incomplete fatty acid β-oxidation will lead to the accumulation of toxic lipid intermediates, causing apoptosis of renal tubular cells. Additionally, lipid peroxidation forms lipid free radicals, promotes endoplasmic reticulum stress and excessive production of reactive oxygen species, ultimately leading to cell dysfunction and death, exacerbating the progression of diabetic nephropathy (Milne et al., 2005; Morrow, 2005; Stadler et al., 2008; Stadler et al., 2015). *In vitro* studies have revealed that incubating human proximal tubular epithelial cells with palmitic acid can induce abnormal intracellular lipid accumulation, and the resulting lipotoxicity can cause damage to cell mitochondria, cytoskeleton damage, and cell apoptosis (Jiang, 2020). This is by the results of this study where palmitic acid causes intracellular lipid deposition in HK-2 cells and leads to cell death.

CD36 is predominantly involved in fatty acid uptake in the kidney. The expression of CD36 is closely related to metabolic disorders such as obesity, insulin resistance, impaired glucose tolerance, and fatty liver disease (Coburn et al., 2001; He et al., 2011; Kennedy and Kashyap, 2011), and CD36 is more closely associated with type 2 diabetes (Aitman et al., 1999). Similarly, studies have indicated that CD36 is highly expressed in renal tubular epithelial cells in diabetic kidney disease (Feng et al., 2017), and renal tubular epithelial cells rely on CD36 to take up fatty acids as the main energy source (Griffin et al., 2001; Liang et al., 2004; Sampson et al., 2003). Experimental investigations have shown that renal tubular epithelial cells that specifically overexpress human CD36 have increased intracellular lipid and triglyceride accumulation, and the main fatty acid subtype is long-chain fatty acids such as stearic acid and palmitic acid taken up by CD36. Increased expression of CD36 will mediate the sequential activation of src kinase, pro-apoptotic p38 MAPK, and caspase 3 in renal tubular epithelial cells, thereby inducing apoptosis of renal tubular epithelial cells (Hao et al., 2020; Susztak et al., 2005). In diabetic nephropathy, there is a strong correlation between the upregulated expression of CD36 and the increased lipid accumulation and apoptosis in renal tubular epithelial cells. Blocking CD36 can significantly inhibit CD36-dependent fatty acid uptake, intracellular lipid droplet growth, and weight gain in mice on a high-fat diet (Hao

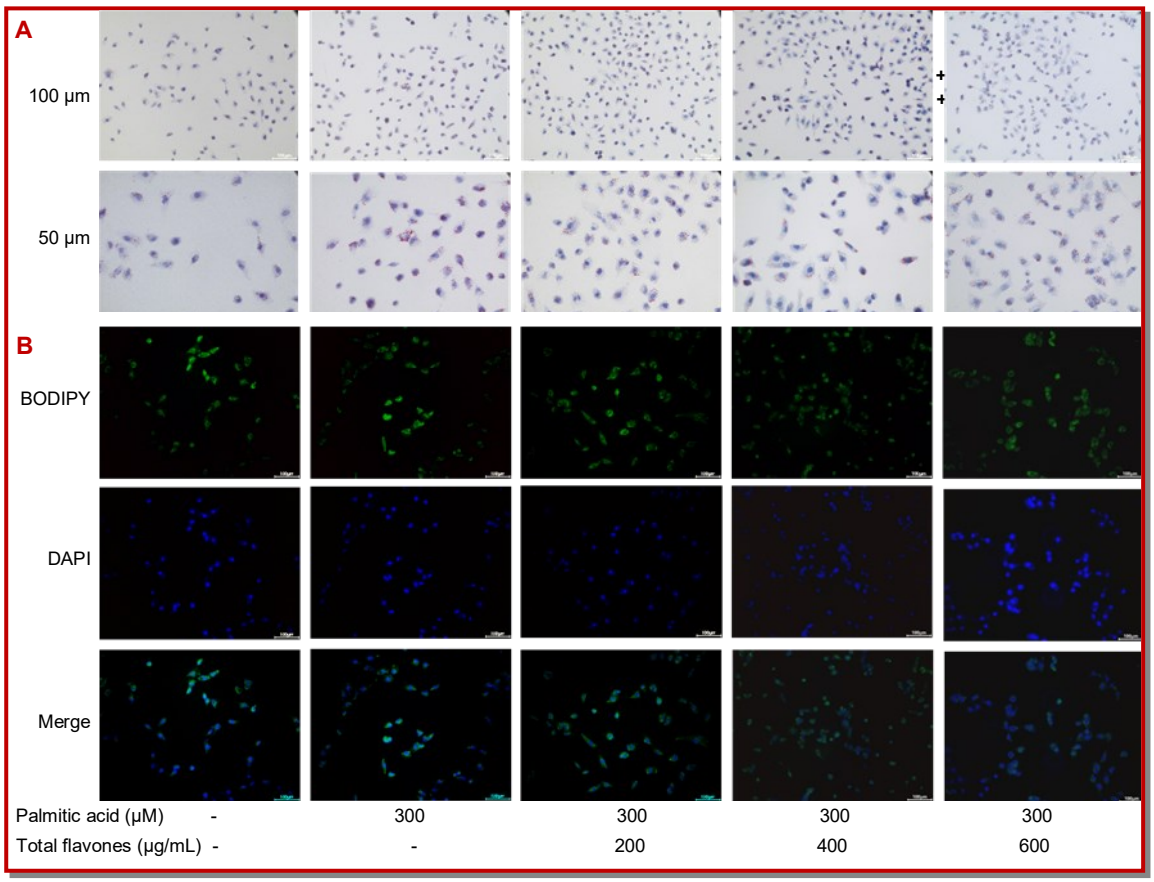

Figure 3: Palmitic acid at a concentration of 300 μM was employed to induce damage in HK-2 cells. Total flavones of *A. manihot* at different concentrations were simultaneously added to the cells and incubated for 24 hours. Oil red O was utilized to stain intracellular fat, and hematoxylin was used to stain the nucleus. The stained cells were observed under an optical microscope (at 200x, 400x; scale bar = 100 μm, 50 μm)(A). Intracellular fat was stained using the BODIPY probe, and the nucleus was stained with DAPI (B). The stained cells were examined using a fluorescence microscope (at 200x; scale bar = 100 μm)

et al., 2020; Heit et al., 2013).

Currently, studies have found that the main active constituents of *A. manihot* are flavonoids, mainly including hyperoside, isoquercitrin, quercetin monoglucuronide, quercetin-3'-O-glucoside, quercetin, and myricetin (Xue et al., 2011). This is consistent with the results of this study. Research has shown that total flavones of *A. manihot* can ameliorate lipid metabolism disorders by reducing endoplasmic reticulum stress, enhancing autophagic activity and regulating mitochondrial function, and reducing renal damage and lipid accumulation in patients with diabetic kidney disease (Ge et al., 2016; Li et al., 2016). This study mainly focuses on the protection of the kidney by total flavones of *A. manihot* through reducing lipid deposition. However, other pharmacological effects of total flavones of *A. manihot* require further evaluation.

Conclusion

Total flavonoids of *A. manihot* can decrease fatty acid uptake by inhibiting the expression of CD36, thereby mitigating intracellular lipid deposition and apoptosis in HK-2 cells induced by palmitic acid and providing a novel therapeutic avenue for ameliorating renal tubular injury in kidney diseases.

Financial Support

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Ethical Issue

Cell lines derived from expansion of primary cell cultures *in vitro* were not relevant material, as all of the original cells were divided and so the cell line had been created outside of the human body. The storage and use of cell lines created from primary human tissue, for research purposes, did not require an ethical approval.

Conflict of Interest

Authors declare no conflict of interest

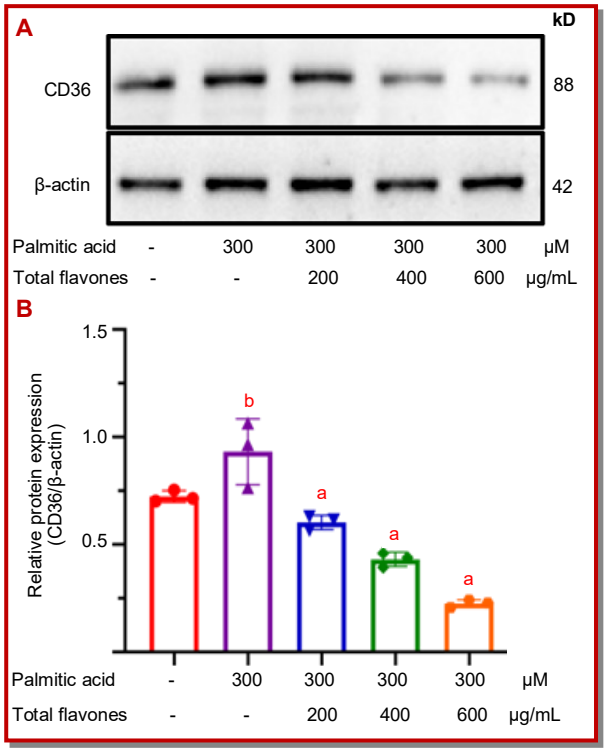

Figure 4: Palmitic acid (300 μM) was employed to induce damage in HK-2 cells (incubated for 24 hours). Total flavones of *A. manihot* at different concentrations were simultaneously added to the cells (incubated for 24 hours). The western blot assay demonstrated the expression of HK-2 cells in different groups (the control group, palmitic acid group, palmitic acid (300 μM) + total flavones of *A. manihot* at different concentrations) (A). Semi-quantitative optical density analysis was conducted using Image J (B). Data are means ± SD. [a]p<0.01 compared to the palmitic acid group. [b]p<0.05 compared to the control group

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Author Info
Enchao Zhou (Principal contact)
e-mail: zhouenchao@njucm.edu.cn
First two authors contributed equally