Effects and mechanism of Kudingcha on obesity

Xiaohui Sui
Shandong University of Traditional Chinese Medicine
Jia Liu
Qilu Hospital, Shandong University
Li Gong (38174606@qq.com)
Qilu Hospital, Shandong University
Lei Zhang
The First Affiliated Hospital of Shandong First Medical University&Shandong Provincial QianFoShan Hospital

Research Article

Keywords: Obesity, Network Pharmacology, Quercetin, Kaempferol, Kudingcha

Posted Date: November 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2225142/v1

Abstract

Obesity is the world's most high-profile public health problem. Kudingcha has been used in traditional medicine because of its antioxidant, anti-inflammatory and weight loss properties. Quercetin and kaempferol are the main components rich in Kudingcha that are effective against obesity. Therefore, we used the method of network pharmacology to study the effects of quercetin and kaempferol to HepG2 cells on obesity. We obtained 20 hub genes through PPI results and the Cytoscape plug-in cytoHubba. The top 10 genes were found eventrully by GO and KEGG enrichment analysis. The results showed that the insulin resistance, PI3K-Akt signaling pathways and adipocytokine signaling pathway might be the central pathways associated with the treatment of obesity by kudingcha. Next, we used Real-Time PCR to detect the expression levels of ISNR, SLc2a4 and APOE genes to study its mechanism. The results showed that compared with the blank group, the expression of ISNR and SLc2a4 increased and the expression of APOE decreased. Our experiments confirmed that Kudingcha contains compounds that can improve insulin resistance and obesity. This can provide a reference for further research and application of Kudingcha in the treatment of obesity.

Introduction

In the past three decades, the rate of obesity has shown a significant increasing trend, which is a common problem all over the world [1–2]. A large number of studies have shown that obesity is not only related to the occurrence of a variety of diseases, such as diabetes, hypertension, cardiovascular disease, chronic obstructive pulmonary disease, obstructive sleep apnea, kidney stones, sexual dysfunction and cancer [3–5], but also affects people's quality of life in social, family, psychological and other aspects[6–7]. Effective prevention and treatment of obesity is an important problem we are facing at present.

For hundreds of years, Kudingcha has been used in traditional medicine because of its antioxidant, anti-inflammatory and weight loss properties. Kudingcha is a plant belonging to the genus Ilex in the Ilex family. In China, it has a long drinking history as a substitute tea for natural plants. Modern pharmacological studies have found that Kudingcha contains triterpenoids, flavonoids, semi terpenoids, polyphenols, alkaloids and other substances, which have biological effects such as weight loss, hypoglycemia, blood lipid regulation, blood pressure reduction, cardiovascular and cerebrovascular protection, antioxidation and so on [8–10]. Therefore, we used the method of network pharmacology to predict and analyze the target and related mechanism of Kudingcha on obesity. Quercetin and kaempferol are the main components rich in Kudingcha that are effective against obesity. Next, we studied the effects of quercetin and kaempferol on HepG2 cells, so as to verify our prediction analysis.

This study further explores the pharmacological effects of Kudingcha, in order to provide a theoretical basis for Kudingcha to prevent and treat obesity from reducing the fat storage capacity of visceral adipocytes.

Methods

Prediction Of The Active Ingredients Of Kudingcha

To determine the chemical ingredients of kudingcha, we searched the Traditional

Chinese Medicine Systems Pharmacology (TCMSP) (http://tcmspw.com/tcmsp.php) and the Encyclopedia of Traditional Chinese Medicine (ETCM) databases (http://www.tcmip.cn/ETCM/). In this study, molecules with an oral bioavailability (OB) value ≥ 30% and drug-likeness (DL) index ≥ 0.18 were identified as meaningful active ingredients[11].

Prediction Of Targets Of Kudingcha

The active ingredients were analyzed using the TCMSP database to identify the known drug targets. The two-dimensional structure of the compound obtained from PubChem was imported into the SwissTargetPrecision database, and a threshold (probability > 0.6) was set to obtain a possible target for each combination[11].

Prediction Of Endometriosis Targets

The keyword “obesity” was searched in three different disease gene databases: (1) GeneCards Human Gene database (https://www.genecards.org/); (2) DisGeNET database (https://www.disgenet.org/home/); (3) Online Mendelian Inheritance in Man (OMIM) database (https://omim.org/). UniProt (http://www.UniProt.org) was used to obtain the gene symbols of all targets (obesity and kudingcha), and the information was used for subsequent network pharmacological data analysis[12].

Screening For Key Targets (Dup: Abstract ?)

The Venny R package was employed to map the targets of kudingcha and the known therapeutic targets of obesity to build a Venny diagram. We combined the crossed targets of kudingcha and obesity after deleting the duplicate targets of obesity. These cross targets were defined as critical targets for the treatment of obesity.

Drug-ingredient-target Interaction Network

We used the Cytoscape software (version 3.7.2, Boston, MA, USA) to construct the drug-ingredient-target interaction network.

Protein-protein Interaction (Ppi) Network Construction

The key targets were subjected to PPI analysis using the STRING database (https://string-db.org/), and the species "Homo sapiens" was selected to generate a PPIbnetwork. After processing the data by R

software (R version 4.1.2), we obtained the top 30 hub genes. Subsequently, we imported the hub gene data into the Cy software and obtained the HUB gene PPI network diagram[12].

Gene Ontology (Go) And Kyoto Encyclopedia Of Geld Genomes (Kegg) Analyses Of Hub Genes

We imported 30 critical genes into the Bioconductor library of R software to perform GO and KEGG pathway enrichment analyses. The screening criteria were set at $P < 0.05$, $Q < 0.05$, and the restricted species was Homo sapiens. GO and KEGG enrichment analyses revealed the top 15 results. Bar and bubble graphs represent the visualization, and a pathway atlas is presented for related pathways that meet clinical significance.

Cell Culture

The human liver cancer cell line HepG2 was used in this study. HepG2 is a hepatoma cell line, which has the fat storage capacity of hepatocytes and can form fat droplets that are easy to observe. The HepG2 cells were obtained from Dr Ying Zhang (Qilu Hospital). HepG2 cells were cultured in DMEM (Life Technologies, USA) containing 1% penicillin/streptomycin (Gibco, USA) and 10% FBS (Gibco, USA) in a humidified atmosphere containing 5% CO2 at 37°C. Cells were passaged when they reached 85% confluence and stored in liquid nitrogen for long-term preservation.

Cell Viability Assay (Dup: Abstract ?)

HepG2 cells were seeded in 96-well plates at a density of 5000 cells per well. When the confluence of cells was 80%, the culture medium was changed to DMEM medium containing 150μM palmitic acid (without FBS). Eight hours later, kaempferol or quercetin was added to the culture medium to make the concentration of quercetin or kaempferol 0, 2.5, 5, 10, 20 and 40 μmol /L, and the culture medium was cultured for 48 h. Then 10 μL solution of sterile Cell Counting Kit (CCK)8 (Bimake, USA) was added to each well and incubated for another 1.5h at 37°C. Absorbance was measured at 450 nm using universal microplate reader (ELx 800, BioTek Instruments, Winooski, VT). In order to exclude the influence of drug-induced cell death on the experiment, the concentration of two drugs with cell viability greater than 90% (IC10) was selected for further study.

Oil Red Staining

HepG2 cells were cultured for 8h in DMEM complete medium containing palmitic acid, and either kaempferol or quercetin. After 24h, discard the original culture medium, wash it with PBS for 3 times, put it into the fume hood to dry. And then, add 500μl of 4% paraformaldehyde to each hole, fix it for 20min, dye it with oil red O staining solution for 10-15min, cover the 24 hole plate during dyeing to prevent dye

precipitation caused by solvent volatilization. After that, wash the excess dye with 60% isopropanol, next wash it with PBS for 3 times. Take photos under 20 times magnification of the microscope. Add 300μl DMSO to each hole to make the oil red dye completely dissolved in DMSO, 100μl of the above solution was added to 96 well plate. Colorimetric absorbance at 450nm was measured by microplate reader.

Quantitativepcr(Qpcr)

Total RNA was extracted from HepG2 cells by using RNeasy mini Kit (RNA Fast 200, China). An amount of 1 μg RNA was reverse-transcribed into cDNA by using SureScript First-Strand cDNA Synthesis Kit (GeneCopoeia, USA). PCR amplification was performed by using the SYBR PCR mix (Takara Bio. Inc., Kyoto, Japan). The primers for APOE were 5'-CGAGGTGTAGGTTATGTTC – 3' (forward) and 5'-TACGCAACTTACGCAAAT-3' (reverse); primers for Slc2a4 were: 5'-CCTGCCCGAAAGAGTCTAAAGC-3' (forward); and 5'- CTAAGAGCACCGAG

ACCAACG-3'(reverse); primers for INSR were: 5'-ACGGTCAATGAGTCAGCCAG

TC-3' (forward) and 5'-ATCTCCATGAGCCATCAGTTCCA-3'(reverse). Primers for GADPH were: 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAA

GACGCCAGTGGA-3'(reverse).

Statistical Analysis

SPSS 22.0 was used to analyze the data. Quantitative data are presented as the mean ± SD of at least three independent experiments. One-way ANOV A were employed to compare the means. Differences were considered statistically significant if the p-value was < 0.05 or < 0.01.

Results

Establishment Of Flow Chart

For the convenience of understanding, we set up a flowchart according to the content of the whole article (Fig. 1).

The Candidate Ingredients And Targets Of Kudingcha

First, we screened the candidate ingredients according to the OB value and DL index conditions. Nine candidate ingredients of kudingcha were obtained from the TCMSP and ETCM databases, as shown in Table 1. According to the TCMSP, ETCM, and SwissTargetPrecision databases, we identified a total of 180 potential targets after deduplication. As shown in Fig. 2, we built an “herbal-compound-target” network diagram comprising 256 nodes and 889 edges.

Table 1
Main chemical composition.

Mol ID	Molecule Name	OB(%)	DL
MOL000211	Mairin	55.38	0.78
MOL000358	beta-sitosterol	36.91	0.75
MOL000363	amyrin Palmitate	32.68	0.3
MOL000422	kaempferol	41.88	0.24
MOL000492	(+)-catechin	54.83	0.24
MOL006504	(-)-Catechin gallate	53.57	0.75
MOL006542	(1R,2S,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-1,10-dihydroxy-1,2,6a,6b,9,9,12a-heptamethyl- 2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	35.46	0.73
MOL006554	Taraxerol	38.4	0.77
MOL000098	quercetin	46.43	0.28

Prediction Of Therapeutic Targets Of Obesity

We searched the GeneCards Human Gene Database, DisGeNET database, and Online Mendelian Inheritance in Man database for “Obesity”. A total of 430 targets were identified after deduplication.

Screening For Key Targets

Using the Venny R package, we eventually obtained 40 key targets to establish a Venn diagram as shown in Fig. 3.

Construction Of The Ppi Network And Selection Of Hub Genes

As depicted in Fig. 4A and Fig. 4B, we obtained the PPI network using the STRING database and the restricted species Homo sapiens. We obtained 20 hub genes through PPI results and the Cytoscape plug-in cytoHubba (Fig. 4C).

Go And Kegg Pathway Enrichment Analyses

We imported 20 critical genes into the Bioconductor library of the R software for GO and KEGG pathway enrichment analyses. The top 10 results, circle and bubble graphs were obtained (Fig. 5).

GO analysis revealed that negative regulation of lipid localization, negative regulation of lipid storage,cellular response to lipid may be vital biological processes related to the treatment of obestiy by kudingcha. KEGG analysis showed that the insulin resistance, PI3K-Akt signaling pathways and adipocytokine signaling pathway might be the central pathways associated with the treatment of obesity by kudingcha.

Establish A Cellular Model Of Obesity

A medium rich in palmitic acid was used to simulate the visceral high fat environment of obesity and stimulate the formation of lipid droplets in HepG2 cells. In order to select the suitable palmitic acid concentration, we used the oil red O staining to determine the fat-forming effect .

As shown in Fig. 6, we found that DMSO decolorization after cell oil red O staining in the environment of 150 μM palmitic acid, the absorbance reached the highest under microplate reader. Therefore, 150μM palmitic acid was selected for subsequent experiments.

Cell Viability Assay

Cell viability was assessed by (CCK)8 method .Various concentrations of quercetin or kaempferol without FBS were added into the culture medium to determine a suitable concentration for the model. As shown in Fig. 7, the highest concentration of the two drugs to make Hepg2 cell viability greater than 90% was about 5 μM. We eventually chose 5 μM quercetin and 5μM kaempferol for further experiments.

Effects Of Quercetin And Kaempferol

To determine the effects of quercetin and kaempferol on Hepg2 cells treated with palmitic acid, oil red O were used to stain cells. We found that no matter whether quercetin or kaempferol was intervened, the adipogenesis of cells was significantly lower than that of the control group, and the 5 μm quercetin + 5 μm kaempferol group had the least adipogenesis (Fig. 8).

The Mechanism Of Quercetin And Kaempferol Treated With Obesity

In order to study the mechanism of quercetin and kaempferol in the treatment of obesity, we used Real-Time PCR to detect the expression levels of ISNR, SLc2a4 and APOE genes. The results showed that compared with the control group, the expression of ISNR and SLc2a4 increased and the expression of APOE decreased (Fig. 9) in the three groups treated with quercetin and kaempferol, and the effects of the combination of quercetin and kaempfero were more obvious than that of the two groups treated alone.

Discussion

Kudingcha, as a daily drink of Chinese people, is often used as a traditional Chinese medicine or daily dietary supplement to reduce body weight and blood lipids[13]. However, its mechanism of action is still lacking in-depth research. Then, we used network pharmacology to analyze the main components of Kudingcha and its potential targets for obesity treatment, and constructed an "activeingredient-target-pathway" network to further investigate the mechanism of action. We screened two compounds in Kudingcha which were most closely related to obesity for adipogenesis assay in vitro. The effects of the two compounds on the expression of genes related to fat metabolism and insulin resistance were determined by RT-PCR.

Previous studies have shown that obesity is related to the abnormal expression of IL-6, AKT1, ADRB2[14–19].Interestingly, these three target proteins showed higher degrees in the protein protein interaction (PPI) network. The abnormal expression of IL6 is involved in adipose tissue and leads to glucose metabolism disorder and eventually leads to obesity[14–16]. It is reported that overexpression of Akt1 can promote adipogenesis[16–17]. Different polymorphic forms, point mutations, and downregulation of ADRB2 is associated with obesity[18–19]. Therefore, the compounds in Kudingcha may regulate the abnormal expression of IL-6,AKT1,ADRB2, thus improve abnormal metabolism, reduce fat accumulation and improve obesity.

The results of the KEGG enrichment analysis showed that the insulin resistance are the pathway with the highest enrichment. This result further confirms that Kudingcha can improve metabolism and obesity. In addition, we also enriched adipocytokine signaling pathway, FoXO signaling pathway and other signaling pathways related to obesity and fat accumulation in KEGG. The results of the GO enrichment analysis showed that negative regulation of lipid storage, negative regulation of lipid localization is the most enriched biological process, it also indicates that Kudingcha has a negative regulatory effect on lipid storage process.

In order to verify the results of network pharmacological analysis, we carried out experiments on it in vitro. Through PPI analysis, we know that the main targets are quercetin and kaempferol, the two most common flavonol glycosides. Previous studies have shown that quercetin and kaempferol play a significant role in anti-inflammation, anti-tumor and reducing blood lipids[20–21]. Therefore, we chose these two monomers to verify the positive effect of Kudingcha on obesity. We simulated the hyperlipidemic environment with palmitic acid to form fat droplets in Hepg2 cells, which represented the fat accumulation process. Two kinds of monomers were added to intervene and observed with oil red o staining. DMSO was used to extract color dyes for quantitative analysis. The results showed that the two drug monomers stimulated the cells to form fewer fat droplets, lower absorbance after oil red o staining, and the combination of the two drugs. Compared with single compound, the intracellular fat droplets were less and the absorbance was lower after 24 hours of culture. This proves that the two monomer components can inhibit lipidstorage, and the joint use of the two components makes the effect of inhibition lipid storage more obvious.

In order to further understand the mechanism of compounds in Kuding tea in improving obesity, we used RT-PCR to detect the changes of fat metabolism related gene RNA. The results showed that the expression of APOE increased in quercetin or kaempferol group. APOE is an enzyme involved in activating fat hydrolysis. Some studies have shown that the lack of APOE can lead to lipid metabolism disorders leading to elevated blood lipids and fat deposition, which may be one of the causes of obesity[22–23]. Insulin resistance is another important cause of obesity[24]. The decrease of ISNR and SLc2a4 is a sign of insulin resistance, which leads to impaired glucose transport and ultimately obesity[25–26]. After 5 μm kaempferol treatment, both ISNR and SLc2a4 increased significantly compared with the control group. However, there was no significant change in group which treated with 5 μm quercetin compared with the control group. So we examined the 5 μm quercetin + 5μ m

kaempferol treatment group. Surprisingly, when quercetin was used in combination with kaempferol, ISNR and SLc2a4 increased significantly compared with the control group, and the trend was more significant than that of kaempferol alone. This may indicate that quercetin can enhance the effect of kaempferol on insulin resistance. Therefore, our experiments show that Kudingcha contains compounds that can improve insulin resistance and obesity. When quercetin and kaempferol were used together, the above results were more significant than those used alone. This can provide a reference for further research and application of Kudingcha in the treatment of obesity.

Declarations

Ethics approval and consent to participate

The study protocols were performed according to Declaration of Helsinki.

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We have contacted Kanehisa Laboratories. We do not directly use these KEGG Pathway map “images” in the article, we need not obtain copyright permission of KEGG. However, they believe that we have written our article using their data, they kindly ask us to cite the following articles in our article [27–29].

Consent for publication

Not applicable.

Availability of data and materials

All data and materials that support the findings of this study are included in this article. If the readers need further datasets analyzed during the current study, it is available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

This research is supported by grants from the China Postdoctoral Science Foundation (2019M652410).

Authors' contributions

Xiaohui Sui conceived and designed the study; Jia Liu analyzed the data; Lei Zhang wrote the manuscript; Li Gong and Lei Zhang supervised the whole process. All authors reviewed the manuscript.

Acknowledgements

We thank everyone who contributed to this article.

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Figures

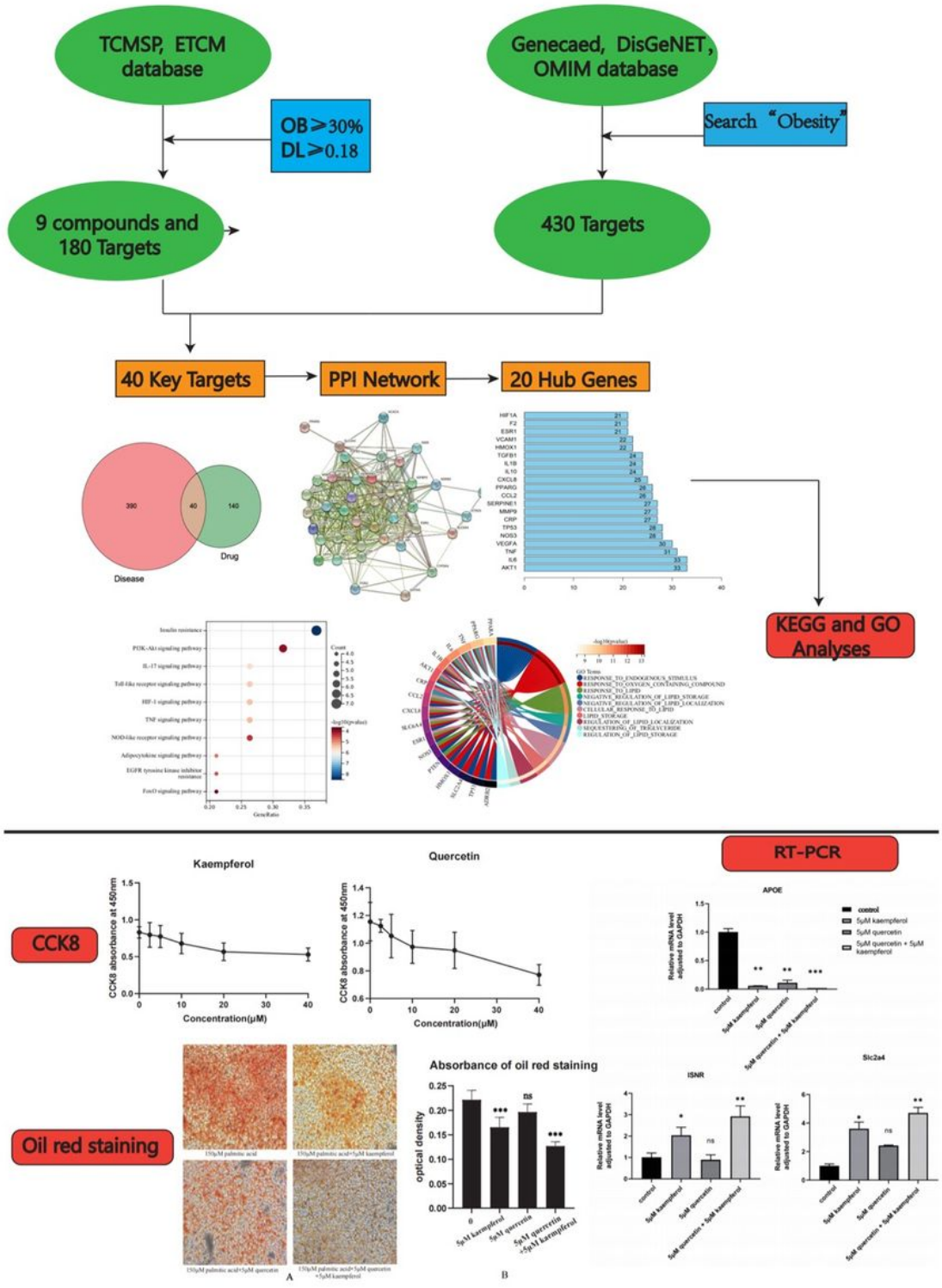

Figure 1

The flow chart of the study.

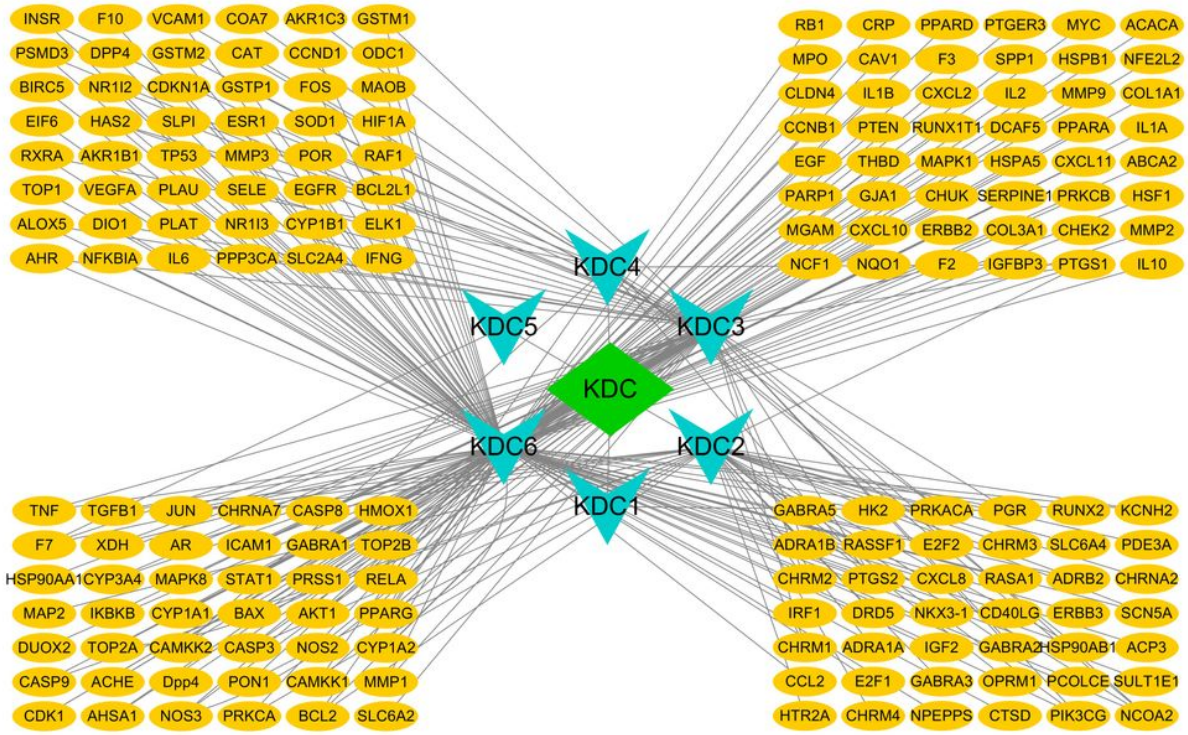

Figure 2

Kudingcha-compound-target genes network. Green nodes represent kudingcha, yellow nodes represent target genes, and blue nodes represent six kinds of compounds in kudingcha. KDC1 is Mairin, KDC2 is beta-sitosterol, KDC3 is kaempferol, KDC4 is (+)-catechin, KDC5 is (-)-catechin gallate, and KDC6 is quercetin.

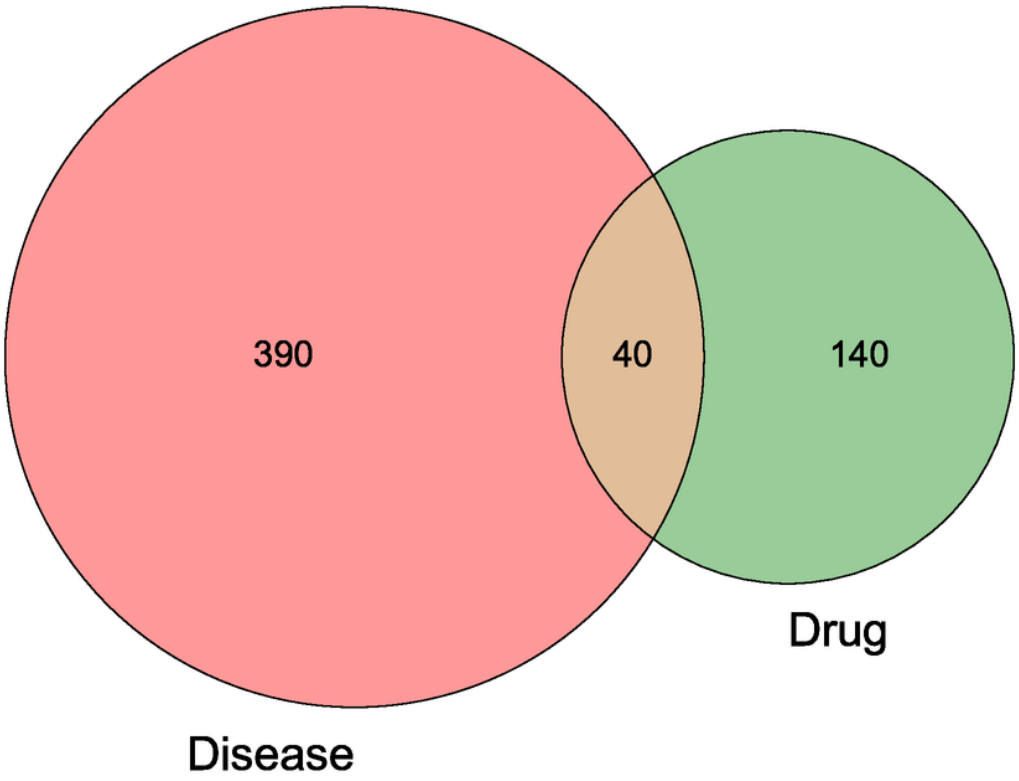

Figure 3

Venn diagram of targets of active ingredients of kudingcha and those related to obesity.

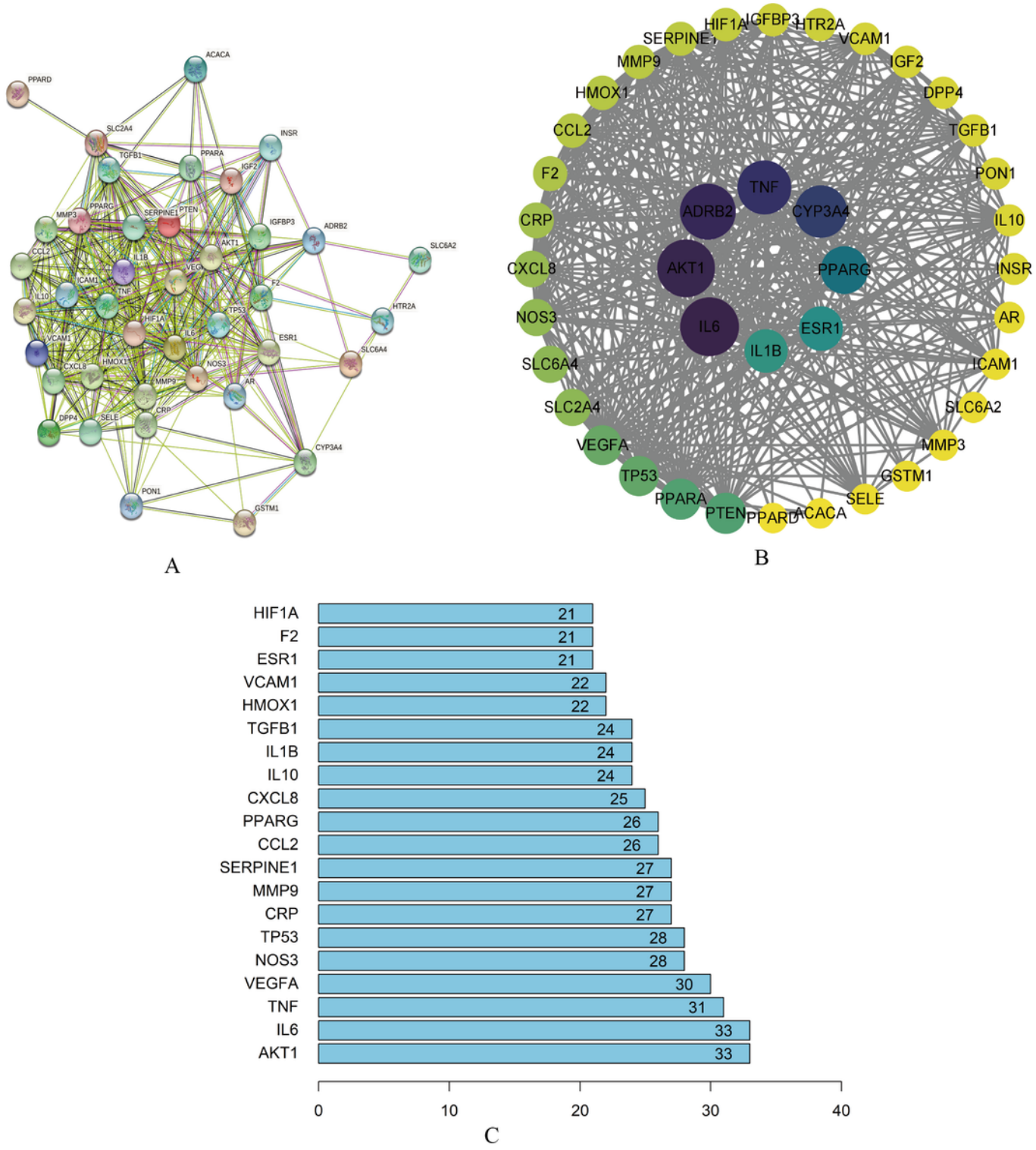

Figure 4

Construction of the PPI network and selection of HUB genes.

(A) Protein-protein interaction (PPI) network of the common targets of active ingredients of kudingcha and obesity. (B) Hub gene interactions in the protein-protein interaction (PPI) network: 40 significant hubs according to the degree value; from large to small, the color gradually changes from purple to green. (C) 20 significant hubs and degree value.

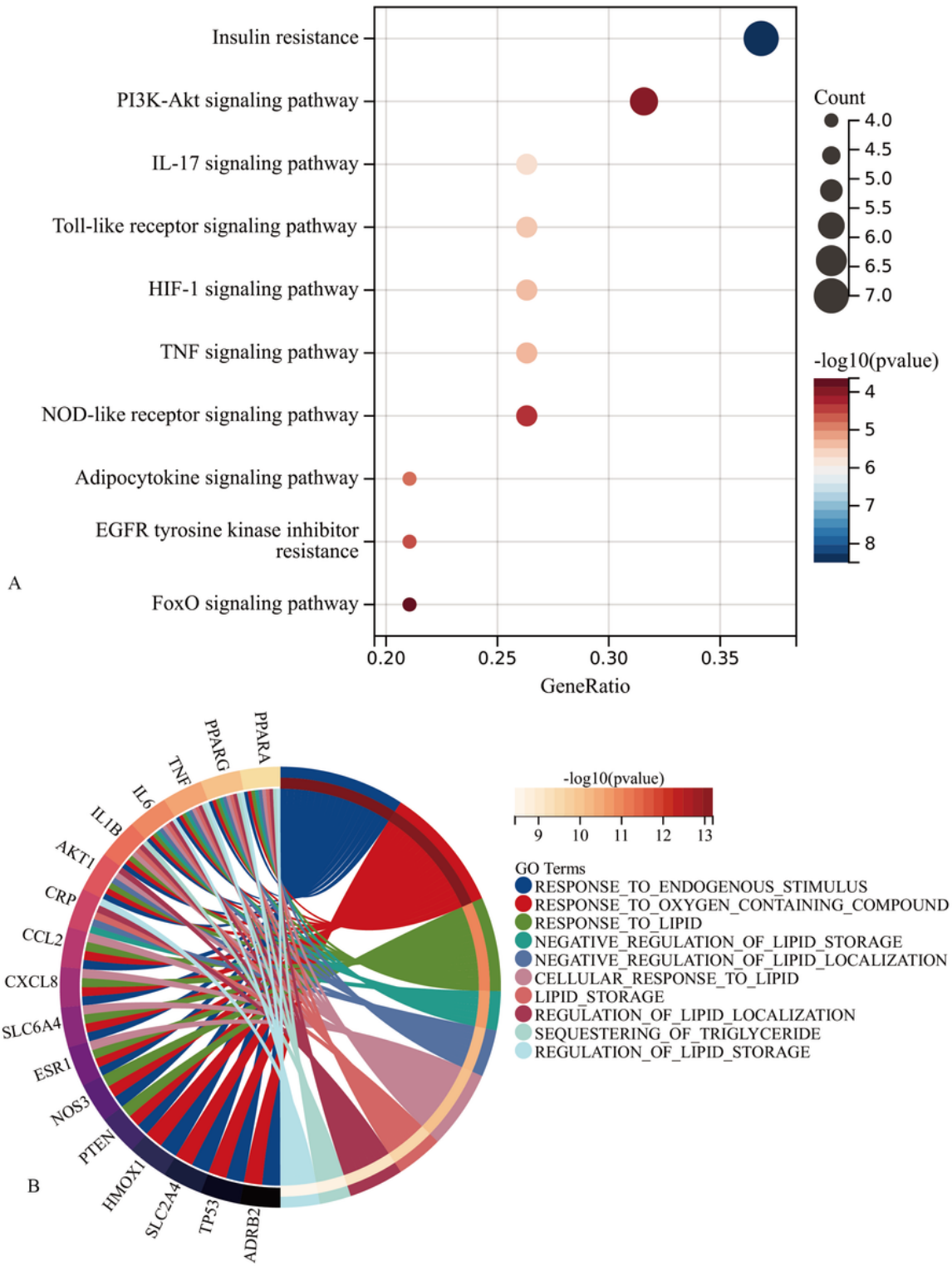

Figure 5

KEGG and GO analyses of the 20 hub genes. (A) KEGG analyses of the 20 hub genes. (B) GO analyses of the 20 hub genes.

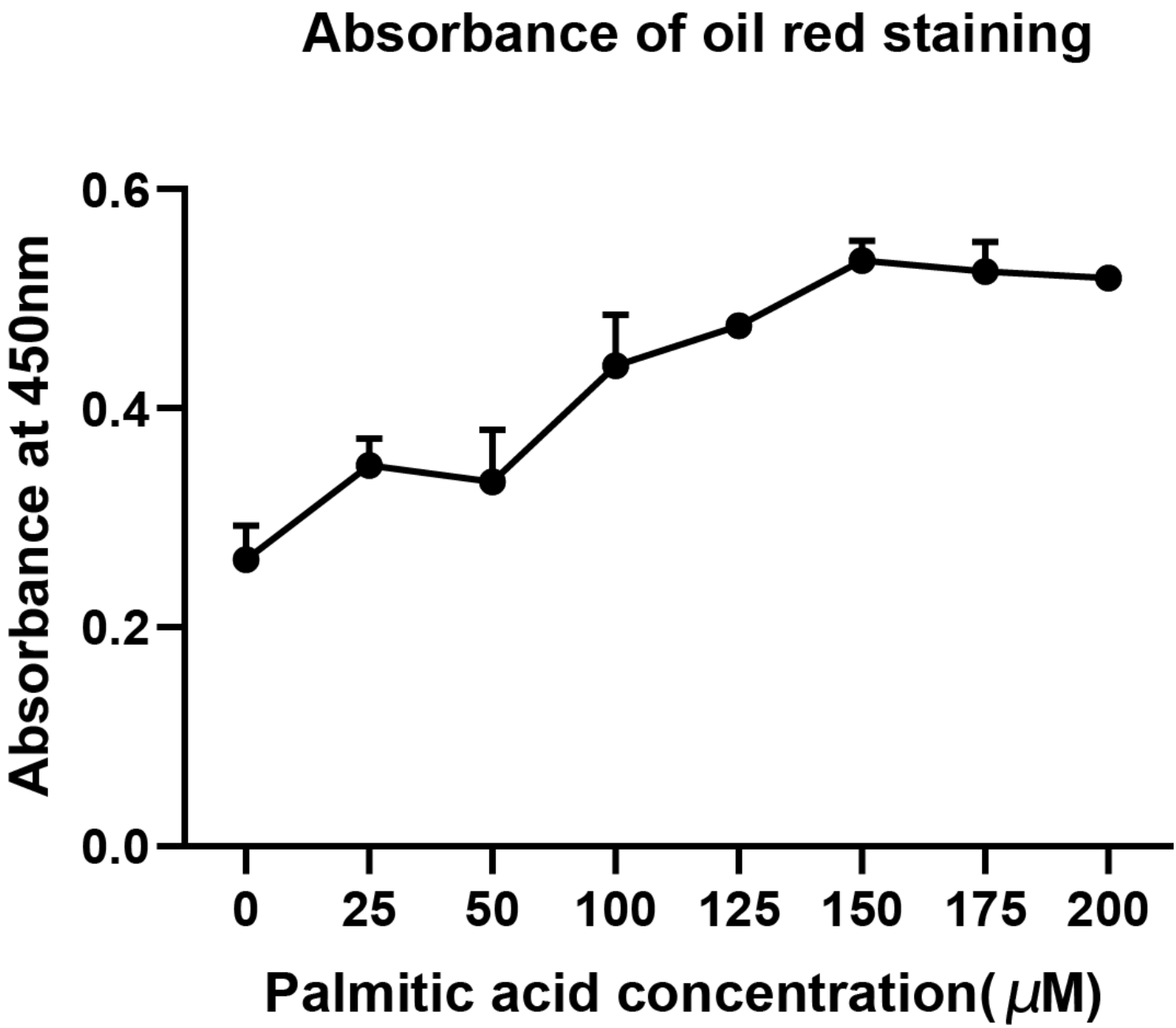

Figure 6

Changes of oil Red o staining absorbance of HepG2 cells cultured with different concentrations of palmitic acid. Data represent means ± SD, *n*=3.

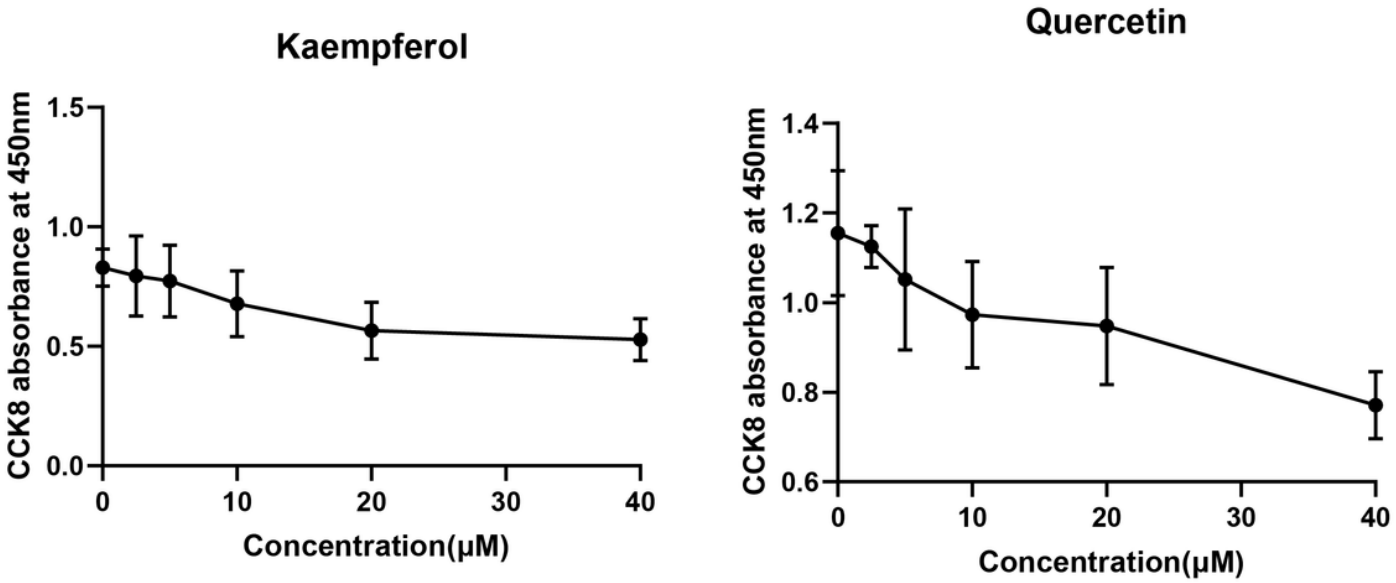

Figure 7

Effect of quercetin or kaempferol at different concentrations on the absorbance of CCK8 at 450nm. Data represent means ± SD, *n*=3.

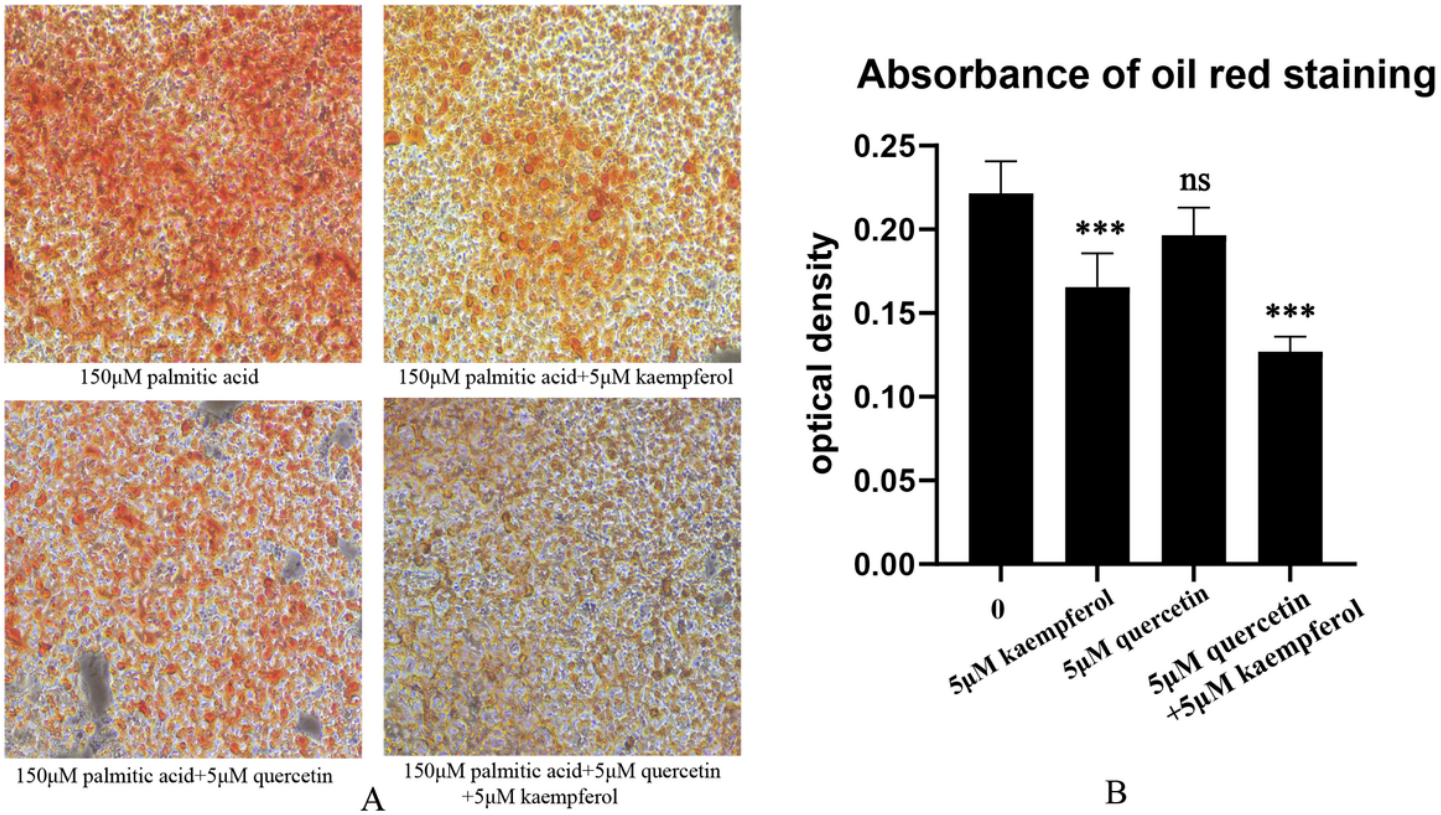

Figure 8

Effects of quercetin and kaempferol on Hepg2 cells .

(A) Cells of different treatment groups were stained with oil red at 20 times magnific-

ation. (B) The absorbance of the cells in different treatment groups was determined by oil red staining spectrophotometer. Data represent means ± SD, n=3. ***P<0.001 versus the control group, ns: P>0.05 versus control group.

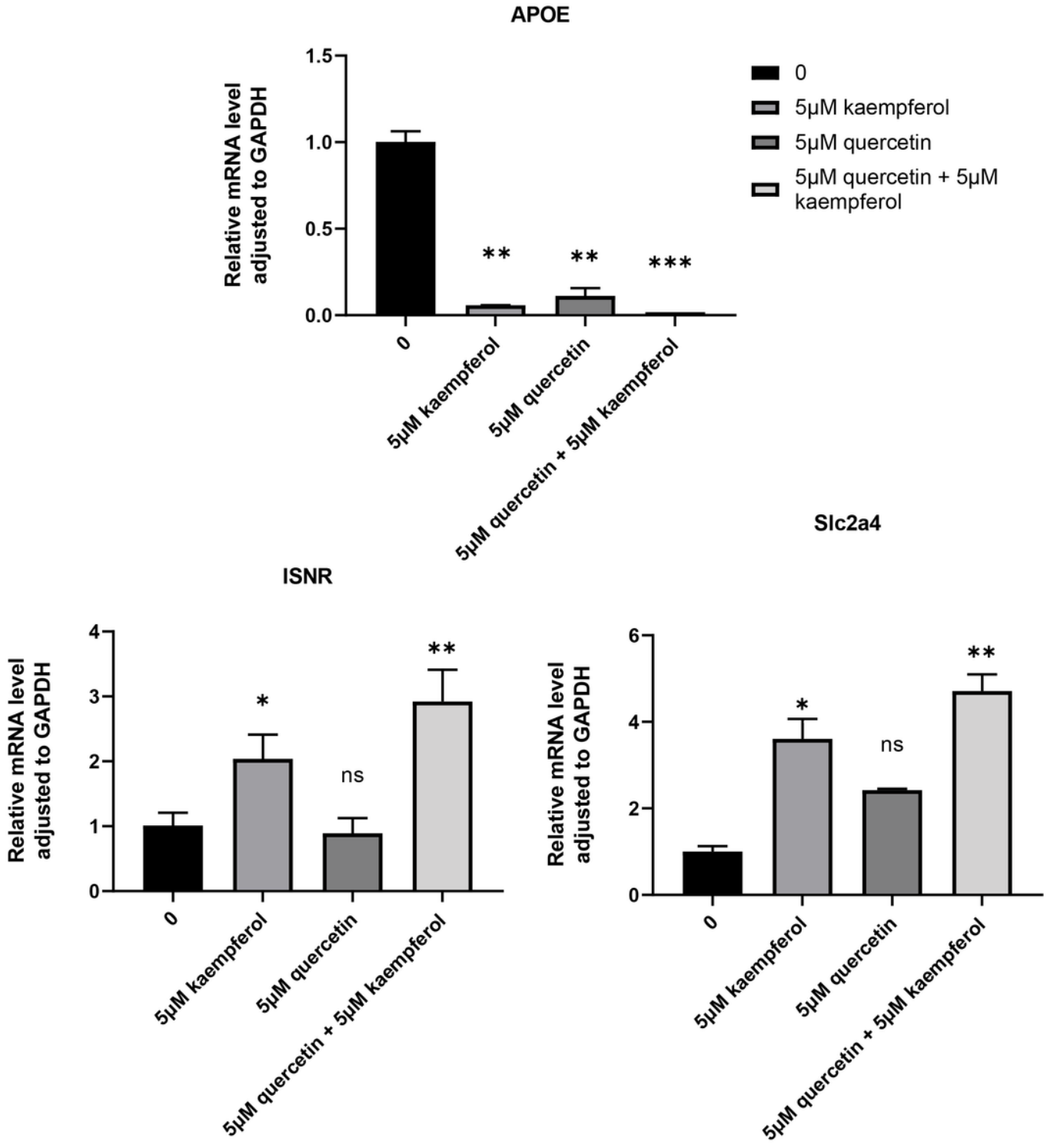

Figure 9

Levels of ISNR, SLc2a4 and APOE genes were changed by quercetin and kaempferol. Data represent means ± SD, *n*=3. **P*<0.05 versus control group, ***P*<0.01 versus the control group, ns: *P*>0.05 versus control group.