Article

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Effects of Four Main Active Flavonoids of *Coreopsis tinctoria* Nutt. on Oleic Acid-Induced Lipid Metabolism and Oxidative Stress in HepG2 Cells

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Background: Traditional Chinese medicines exhibit tremendous beneficial effects on the control of hyperlipidemia and hyperlipidemia-associated disorders. In the present study, we investigated the effects of four *Coreopsis tinctoria* Nutt. extracts, including luteolin, marein, naringenin (NGN) and chlorogenic acid (CQA), on lipid accumulation and oxidative stress induced by oleic acid (OA) in HepG2 cells.
Methods: Oleic acid was employed to create a high-lipid milieu in a cellular setting *in vitro* using HepG2 cells. After treatment by luteolin, marein, NGN, and CQA, cell counting kit-8 assay was used for measuring cell viability. Lipid accumulation, lipid metabolism and oxidative stress were examined by means of enzyme-linked immunosorbent assay, Oil red O staining, quantitative real-time polymerase chain reaction (qRT-PCR) and 2′,7′-dichlorodihydro fluorescein diacetate assays. Western blot and qRT-PCR assays were applied to determine the expression of genes and proteins, respectively.
Results: In OA-treated HepG2 cells, the administration of the four active flavonoids of *Coreopsis tinctoria* Nutt. (luteolin, marein, NGN and CQA) enhanced cell viability ($p < 0.05$ or $p < 0.01$); reduced lactate dehydrogenase releasing, lipid deposition and production of triglyceride, total cholesterol and low-density lipoprotein-cholesterol ($p < 0.05$ or $p < 0.01$); and elevated high-density lipoprotein-cholesterol production ($p < 0.05$ or $p < 0.01$ or $p < 0.001$). Moreover, after luteolin, marein, NGN or CQA treatment, the expression of lipid metabolism-related genes including 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), low-density lipoprotein receptor (*LDLR*) and apical sodium-dependent bile acid transporter (*ASBT*) was downregulated ($p < 0.01$ or $p < 0.001$) but the expression of cytochrome P450 family 7 subfamily A member 1 (*CYP7A1*) was upregulated ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) in OA-treated HepG2 cells. Similarly, luteolin, marein, NGN or CQA treatment greatly enhanced the anti-oxidant activities ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) and decreased reactive oxygen species production ($p < 0.01$ or $p < 0.001$) in OA-treated HepG2 cells. Sterol regulatory element-binding protein, a major transcription factor that moderates the biosynthesis of fatty acid, cholesterol and triglyceride, was also inhibited after luteolin, marein, NGN or CQA treatment ($p < 0.05$ or $p < 0.01$ or $p < 0.001$).
Conclusion: These findings demonstrated that luteolin, marein, NGN or CQA can effectively reduce OA-induced oxidative stress and lipid accumulation, corroborating their potential in hyperlipidemia treatment.

Keywords: hyperlipidemia; lipid metabolism; oxidative stress; sterol regulatory element-binding proteins

Introduction

Hyperlipidemia is a pathological condition caused by dysregulated lipid metabolism, marked by the increase of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) and the decrease of high-density lipoprotein cholesterol (HDL-C) in plasma [1, 2]. According to epidemiological studies, hyperlipidemia stands as one of the risk factors of liver disease, kidney disease and cardiovascular disease such as atherosclerosis and aortic valve disease [3–5]. Statins remain the major treatment of hyperlipidemia, contributing to the significant reduction in the morbidity and mortality caused by metabolic disease and cardiovascular disease. However, drug resistance, lack of adherence and adverse events limit the application of statins in hyperlipidemia treatment [6–8]. Therefore, developing safe and effective hyperlipidemia treatments is an important clinical imperative.

A growing body of studies has revealed that traditional Chinese medicines (TCMs) present immense cura-

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tive effects in the treatment of hyperlipidemia-associated disorders by modulating lipid metabolism [9–11]. TCMs or their extracts boast widespread clinical applications due to low toxicity and definite curative effects [12]. For instance, curcumin, an extract of *Curcuma longa* Linn., could greatly improve fat deposition in hepatic tissues and hyperlipidemia in high-fructose-fed rats [13]. In non-alcoholic fatty liver disease rat model, extracts from lotus leaf, Notoginseng, Danshen and hawthorn have been shown to effectively ameliorate liver damage and lipid profiles [14]. Wang *et al*. [15] reported that *Astragalus membranaceus* regulates lipid metabolism by targeting cyclin D1, vascular endothelial growth factor A, and serine/threonine kinase 1, thus improving acquired hyperlipidemia.

Coreopsis tinctoria Nutt. is a medicine-food plant that has been traditionally used to treat hyperlipidemia, diabetes and hypertension. It has been reported that *Coreopsis tinctoria* Nutt. is rich in amino acids, volatile oils, flavonoids and other active compounds [16,17]. The extracts of *Coreopsis tinctoria* Nutt. can effectively reduce serum levels of TC, LDL and TG in mouse models of type 2 diabetes [18]. Nevertheless, the pharmacological mechanisms of the active compounds of *Coreopsis tinctoria* Nutt. underlying the improvement of hyperlipidemia and hyperlipidemia-related disorders remains to be elucidated. As disclosed in the literature, luteolin, marein, naringenin (NGN) and chlorogenic acid (CQA) are main active compounds of *Coreopsis tinctoria* Nutt. [17,19]. In the current study, we aimed to investigate the role of luteolin, marein, NGN and CQA in improving oxidative stress and lipid excess induced by oleic acid (OA) in HepG2 cells.

Sterol regulatory element-binding proteins (SREBPs), which are the major basic-helix-loop-helix leucine zipper transcription factors, play a crucial role in regulating fatty acids synthesis, cholesterol synthesis, oxidative stress and lipid metabolism [20]. Mammalian SREBPs include SREBP-1a, SREBP-1c and SREBP-2. The expression of these SREBPs and their activity can be inhibited by the induced AMP-activated protein kinase (AMPK) [21,22]. According to several reports, marein could reduce lipid deposition by inhibiting AMPK signaling, while NGN and CQA could inhibit SREBPs expression [23–25]. Lim *et al*. [26] indicated that luteolin inhibits SREBPs signaling pathway. These reported findings all point to a possibility that these *Coreopsis tinctoria* Nutt. extracts have an inhibitory effect on lipid accumulation and lipid metabolism-associated protein expression in OA-treated liver cells by inhibiting SREBPs signaling.

In this study, we explored the roles and mechanisms of the four extracts of *Coreopsis tinctoria* Nutt., namely luteolin, marein, NGN and CQA, on oxidative stress, lipid metabolism and lipid accumulation in OA-treated HepG2 cells, thus providing experimental basis for exploring the efficacy of *Coreopsis tinctoria* Nutt. in the treatment of hyperlipidemia and related disorders.

Materials and Methods

Cell Culture

HepG2 cells (SCSP-510), obtained from Shanghai Cell Bank Type Culture Collection Committee (Shanghai, China), were first identified by means of short tandem repeat (STR) profiling. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; D5030, Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (16000044, Gibco, Grand Island, NY, USA) in an incubator infused with 5% CO_2 and set at 37 °C. No mycoplasma infection was detected in routine mycoplasma test.

Administration of HepG2 Cells

HepG2 cells were initially treated with various concentrations of luteolin (0, 5, 10, 20, 30, 40 µM), marein (0, 0.1, 1, 5, 10, 20 µM), CQA (0, 50, 100, 200, 300, 500 µM), and NGN (0, 25, 50, 100, 200, 300 µM) for 24 h to determine the optimal doses. To determine the optimal dosage of OA for cell treatment, a range of concentrations was utilized: 0, 0.1, 0.2, 0.5, 1.0, 1.5, or 2.0 mM. Subsequently, the HepG2 cells were treated with the optimal dose of OA to mimic cell damage seen in hyperlipidemic milieu. Then, the cells were allocated into 6 groups designed for exposure to different treatments: control group (HepG2 cells without any treatment), OA group (HepG2 cells treated with 2.0 mM OA for 24 h), OA + luteolin group (OA-induced HepG2 cells treated with 30 µM luteolin for 24 h), OA + marein group (OA-induced HepG2 cells treated with 10 µM marein for 24 h), OA + CQA group (OA-induced HepG2 cells treated with 300 µM CQA for 24 h), OA + NGN group (OA-induced HepG2 cells treated with 200 µM NGN for 24 h).

CCK-8 Assay and Lactate Dehydrogenase (LDH) Measurement

The CCK-8 assay was used to assess the potential cytotoxic effects of the flavonoids at various concentrations on HepG2 cells, a step crucial for determining the optimal dosages for further experiments. After plating a 96-well plate with 5×10^3 HepG2 cells per well, each well was supplemented with 10 µL of cell counting kit-8 solution (CCK-8, BC0685; Solarbio, Shanghai, China) and incubated for 1 h at 37 °C under the atmosphere of 5% CO_2. Then, a microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, Waltham, MA, USA) was employed to determine the absorbance at 450 nm.

The LDH assay is a reliable method for detecting cytotoxicity that results in the loss of cell membrane integrity and identifying cytotoxic effects of the flavonoids at various concentrations on HepG2 cells. After treatment, the cell supernatants were harvested to assess the LDH level using an LDH enzyme-linked immunosorbent assay (ELISA) kit (SP10961, Wuhan Saipei Biotechnology Co., Ltd., Wuhan,

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Table 1. Sequences of primers used in qRT-PCR.

Gene	Primer sequences	
	Forward (5′–3′)	Reverse (5′–3′)
HMGCR	TGATTGACCTTTCCAGAGCAAG	CTAAAATTGCCATTCCACGAGC
LDLR	CAGCTACCCCTCGAGACAGA	CACTGTCCGAAGCCTGTTCT
CYP7A1	GAGAAGGCAAACGGGTGAAC	GGATTGGCACCAAATTGCAGA
ASBT	GGCCTTGGTGATGTTCTCCA	GAGTAACCCGCCACAGGAAA
SREBP1	TTCGCTTTCTGCAACACAGC	AAGGAGACGAGCACCAACAG
SREBP2	CCTCACCACCCCTATCCAGA	ATAGAGGGCTTCCTGGCTCA
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT

Note: qRT-PCR, quantitative real-time polymerase chain reaction; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase ; *LDLR*, low-density lipoprotein receptor; *CYP7A1*, cytochrome P450 family 7 subfamily A member 1; *ASBT*, apical sodium-dependent bile acid transporter; *SREBP1*, sterol regulatory element-binding protein 1; *SREBP2*, sterol regulatory element-binding protein 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

China) according to the manufacturer's protocol. A microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, Waltham, MA, USA) was applied to measure the absorbance at 450 nm.

Oil Red O Staining

The cells in each group were rinsed with phosphate-buffered saline. Next, upon fixing with 4% paraformaldehyde at ambient temperature for 30 min, the cells were treated with Oil red O solution (dissolved in 400 µL isopropanol; G1262, Solarbio, Beijing, China) for 15 min. Then, a light microscope (DM3000, Leica, Wetzlar, Germany) was used to photograph the stained cells at 200× magnification.

Measurement of Lipid Profiles

Treated HepG2 cells were obtained for lipid profile measurements. The expression levels of TG, TC, LDL-C or HDL-C were examined by TG content assay kit (BC0625, Solarbio, Beijing, China), TC content assay kit (BC1985, Solarbio, Beijing, China), LDL-C (BC5335, Solarbio, Beijing, China) or HDL-C (BC5325, Solarbio, Beijing, China), respectively. All experiments were performed in accordance with the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction Assay

TRIzol reagent (9108, TaKaRa, Tokyo, Japan) was applied to isolate the HepG2 cells to obtain the total RNA. Then, PrimeScript RT Master Mix transcription kit (RR036A, TaKaRa, Tokyo, Japan) was employed to reverse-transcribe total RNA into complementary RNA (cDNA). Afterward, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted with SYBR Green reagent (RR420, TaKaRa, Tokyo, Japan), and gene expression was measured using Sequence Detection System (ABI prism 7700, Applied Biosystems, Waltham, MA, USA). All steps were performed according to the manufacturer's instructions. A $2^{-\Delta\Delta Ct}$ method was utilized to quantify the gene expression. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as loading control. Primer sequences of genes measured in this experiment are given in Table 1.

Western Blot Assay

Radioimmunoprecipitation assay (RIPA; P0013B, Beyotime Biotechnology, Shanghai, China) was employed to extract total proteins from the HepG2 cells, followed by quantification using bicinchoninic acid assay (BCA) kit (P0012, Beyotime Biotechnology, Shanghai, China). Then, upon the separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred from gel to polyvinylidene fluoride (PVDF) membranes. After blocking, primary antibodies were added for incubation overnight at 4 °C. The primary antibodies used included anti-3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, 1:1000, ab242315, Abcam, Cambridge, MA, USA), low-density lipoprotein receptor (LDLR, 1:1000, ab30532, Abcam, Cambridge, MA, USA), SREBP1 (1:1000, ab28481, Abcam, Cambridge, MA, USA), SREBP2 (1:1000, ab30682, Abcam, Cambridge, MA, USA) or β-actin (1:2000, ab8226, Abcam, Cambridge, MA, USA). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:2000, ab288151 or ab97040, Abcam, Cambridge, MA, USA) at ambient temperature for 1 h. Ultimately, protein bands were developed using enhanced chemiluminescence (RPN2105, Amersham, Arlington Heights, IL, USA) and analyzed by Image J software (version 1.8, LOCI, University of Wisconsin, Madison, WI, USA).

Detection of Reactive Oxygen Species

The intracellular reactive oxygen species (ROS) level in HepG2 cells was determined by using 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA) probe

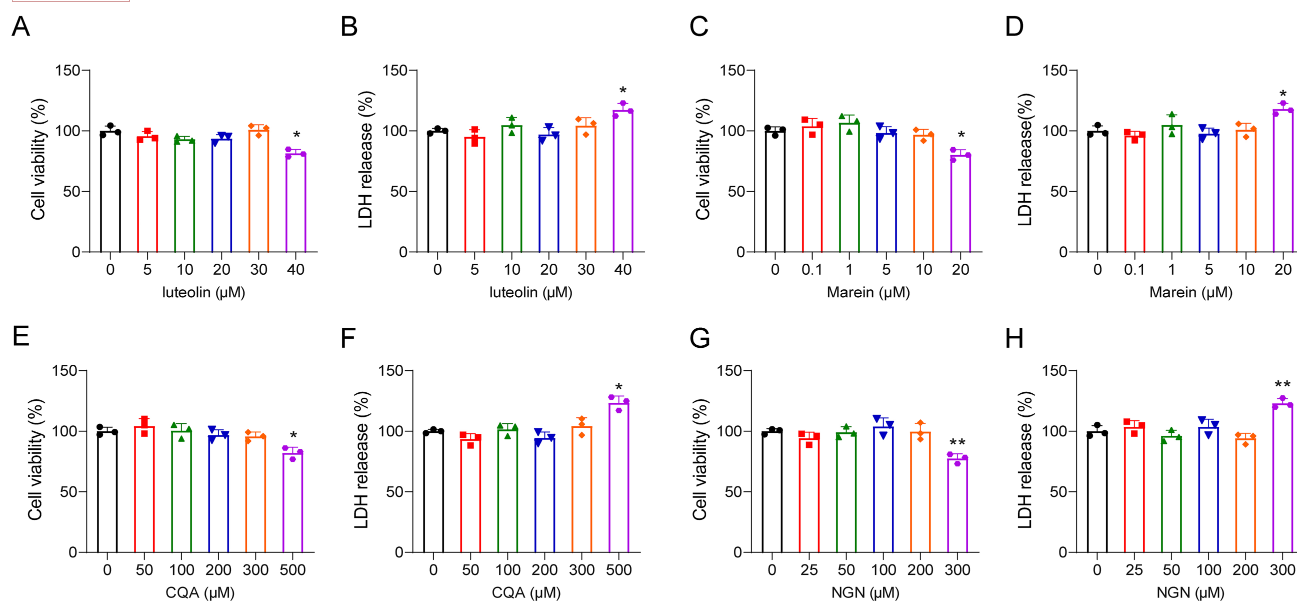

Fig. 1. Cytotoxic effects of *Coreopsis tinctoria* Nutt. extracts on HepG2 cells. (A,B) HepG2 cells were treated with 0, 5, 10, 20, 30 or 40 µM of luteolin for 24 h. CCK-8 assay was employed to measure cell ability, and LDH level was determined by ELISA. (C,D) HepG2 cells were treated with 0, 0.1, 1, 5, 10 or 20 µM of marein for 24 h. Then, cell viability was determined by CCK-8 assay, and LDH level was determined by ELISA. (E,F) HepG2 cells were treated with 0, 50, 100, 200, 300 or 500 µM of CQA for 24 h. Cell viability was measured using CCK-8 assay, and LDH level was determined by ELISA. (G,H) HepG2 cells were treated with 0, 25, 50, 100, 200, or 300 µM of NGN for 24 h. Cell viability and LDH level was determined using CCK-8 assay and ELISA, respectively. Data are expressed as mean ± standard deviation. N = 3 per group, *$p < 0.05$, **$p < 0.01$. Abbreviations: CCK-8, cell counting kit-8; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; CQA, chlorogenic acid; NGN, naringenin.

(ab113851, Abcam, Cambridge, MA, USA). Briefly, the cells were inoculated into a 96-well plate, followed by incubation with 10 µM DCFH-DA at 37 °C for 30 min. Subsequently, upon rinsing with fresh medium, the cells were further observed and imaged under a fluorescent microscope (DM3000, Leica, Wetzlar, Germany).

Detection of Oxidative Stress

After treatment, the culture supernatants of HepG2 cells were collected by centrifugation. Then, malondialdehyde (MDA) concentration and the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were measured by MDA Content Assay Kit (BC0025, Solarbio, Bejing, China), GSH-Px Activity Assay Kit (BC1195, Solarbio, Bejing, China), and SOD Activity Assay Kit (BC0175, Solarbio, Bejing, China), respectively. All procedures were carried out in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9.0 software (GraphPad company, La Jolla, CA, USA). Results are expressed as mean ± standard deviation. Student's *t*-test was employed to analyze comparison of quantitative variables between independent groups, and one-way analysis of variance (ANOVA) was applied for comparing quantitative variables among three and more groups. Results with $p < 0.05$ were considered statistically significant. All experiments were conducted in triplicate.

Results

Cytotoxic Effects of Four Active Ingredients from Coreopsis tinctoria Nutt. Extracts on HepG2 Cells

In the initial part of this study, the cytotoxic effects of luteolin, marein, NGN, and CQA on HepG2 cells were tested. Luteolin at a dose of 5, 10, 20 or 30 µM had no effects on HepG2 cell viability and release of LDH ($p >$ 0.05), while 40 µM of luteolin could suppress HepG2 cell viability ($p < 0.05$) and promote release of LDH ($p < 0.05$) (Fig. 1A,B). Marein at a dose of 0.1, 1, 5 or 10 µM were not cytotoxic to HepG2 cells and had no effects on LDH release ($p > 0.05$), but the cell viability was significantly suppressed ($p < 0.05$) and the release of LDH was promoted upon raising the concentration of marein to 20 µM ($p <$ 0.05) (Fig. 1C,D). Within the concentration range of 50 to 300 µM, CQA showed no obvious toxic effect on HepG2 cell ($p > 0.05$), while at the concentration of 500 µM, CQA significantly reduced cell viability ($p < 0.05$) and promoted LDH release ($p < 0.05$) (Fig. 1E,F). At the concentration from 25 to 200 µM, NGN had no influence on HepG2 cell viability and release of LDH ($p > 0.05$), but the cell viability was significantly suppressed ($p < 0.01$) and the re-

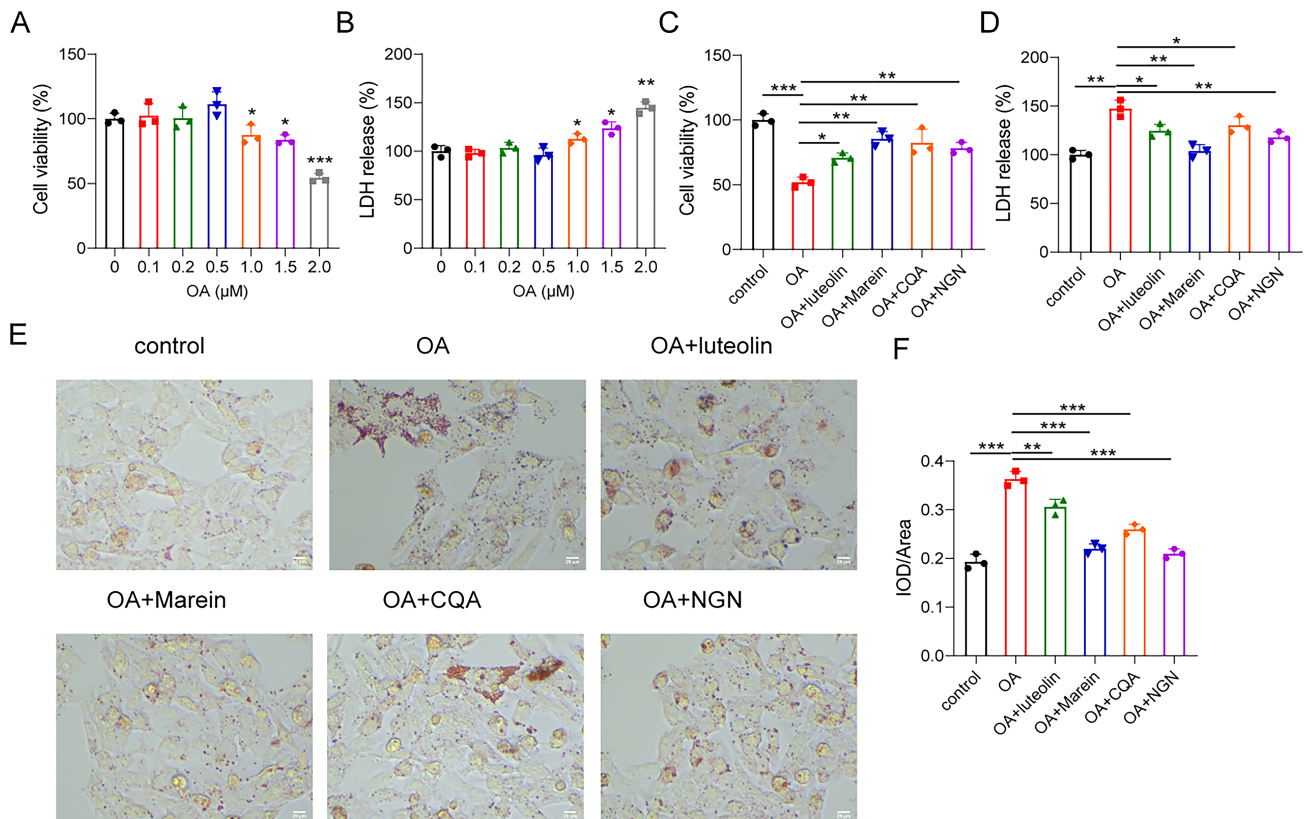

Fig. 2. Effects of *Coreopsis tinctoria* Nutt. extracts on oleic acid (OA)-induced lipid accumulation in HepG2 cells. (A,B) HepG2 cells were treated with 0, 0.1, 0.2, 0.5, 1.0, 1.5 or 2.0 mM of OA for 24 h. The cell viability and LDH level were determined by CCK-8 assay and ELISA, respectively. HepG2 cells induced by OA were treated with luteolin, marein, CQA or NGN for 24 h. (C) HepG2 cell viability measured by CCK-8 assay. (D) LDH level detected by ELISA. (E) Representative image of Oil red O stained cells under microscope. Scale bar = 25 µm. (F) Integrated optical density (IOD) values at 520 nm of Oil red O stains captured in HepG2 cells. Data are expressed as mean ± standard deviation. n = 3 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

lease of LDH was promoted ($p < 0.01$) by 300 µM NGN (Fig. 1G,H). These results established the safe dosages of luteolin (30 µM), marein (10 µM), CQA (300 µM), and NGN (200 µM) in the context of HepG2 cells, which were used in the subsequent experiments.

Luteolin, Marein, CQA and NGN Greatly Ameliorated OA-Induced Lipid Accumulation in HepG2 Cells

HepG2 cells were treated with OA to simulate *in vitro* high-lipid milieu in cellular setting. As exhibited in the results, OA within the concentration range of 0–0.5 mM was not cytotoxic to HepG2 cells ($p > 0.05$), but beyond this concentration range, the inhibitory effect of OA treatment on cell activity became evident ($p < 0.05$ or $p < 0.001$) in a concentration-dependent fashion (Fig. 2A). On the contrary, the level of LDH was markedly elevated when the concentration of OA exceeded 1.0 mM ($p < 0.05$ or $p < 0.01$) (Fig. 2B). Hence, 2.0 mM OA was selected for subsequent experiment. To explore the beneficial roles of luteolin, marein, CQA and NGN in lipid accumulation, OA-treated HepG2 cells were treated with these four extracts. OA treatment significantly inhibited HepG2 cell viability ($p < 0.001$) and aggravated LDH production ($p < 0.01$), which could be partly alleviated by luteolin, marein, CQA or NGN treatment ($p < 0.05$ or $p < 0.01$) (Fig. 2C,D). As revealed in Fig. 2E,F, OA treatment resulted in significant lipid accumulation in HepG2 cells ($p < 0.001$), which could however be reversed by luteolin, marein, CQA or NGN ($p < 0.01$ or $p < 0.001$). In summary, luteolin, marein, CQA or NGN could effectively mitigate cell injury and ameliorate lipid accumulation induced by OA in HepG2 cells.

Luteolin, Marein, CQA or NGN Altered Lipid Synthesis in OA-Induced HepG2 Cells

The production of TG, TC and LDL-C was markedly elevated in the OA groups ($p < 0.001$), while the synthesis of HDL-C was reduced ($p < 0.001$) as opposed to the control group. However, all these changes were alleviated upon luteolin, marein, CQA or NGN treatment ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) (Fig. 3A–D). Moreover, the mRNA expression of *HMGCR*, *LDLR* and apical sodium-dependent bile acid transporter (*ASBT*) measured by qRT-PCR assay was dramatically elevated in OA-treated HepG2

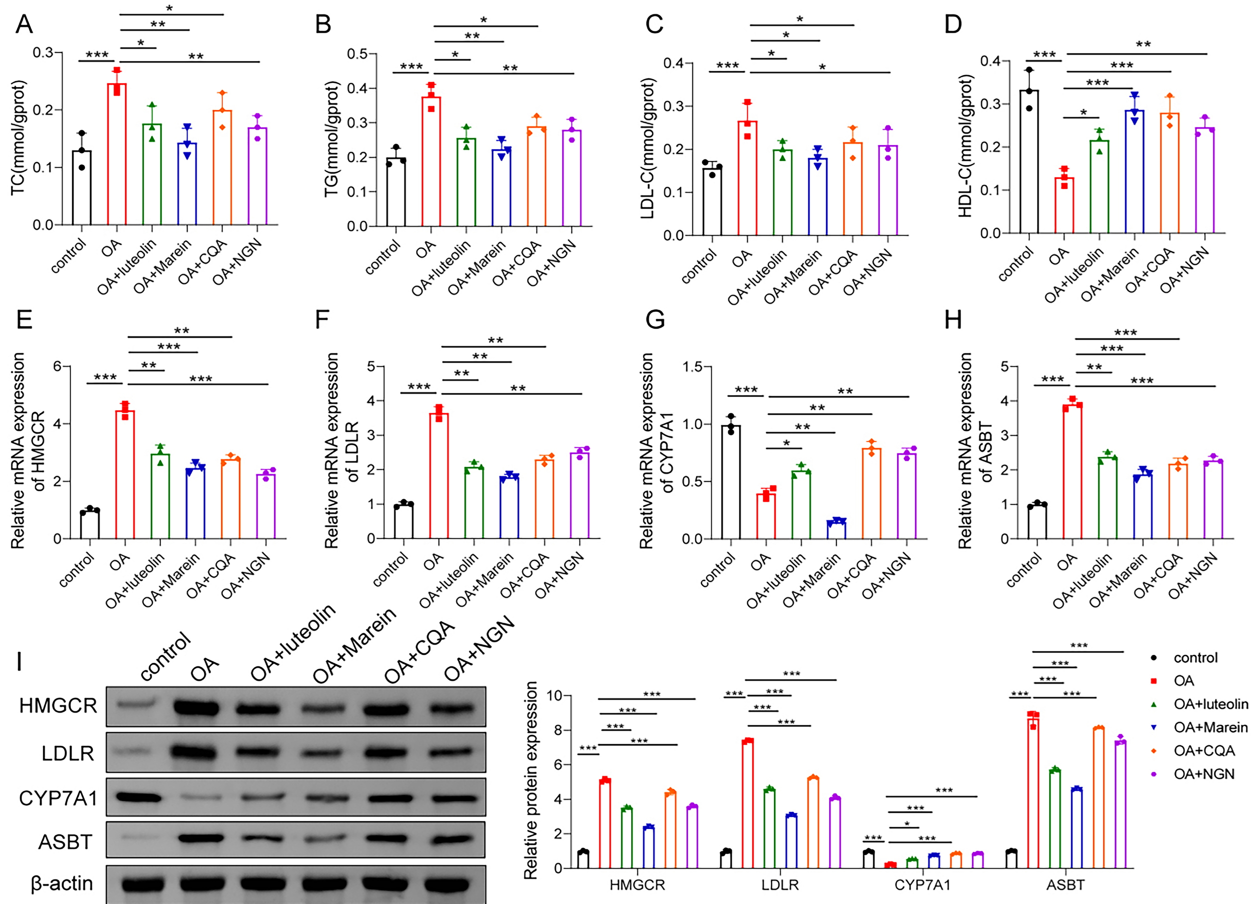

Fig. 3. Effects of *Coreopsis tinctoria* Nutt. extracts on OA-induced altered lipid metabolism in HepG2 cells. The HepG2 cells induced by OA were treated with luteolin, marein, CQA or NGN for 24 h. (A–D) ELISA was performed to analyze lipid profiles (TG, TC, HDL-C or LDL-C) in HepG2 cells. (E–H) The mRNA levels of genes related to lipid metabolism including *ASBT*, *LDLR*, *CYP7A1* and *HMGCR* were measured by qRT-PCR. (I) Western blot was applied to detect the levels of HMGCR, LDLR, CYP7A1 and ASBT proteins. Data are expressed as mean ± standard deviation. n = 3 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

cells ($p < 0.001$), but such an increase could be effectively suppressed by luteolin, marein, CQA or NGN treatment ($p < 0.01$ or $p < 0.001$) (Fig. 3E,F,H). Additionally, the low expression of cytochrome P450 family 7 subfamily A member 1 (*CYP7A1*) in OA-induced HepG2 cells, as compared to the control group ($p < 0.001$), could be reversed by luteolin, CQA or NGN treatment ($p < 0.05$ or $p < 0.01$) but not marein treatment (Fig. 3G). Western blot assay indicated that OA enhanced expression of HMGCR, LDLR, and ASBT protein and reduced CYP7A1 expression in HepG2 cells ($p < 0.001$); however, these protein expression trends could be partially reversed following luteolin, marein, CQA or NGN treatment ($p < 0.05$ or $p < 0.001$) (Fig. 3I). Taken together, luteolin, marein, CQA or NGN treatment could alter lipid synthesis in HepG2 cells treated with OA.

Luteolin, Marein, CQA or NGN Inhibited OA-Induced Oxidative Stress in HepG2 Cells

Lipid accumulation has been reported to result in a series of deleterious events, such as reactive oxygen species production and the ensuing oxidative stress [27,28]. In this study, we measured the oxidative stress level of OA-induced HepG2 cells after luteolin, marein, CQA or NGN administration. As displayed in Fig. 4A, ROS level was significantly upregulated in OA group in contrast to the control group ($p < 0.001$), a condition that could be partially rescued by luteolin, marein, CQA or NGN treatment ($p < 0.01$ or $p < 0.001$). The OA group also exhibited increased MDA concentration ($p < 0.001$) and inhibited SOD and GSH-Px activities ($p < 0.001$), but these events characteristic of oxidative stress could be strikingly ameliorated by luteolin, marein, CQA or NGN treatment ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) (Fig. 4B–D). Overall, luteolin, marein, CQA or NGN had a conspicuous suppressive influence on oxidative stress induced by OA in HepG2 cells.

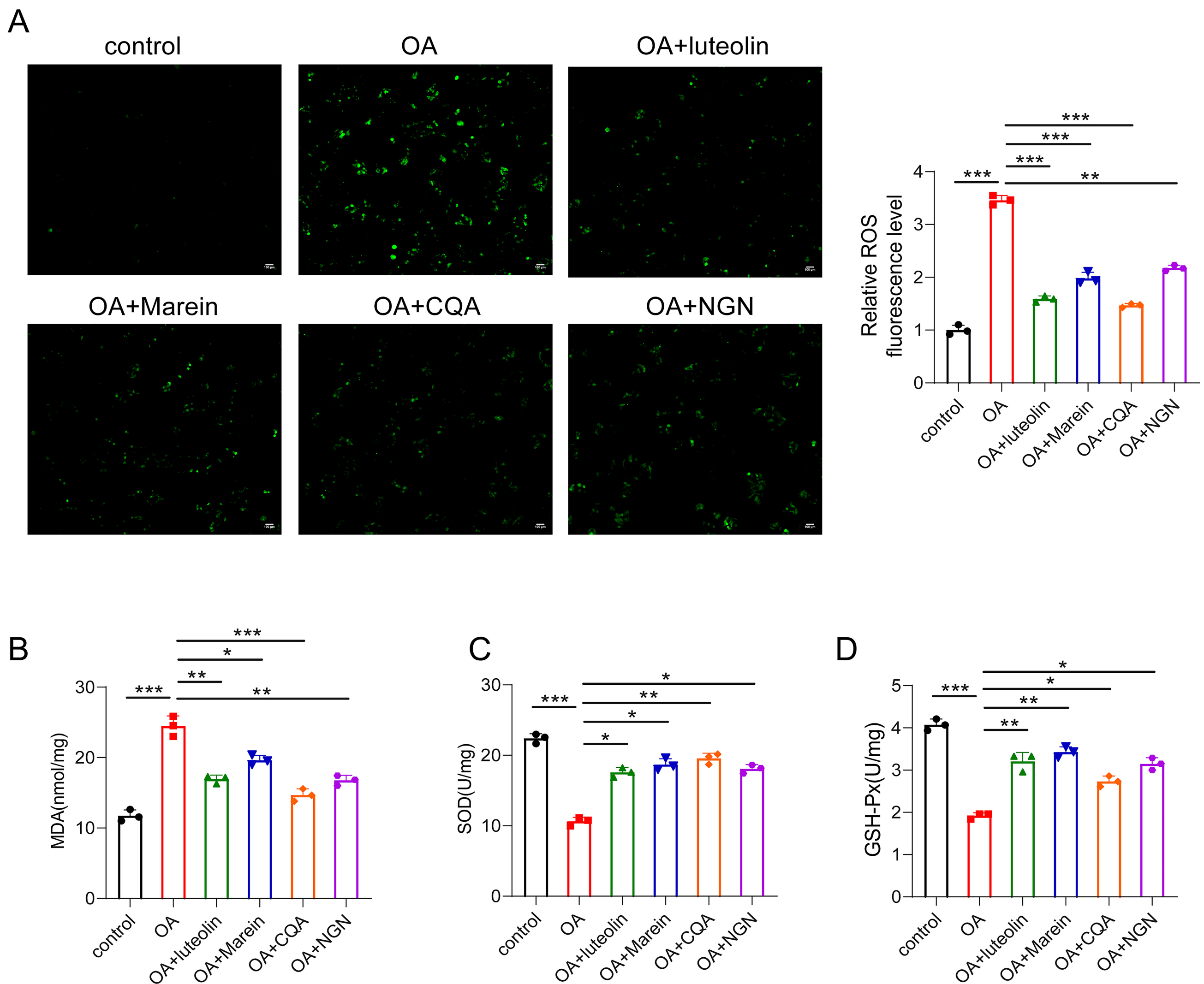

Fig. 4. Effects of *Coreopsis tinctoria* Nutt. extracts on OA-induced oxidative stress in HepG2 cells. HepG2 cells induced by OA were treated with luteolin, marein, CQA or NGN for 24 h. (A) ROS level was determined using DCFH-DA staining. Scale bar = 100 µm. (B–D) ELISA was carried out to examine the activities of GSH-Px and SOD as well as the concentration of MDA. Data are expressed as mean ± standard deviation. n = 3 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: ROS, reactive oxygen species; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; DCFH-DA, 2′,7′-dichlorodihydro fluorescein diacetate.

Luteolin, Marein, CQA or NGN Inactivated SREBPs-Related Proteins in OA-Treated HepG2 Cells

SREBPs play an important role in lipid metabolism [29]. In this study, we explored the effects of luteolin, marein, CQA or NGN on SREBPs expression in OA-treated HepG2 cells. According to our data, the *SREBP1* and *SREBP2* mRNA levels were promoted in OA-treated HepG2 cells ($p < 0.001$), but luteolin, marein, CQA or NGN treatment could obviously reverse the elevated expression of the *SREBP1* and *SREBP2* ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) (Fig. 5A,B). Besides, expression of SREBP1 and SREBP2 protein was also elevated in the OA group ($p < 0.001$), an increase that could be partially rescued by luteolin, marein, CQA or NGN treatment ($p < 0.05$ or $p < 0.001$) (Fig. 5C). Taken together, luteolin, marein, CQA or NGN markedly inhibited the expression of SREBPs in OA-induced HepG2 cells.

Discussion

The incidence and mortality of hyperlipidemia and its complications are presenting an increasing trend worldwide. Accumulating clinical and experimental evidence indicates that TCM has excellent therapeutic effect on hyperlipidemia. For example, Lingguizhugan has been shown to reduce serum TC, TG, HDL-C, LDL-C levels and body weight, presenting a therapeutic benefit in patients with hyperlipidemia and obesity [30]. As demonstrated by Yao *et*

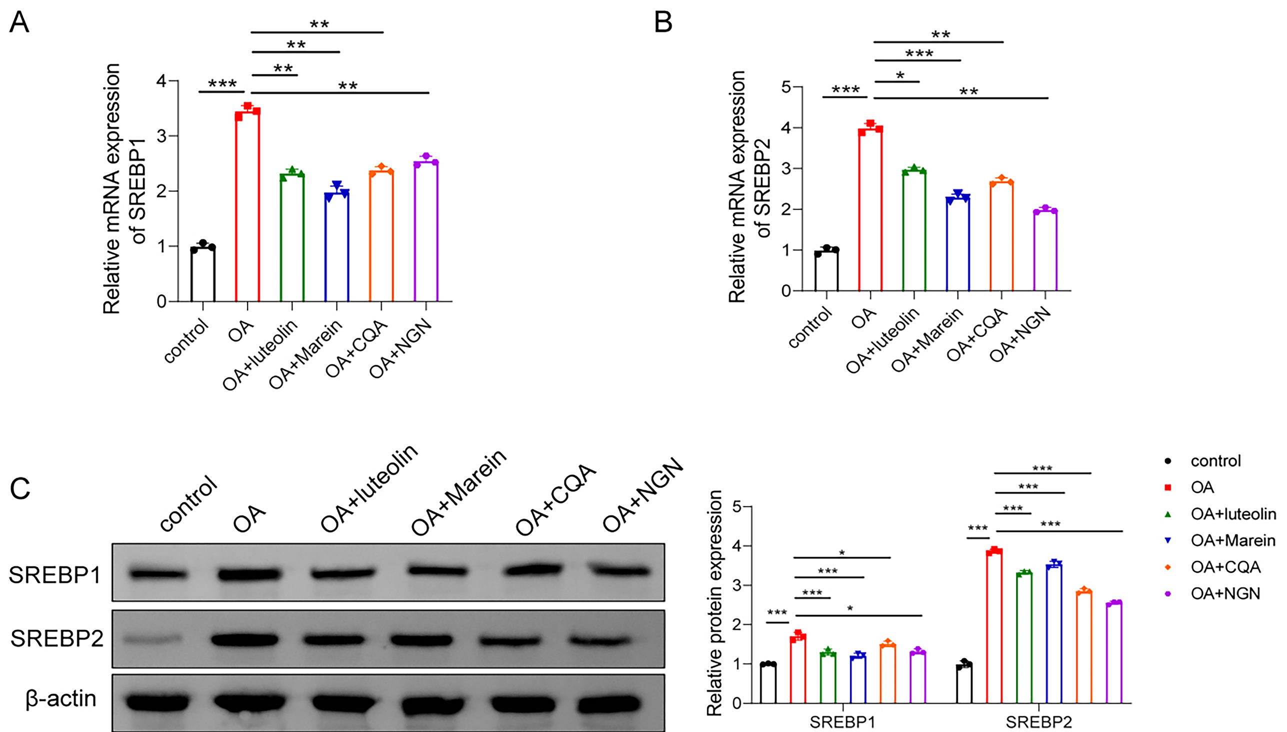

Fig. 5. Effects of *Coreopsis tinctoria* Nutt. extracts on expression of SREBPs in OA-induced HepG2 cells. HepG2 cells induced by OA were treated with luteolin, marein, CQA or NGN for 24 h. (A,B) qRT-PCR was employed to analyze the mRNA levels of *SREBP1* and *SREBP2*. (C) Western blot was performed to determine the expression level of SREBP1 and SREBP2 proteins. Data are expressed as mean ± standard deviation. n = 3 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

al. [31], modified Lingguizhugan decoction alleviates hyperglycemia, obesity, hyperlipidemia, hypertension, insulin resistance and hepatic injury induced by high-fat diet. Ge *et al*. [32] verified that Wuwei Qingzhuo San has hypolipidemic effect in the mice administered with high-fat diet by regulating metabolic disorders.

In this study, the therapeutic effects of four active ingredients from *Coreopsis tinctoria* Nutt. on hyperlipidemia were investigated. Our results revealed that luteolin, marein, CQA or NGN reduced lipid deposition and suppressed oxidative stress and altered lipid metabolism in OA-treated HepG2 cells, probably mediated by a mechanism involving SREBPs signaling.

Coreopsis tinctoria Nutt., an annual herb in genus *Coreopsis*, boasts more than 120 chemical constituents and possesses a wide range of properties such as anti-hypertensive, anti-cancer, anti-inflammatory, anti-oxidative stress activities [33]. Wang *et al*. [34] reported that NGN, one of the active ingredients of *Coreopsis tinctoria* Nutt., reduced deposition of lipid in hepatocytes. Additionally, luteolin, marein and CQA have been demonstrated to regulate lipid metabolism. For instance, Yan *et al*. [35] revealed that the anti-diabetic effect of CQA is implemented through the suppression of hepatic lipid metabolism by reducing TG synthesis and fatty acid oxidation. Zhu *et al*. [36] indicated that luteolin combined with lycopene treatment could improve lipid prolife by modulating Silent Information Regulator 2 Homolog 1 (SIRT1)/Mitogen-Activated Protein Kinase (MAPK) signaling, thus effectively attenuating non-alcoholic fatty liver disease. In this study, our data showed that luteolin, marein, NGN and CQA effectively reduced the accumulation of lipid and synthesis of TG, TC, and LDL-C, and increased synthesis of HDL-C in OA-treated HepG2 cells. In addition, HMGCR, LDLR, CYP7A1 and ASBT are important mediators in lipid metabolism. CYP7A1 and HMGCR are rate-limiting enzyme in bile acid synthesis and cholesterol synthesis, respectively [37]. ASBT is involved in regulating reabsorption of bile acids and LDLR is responsible for transporting cholesterol [38]. We found that the expression of HMGCR, LDLR and ASBT were downregulated but CYP7A1 expression was upregulated by luteolin, marein, NGN and CQA treatment in OA-treated HepG2 cells, further validating the therapeutic potential of the four active ingredients of *Coreopsis tinctoria* Nutt. in hyperlipidaemia.

Abnormal lipid metabolism results in lipid deposition, which then induces ROS overproduction and the ensuing oxidative stress. The production of ROS is regulated by multiple players such as MDA, SOD and GSH-Px [39,40]. Oxidative stress is closely associated with hyperlipidemia. It has been demonstrated that hampering the progression of oxidative stress may improve hyperlipidemia, atherosclerosis and other cardiovascular diseases [41,42]. In the present study, OA-induced elevation of ROS and MDA levels as

well as decline in GSH-Px and SOD activities in HepG2 cells were found to be reversed by luteolin, marein, NGN or CQA treatment. SREBP family proteins (SREBP1a, SREBP1c and SREBP2) have been demonstrated to exert anti-oxidative activities and regulate cholesterol synthesis and lipid metabolism [29]. Specifically, SREBP2 positively regulates LDLR and HMGCR expression and mediates cholesterol uptake [43]. Xie *et al*. [44] revealed that inhibition of FKBP38-mTOR-SREBPs signaling could improve lipid accumulation and hyperlipidemia induced by OA *in vivo*. Our results proved that treatment of luteolin, marein, NGN or CQA could inhibit upregulation of SREBP1 and SREBP2 expression in OA-treated HepG2 cells. Taking these findings into consideration, it is plausible that SREBPs signaling underpins the mechanism underlying the protective roles of luteolin, marein, NGN or CQA in lipid accumulation and oxidative stress induced by OA.

This study emphasizes the effectiveness of TCM in treating hyperlipidemia, inspired by examples such as Lingguizhugan and modified Lingguizhugan decoction, which have shown beneficial effects on lipid profiles and obesity. In the context of clinical drug development, compounds or derivatives, after drug designing and synthesis, are subjected to rigorous testing on their bioavailability, efficacy, and safety. Advanced drug delivery systems (e.g., nanoparticles, liposomes) could also be explored to enhance cellular uptake and targeted delivery. These flavonoids hold potential as adjunct therapies to existing hyperlipidemia treatments (e.g., statins) owing to the resulting synergistic effects, which aid in reducing drug doses and associated side effects.

Several limitations of this study should be acknowledged. Firstly, only one cell type was investigated in this study. HepG2 cells represent a widely accepted cellular model for studying liver-related metabolic processes, but a broader array of cell types and models are available for studying the therapeutic roles and mechanisms of active flavonoids in hyperlipidemia and associated disorders. Secondly, the mechanisms of the active flavonoids are not fully investigated; therefore, an in-depth investigation into the molecular mechanisms through which these flavonoids exert their lipid-lowering and antioxidant effects is warranted. Thirdly, the *in vitro* findings revealed in this study have not been validated through *in vivo* approach, for instance, verifying the efficacy and safety of the active flavonoids in animal models of hyperlipidemia. Additionally, the effect of marein on the gene or protein expression of CYP7A1 deserves further validation by *in vivo* experiments on the grounds of its contradictory effect on CYP7A1 gene and protein expression in HepG2 cells. Future research should focus on utilizing animal models to experimentally confirm the safety, efficacy, and mechanisms of action of these active flavonoids, in a bid to contribute to the development of novel hyperlipidemia treatments.

Conclusion

In conclusion, luteolin, marein, NGN and CQA isolated from *Coreopsis tinctoria* Nutt. hold promise as potential agents to alleviate hyperlipidemia through reducing lipid deposition and inhibiting lipid metabolism shift and oxidative stress possibly by means of SREBPs suppression. Nevertheless, the beneficial effects of luteolin, marein, NGN and CQA on oxidative stress, lipid accumulation, and lipid metabolism observed in this study still require more robust validation testing using *in vivo* approach.

Abbreviations

AMPK, AMP-activated protein kinase; CQA, chlorogenic acid; CYP7A1, cytochrome P450 family 7 subfamily A member 1; GSH-Px, glutathione peroxidase; HDL-C, high-density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL-C, low-density lipoprotein cholesterol; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; MDA, malondialdehyde; NGN, naringenin; OA, oleic acid; ROS, reactive oxygen species; SREBPs, sterol regulatory element-binding proteins; SOD, superoxide dismutase; TCMs, traditional Chinese medicines; TC, total cholesterol; TG, triglyceride.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Author Contributions

LSZ, LLZ and XRC designed the research study. LSZ, LLZ and XRC performed the research. RFL, YTK, YYC and YCX provided help and advice on experiments. RFL, YTK, YYC and YCX analyzed the data. LLZ and XRC writing the first draft. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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