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Deciphering the potent antioxidant mechanism of *Triadica cochinchinensis* monofloral honey: integrating metabolomics, transcriptomics and network pharmacology to analysis from free radical scavenging and cellular redox regulation

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ABSTRACT: *Triadica cochinchinensis* honey (TCH) is a specialty honey with strong antioxidant activity. However, its key antioxidant components and underlying mechanisms remain unclear. This study systematically evaluated the antioxidant effects and mechanisms of TCH using metabolomics, transcriptomics, and network pharmacology. The results revealed that TCH exhibited higher 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) radical scavenging activity and ferric ion reducing antioxidant power (FRAP) capacity than nine common commercial honeys. Metabolomic analysis identified D-chiro-inositol as a key antioxidant compound, which, together with (-)-gallicocatechin gallate, identified in prior research, constitutes the core antioxidant monomers of TCH (TCMs). TCH and TCMs protected LO2 cells against hydrogen peroxide (H₂O₂)-induced oxidative damage by increasing cell viability, reducing malondialdehyde (MDA) and reactive oxygen species (ROS) levels, enhancing antioxidant enzyme activities and total antioxidant capacity, suppressing apoptosis, and reversing G0/G1 cycle arrest. Transcriptomics and network pharmacology analyses revealed that the potential cellular protective mechanism of TCMs may involve a multi-target antioxidant network by regulating oxidative stress-related genes within key signaling pathways, including mitogen-activated protein kinase (MAPK), tumor necrosis factor (TNF), and epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) resistance pathways, in H₂O₂-damaged cells.

Keywords: honey, *Triadica cochinchinensis*, antioxidant activity, metabolomics, transcriptomics, network pharmacology.

1. Introduction

Honey is a natural sweetener with antioxidant, antibacterial, anti-inflammatory, and wound-healing properties [1]. Its biological properties are influenced by botanical origin, as different floral sources contribute distinct chemical compositions [2]. For example, Mānuka honey exhibits up to 50-fold greater antibacterial activity than many other honey varieties due to its high methylglyoxal content [3]. *Triadica cochinchinensis* honey (TCH) is one of the major commercial honey varieties in China and is characterized by its light amber

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color and distinctive flavor profile ^[4]. It has attracted growing interest due to its unique biochemical compositions and potential medicinal applications ^[5]. TCH exerts marked protective effects against alcoholic liver disease by ameliorating alcohol-induced intestinal flora dysbiosis ^[6]. It has also significantly influenced lipid synthesis and metabolism in the livers of naturally aged mice ^[7]. Polyphenolic compounds, including ellagic acid, gallic acid, salicylic acid, naringenin, rutin, and quercetin have been identified in TCH and are considered to contribute to its antioxidant activity ^[8,9]. Our previous studies identified (-)-gallocatechin gallate (GCG) as a unique flavonoid marker for TCH ^[5]. However, the mechanism underlying the antioxidant activity TCH has not yet been systematically elucidated. Recent advances in apiculture have substantially increased the production of high-quality TCH, underscoring the need for a comprehensive investigation of its bioactive properties.

Several methods have been used to evaluate the antioxidant activity of honey. Chemical assays, such as 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), 2,2'-azino-bis-3-ethylbenzothiazolin-6-sulphonic acid (ABTS) and ferric ion reducing antioxidant power (FRAP) assays, are commonly employed to assess free radical scavenging activity and reducing power ^[10, 11]. In addition, cellular antioxidant activity assays can better reflect the actual antioxidant efficacy of bioactive compounds in living systems ^[12]. For example, *Schefflera oleifera* honey has been reported to reduce reactive oxygen (ROS) levels and inhibit the proliferation of HepG2 cells ^[13]. Moreover, multi-omics technologies have been widely applied to investigate the antioxidant mechanisms of honey. Metabolomics can be used to identify antioxidant-active compounds in honey ^[14]. 7-O-methylchrysin was identified as a potential marker associated with the free radical scavenging activity of *Bauhinia championii* honey ^[15]. Transcriptomics enables the identification of antioxidant-related genes and pathways regulated by honey. Alhagi honey polysaccharides were shown to alleviate alcoholic liver disease in mice by suppressing the expression of oxidative stress marker genes and the nuclear factor kappa-B (NF-κB) pathway ^[16]. Network pharmacology can reveal the interactions among antioxidant compounds, their molecular targets, signaling pathways, and related diseases. Luteoline, fisetin and kaempferol in Tualang honey were reported to potentially regulate atherosclerosis through interactions with key biological targets, including epidermal growth factor receptor (*EGFR*) and AKT serine/threonine kinase 1 (*AKT1*) ^[17].

However, current research on the antioxidant properties of honey has several limitations: 1) it focuses on the overall antioxidant activity of honey, with limited quantitative correlation analysis between activity and chemical components; 2) it mostly utilizes single-omics approaches, with limited use of multi-omics to explore the mechanism of antioxidant activity; and 3) there is limited validation at the cellular level for honey and its antioxidant monomers.

To address these limitations, this study employed chemical assays to compare the *in vitro* antioxidant activities of TCH with those of nine other commercial honey varieties in China. Subsequently, metabolomics was used to analyze the chemical composition of the honeys, to identify characteristic antioxidant monomers in TCH. Next, the protective effects of TCH and its antioxidant monomers (GCG and D-chiro-inositol (DCI)) against hydrogen peroxide (H₂O₂) -induced oxidative stress injury in LO2 cells were investigated. Finally,

transcriptomics and network pharmacology were employed to analyze critical pathways, and target points associated with the observed effects. This study aimed to establish a systematic analytical framework of chemical monomer-cellular response-signaling pathway interactions, providing molecular insight into the antioxidant properties and potential high-value utilization of TCH.

2. Materials and methods

2.1 Chemicals and reagents

The reagents used in this study included: rutin and gallic acid standards (purity $\geq 98\%$) (Weiye, Beijing, China), aluminium chloride (AlCl_3), sodium carbonate (Na_2CO_3), and absolute ethanol (Xilong Scientific, Shantou, China); Folin-Ciocalteu reagent, ABTS free radical scavenging capacity (ABTS+), FRAP kits, DCI, and GCG standards (purity $\geq 98\%$) (yuanye, Shanghai, China); DPPH (Phygene, Fujian, China); Liquid chromatography/mass spectrometry (LC-MS)-grade acetonitrile and methanol (Fisher Scientific, Loughborough, UK); LC-MS-grade formic acid (TCI, Shanghai, China); 2-amino-3-(2-chloro-phenyl)-propionic acid (Aladdin, Shanghai, China); ammonium formate (Sigma-Aldrich, Shanghai, China); and ultrapure water (Milli-Q system, Millipore, Bedford, USA).

LO2 cells were obtained from Cellverse (Shanghai, China). Additional reagents included 0.25% (w/v) Trypsin-ethylenediaminetetraacetic acid (EDTA) solution without phenol red, cell counting kit-8 (CCK-8), phosphate buffered saline (PBS) and Roswell Park Memorial Institute (RPMI) 1640 medium (Solarbio, Beijing, China), and Fetal bovine serum (FBS) (Nova Pharmaceutica, Shanghai, China). Assay kits used in this study included Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), total antioxidant capacity assay (T-AOC) kits (Nanjing Jiancheng, Nanjing, China); ROS assay kits, cell cycle analysis kits and apoptosis analysis kits (Meilunbio, Dalian, China); and Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) apoptosis detection kits (Elabscience, Wuhan, China).

Molecular biology reagents used in this study included the Trans-Zol Up RNA Kit and TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China), and MagicSYBR mixture (Cwbio, Jiangsu, China). Protein analysis reagents included Radio immunoprecipitation assay (RIPA) buffer, 20× tris-buffered saline with Tween-20 (TBST), developing powder, fixer (Solarbio, Beijing, China); Bicinchoninic acid (BCA) protein assay kit (Biosharp, Anhui, China); 3-(N-morpholino) propanesulfonic acid-sodium dodecyl sulfate (MOPS-SDS) running buffer and FuturePAGE™ pre-cast gel (ACE, Nanjing, China); Dual-color prestained protein marker (15–150 kDa) (Epizyme Biotechnology, Shanghai, China); Ponceau S staining solution, Coomassie Brilliant Blue staining solution, QuickBlock™ blocking buffer, QuickBlock™ primary and secondary antibody dilution buffers (Beyotime, Shanghai, China); Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Merck-millipore, Darmstadt, Germany); and Meilunbio® FG Super Sensitive enhanced chemiluminescence (ECL) Reagent (MeilunBio, Dalian, China).

2.2 Sample collection

TCH and nine representative commercial honey varieties commonly consumed in China were selected for this study. TCH was produced in Yongxiu, Jiangxi, China. The comparative honey samples included eight types of monofloral honey: *Brassica campestris* honey (BCH, Zhongxiang, Hubei, China), *Citrus reticulata* honey (CRH, Nanfeng, Jiangxi, China), *Robinia pseudoacacia* honey (RPH, Yan'an, Shaanxi, China), *Vitex negundo* L. var. honey (VNH, Shijiazhuang, Hebei, China), *Lycium chinense* Miller honey (LMH, Zhongning, Ningxia, China), *Litchi chinensis* Sonn honey (LSH, Qinzhou, Guangxi, China), *Eriobotrya japonica* honey (EJH, Suzhou, Jiangsu, China), and *Tilia tuan* honey (TTH, Mudanjiang, Heilongjiang, China), along with one polyfloral honey (POH, Zhongxiang, Hubei, China). All samples were obtained from professional apiaries. All monofloral honey samples were classified based on quantitative melissopalynological analysis in our previous study, with a native pollen rate exceeding 45% in each honey [5]. Samples were stored at -20 °C until analysis.

2.3 Total phenolic content and total flavonoids content determination

The total phenolics content (TPC) was determined using the Folin–Ciocalteu method [18]. Briefly, 2 mL of honey sample supernatant (0.5 g/mL) was mixed with 6 mL of ultrapure water, 0.5 mL of Folin–Ciocalteu reagent, and 1.5 mL of 20% (w/v) Na₂CO₃ solution. The mixture was then diluted to a final volume of 10 mL with ultrapure water. After incubation in the dark at 25 °C for 30 min, the absorbance was measured at 763 nm.

The total flavonoid content (TFC) was determined using the method described in our previous study [4]. Briefly, 7 mL of honey sample supernatant (0.5 g/mL) was mixed with 2 mL of 1% (w/v) AlCl₃ ethanol solution and diluted to a final volume of 10 mL with 95% (v/v) ethanol. After incubation at 25 °C for 10 min, the absorbance was measured at 405 nm.

Calibration curves were constructed using gallic acid and rutin standard solutions at concentrations ranging from 0.01 to 0.5 mg/mL. The calibration equations were $y = 12.624x + 0.0855$ ($R^2 = 0.998$) for gallic acid and $y = 10.295x + 0.0361$ ($R^2 = 0.999$) for rutin. The results were expressed as gallic acid equivalents (GAE) in mg/100 g of honey and rutin equivalents (RE) in mg/100 g of honey.

2.4 Determination of in vitro antioxidant capacities of TCH and nine other honey varieties

The DPPH free radical scavenging activity of honey samples was determined using the method described by Hu [19]. Honey samples were prepared in water at concentrations ranging from 0.003 to 0.1 g/mL. Briefly, 100 µL of honey solution and 100 µL of DPPH ethanol solution (0.08 mg/mL) were added to each well of a 96-well plate. For the control group, 100 µL of the honey solution was mixed with 100 µL of ethanol. For the blank group, 100 µL of DPPH ethanol solution (0.08 mg/mL) was mixed with 100 µL of water.

The reaction mixtures were thoroughly mixed and incubated at 25 °C in dark for 30 min. Absorbance was then measured at 517 nm using a microplate reader.

The DPPH free radical scavenging rate (A) was calculated as follows:

$$A = [1 - (A_1 - A_0) / A_2] \times 100\%$$

where A₁, A₂ and A₀ represent the absorbance of the honey sample, control, and blank group, respectively. The results were expressed as the 50% inhibitory concentration (IC₅₀).

The ABTS⁺ radical scavenging activity and FRAP of honey samples were determined using commercial assay kits. The results of ABTS⁺ were expressed as the IC₅₀ of ABTS⁺, and the FRAP results were presented as mg Trolox kg⁻¹ of honey.

2.5 Untargeted metabolomics analysis of TCH and nine other honey varieties

2.5.1 Sample preparation

Honey samples (100 mg, n = 6) were accurately weighed into 2 mL centrifuge tubes and mixed with 400 µL of methanol by vortexing for 1 min. The samples were then centrifuged at 12,000 rpm for 10 min at 4°C. Following centrifugation, the supernatants were transferred to new 2 mL centrifuge tubes and concentrated to dryness. The residues were reconstituted in 150 µL of 2-chloro-L-phenylalanine solution (4 mg/L) prepared in 80% (v/v) methanol-water. The resulting solutions were filtered through 0.22 µm membranes, diluted 5-fold with 2-chloro-L-phenylalanine solution (4 mg/L) prepared in 80% (v/v) methanol-water, and transferred to sample vials for subsequent analysis.

2.5.2 UHPLC-Q Exactive Orbitrap-MS/MS analysis

The LC analysis was performed using a Vanquish ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, MA, USA). Chromatographic separation was achieved on an ACQUITY UPLC® HSS T3 column (150 × 2.1 mm, 1.8 µm; Waters, Milford, MA, USA). The column temperature was maintained at 40 °C. The flow rate and injection volume were set at 0.25 mL/min and 2 µL, respectively. For LC-electrospray ionization (ESI) (+)-MS analysis, the mobile phase consisted of 0.1% (v/v) formic acid in acetonitrile (C) and 0.1% (v/v) formic acid in water (D). The gradient elution program was as follows: 0–1 min, 2% C; 1–9 min, 2%–50% C; 9–12 min, 50%–98% C; 12–13.5 min, 98% C; 13.5–14 min, 98%–2% C; and 14–20 min, 2% C. For LC-ESI (–)-MS analysis, the mobile phases consisted of acetonitrile (A) and 5 mmol/L ammonium formate (B). The gradient elution program was as follows: 0–1 min, 2% A; 1–9 min, 2%–50% A; 9–12 min, 50%–98% A; 12–13.5 min, 98% A; 13.5–14 min, 98%–2% A; and 14–17 min, 2% A.

Mass spectrometric detection was performed on Q Exactive HF-X (Thermo Fisher Scientific, MA, USA) equipped with an ESI ion source. Simultaneous MS1 and tandem mass spectrometry (MS/MS) data acquisition was conducted in Full MS-data-dependent MS2 (ddMS2) mode. The operating parameters were as follows: sheath gas pressure, 30 arb; auxiliary gas flow rate, 10 arb; spray voltage, 3.50 kV for ESI (+) and –2.50 kV for ESI (–), capillary temperature, 325 °C; MS1 range, m/z 81–1,000; MS1 resolution, 60,000 full width at half maximum (FWHM); number of data dependent scans per cycle, 8; MS/MS resolution, 15,000 FWHM; normalized collision energy, 30%; dynamic exclusion, automatic.

The metabolites were identified by comparing their retention time and mass spectra with those of reference standards. Compound annotation and identification were performed using the public databases Human Metabolome Database (HMDB), MassBank, LipidMaps, mzCloud, Kyoto Encyclopedia of Genes and Genomes (KEGG) together with an in-house compound database. Raw data were processed using the XCMS

package in R for peak detection, filtering, and alignment. Multivariate statistical analyses were performed using the ropls package in R.

2.6 Quantitation of D-chiro-inositol (DCI) in TCH

A 1,000 mg/L DCI standard solution was prepared in 80% (v/v) methanol. The stock solution was subsequently diluted to obtain DCI standard solutions at concentrations of 100, 200, 500 and 1,000 mg/L to construct the standard curve. Briefly, 2 g TCH was accurately weighed and mixed with 10 mL of 80% (v/v) methanol. After vortexing for 5 min, the samples were filtered through a 0.22 μm syringe filter and immediately analyzed by high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) (waters, MA, USA). Chromatographic separation was performed using an XBridge Amide amino (NH_2) column (150 mm $\times$ 2.1mm, 2.5 μm ; Waters, MA, USA). The mobile phases consisted of ultrapure water (A) and acetonitrile (B). The flow rate was 0.3 mL/min, the column temperature was maintained at 35 $^\circ\text{C}$, and the injection volume was 2 μL . The gradient elution program was as follows: 0–10 min, 20% A; 10–14 min, 20%–40% A; 14–16 min, 40% A; 16–18 min, 40–20% A; and 18–21 min, 20% A. The ELSD was operated with a drift tube temperature of 95 $^\circ\text{C}$, a nebulizing gas flow rate of 2.2 L/min, and a gain setting of 5.

2.7. Protective effect of TCH and *Triadica cochinchinensis* honey monomers (TCMs) on H_2O_2 -induced oxidative damage in LO2 cells

2.7.1 Cell culture

LO2 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% carbon dioxide (CO_2). When cell confluence exceeded 80%, cells were digested with 0.25% (w/v) trypsin-EDTA for 2 min and subcultured at a 1:3 split ratio. Cells in the logarithmic growth phase were used for subsequent experiments.

2.7.2 Screening the concentration of H_2O_2

LO2 cells were seeded into 96-well plates at a density of 1×10^5 cells/mL (100 μL per well). After allowing the cells to adhere, the culture medium was replaced with 100 μL of medium containing H_2O_2 at final concentrations of 0, 100, 200, 300, 400, and 500 $\mu\text{mol/L}$. Each group was set in six replicates. The plates were incubated at 37 $^\circ\text{C}$ in 5% CO_2 for 24 h, and cell viability was assessed using the CCK-8 assay.

2.7.3. Determination of the dosage range of TCH and TCMs

TCMs refer to the characteristic antioxidant monomers of TCH, namely DCI and GCG (discovered in our previous studies, [5]). LO2 cells were seeded in the 96-well plates at a density of 1×10^5 cells/mL (100 μL per well). Following adhesion, 100 μL of culture medium containing TCH or TCMs at gradient concentrations were added to each well, with five replicate wells per group. The plates were then incubated at 37 $^\circ\text{C}$ in humidified atmosphere containing 5% CO_2 for 24 h, and cell viability was evaluated using the CCK-8 assay.

TCMs were classified into three groups: (a) DCI; (b) GCG; (c) DCI + GCG. The concentrations of TCMs were determined based on the contents of DCI and GCG in TCH (10 mg/mL of TCH contains 62.7 μg DCI and

0.576 ng GCG), as well as the optimal concentration range of TCH identified from LO2 cell viability assays. Detailed concentrations of all samples are summarized in Table S1.

2.7.4. Effect of TCH and TCMs on H₂O₂-induced oxidative damage in LO2 cells

LO2 cells were randomly divided into four groups for both the TCH and TCMs experiments: (a) control group, in which cells cultured in RPMI-1640 medium under normal conditions; (b) model group, in which cells were exposed to H₂O₂ (500 µmol/L); (c) positive control group, in which cells were pretreated with Vitamin C (VC, 15 µg/mL); and (d) experimental group, in which cells were pretreated with TCH or TCMs. After 24 h of pretreatment with VC, TCH, or TCMs, oxidative stress was induced by exposure to H₂O₂ for 6 h. Cell viability was then assessed using the CCK-8 assay. The optimal VC concentration, low-, medium-, and high-dose TCH groups, and optimal concentrations of DCI, GCG, and DCI + GCG were selected for further analysis. Detailed concentrations of all treatment groups are summarized in Table S2.

2.7.5. Determination of intracellular oxidative stress parameters in LO2 cells treated with TCH and TCMs

LO2 cells were randomly allocated to different experimental groups. For the TCH assays, four groups were established: Control group, H₂O₂ model group (500 µmol/L), VC group (15 µg/mL) and TCH treatment groups (low: 2.5 mg/mL, medium: 5 mg/mL and high: 10 mg/mL). For the TCMs experiment, four groups were also established: Control group, H₂O₂ model group (500 µmol/L), VC group (15 µg/mL) and TCMs treatment groups (DCI (15.67 µg/mL), GCG (0.576 µg/mL), DCI + GCG (15.67 µg/mL+0.144 ng/mL)).

Cells were treated according to the protocol described in 2.7.1. After cell culture, the culture medium from each group was collected into test tubes. Cells were then washed twice with PBS, and 150 µL of cell lysis buffer was added to each well. Subsequently, cells were collected in centrifuge tubes for centrifugation, and the supernatant was collected for subsequent analysis. Intracellular concentrations of MDA, T-AOC, SOD, GSH-Px, and CAT were measured according to the detailed instructions provided by the respective assay kits.

2.7.6. Flow cytometric analysis of intracellular ROS, apoptosis and cycle

Intracellular ROS levels in LO2 cell were measured using a ROS assay kit. Briefly, cells were trypsinized collected and washed with PBS. Subsequently, cells were incubated with 10 µmol/L dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37 °C. After incubation, cells were washed three times with serum-free medium and analyzed using flow cytometry.

Cell apoptosis was assessed using an Annexin V-FITC/PI apoptosis detection kit. Apoptosis was analyzed by flow cytometry (DxFLEX, Beckman Coulter, CA, USA), with 10³-10⁴ LO2 cells analyzed per sample.

Cell cycle distribution was determined using a cell cycle and apoptosis analysis Kit. Briefly, after treatment, cells were trypsinized, collected, and fixed with cold 75% (v/v) ethanol at 4 °C overnight. The cells were then washed with PBS and stained with a staining solution consisting of staining buffer, PI (20×), and Ribonuclease A (20×) at a ratio of 100:5:2. Samples were incubated for 30 min at 37 °C in the dark before cell cycle analysis by flow cytometry.

2.8 Protective mechanisms of TCMs against oxidatively damaged LO2 cells

2.8.1. Transcriptomic analysis

LO2 cells from the cell experiments were harvested for ribonucleic acid sequencing (RNA-seq) analysis. The raw sequencing data were deposited to the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under accession number PRJNA1293822. RNA-seq was performed by Shanghai Xuran Gene (Shanghai, China). Differential expression analysis was performed using the DESeq 2 Bioconductor package. Differentially expression genes (DEGs) were defined based on a threshold of $P < 0.05$ and fold change (FC) > 1.2 . Subsequently, KEGG pathway enrichment analysis was performed using the identified DEGs.

2.8.2. Network pharmacology analysis

The SMILES structures of DCI and GCG were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>, accessed on August 26th 2025) and uploaded to the SwissTargetPrediction platform (<https://www.swisstargetprediction.ch/>, accessed on August 26th 2025) to obtain the compound-related targets^[20]. Target genes associated with “oxidative stress” were collected from the Genecards database (<https://www.genecards.org/>, accessed on August 26th 2025)^[21].

Common targets between TCMs and oxidative stress were identified and imported into Cytoscape software (version 3.9.1) for network visualization and topological analysis. Key targets were identified based on network topology parameters. Subsequently, the selected targets were analyzed using the Metascape platform (<https://metascape.org>, accessed on August 26th 2025) for KEGG enrichment analysis^[22].

2.8.3. Real-time quantitative PCR (RT-qPCR) analysis

Based on the RNA-seq results, five DEGs, including caspase-3 (*CASP3*), Fas cell surface death receptor (*FAS*), component of inhibitor of nuclear factor kappa B kinase complex (*CHUK*), cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*), and heat shock protein family A member 6 (*HSPA6*), which are associated with oxidative damage, were selected for RT-qPCR verification. Total RNA was extracted from the LO2 cells using the TrizolUp Kit. The extracted RNA was reverse-transcribed into complementary DNA using the TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (GenBank accession no.: NM_002046) was used as the internal reference gene.

The primer sequences (Table S3) were designed using the Prime Primer 5.0 software based on mRNA sequence obtained from the NCBI GenBank database and synthesized by Sangon Biotech (Shanghai, China). The RT-qPCR reaction system mixture consisted of 10 μ L ChamQ Universal SYBR qPCR Master Mix, 1 μ L cDNA, 0.4 μ L forward primer, 0.4 μ L reverse primer, and 8.2 μ L RNase free ddH₂O. Thermal cycling conditions were as follows: initial denaturation 95 °C for 10 min, followed by 40 cycles of 95°C for 15s, annealing at T_m for 30s and extension at 72°C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method^[23].

2.8.4. Western blot analysis

LO2 cells were homogenized and lysed in RIPA buffer containing protease inhibitors to obtain total protein extracts. Protein concentrations were determined using the BCA protein assay kit. Proteins were separated by 10% (w/v) MOPS-SDS and transferred onto PVDF membranes. The membranes were then blocked with 5% (w/v) skimmed milk powders in TBST at room temperature for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies, including anti-CASP3, anti-FAS, anti-CHUK, anti-CYP1B1, anti-HSPA6, and anti- β -actin from Abcam (Cambridge, UK). The diluted secondary antibody (1:10,000) was added, and the membranes were incubated at room temperature for 1 h. Protein signals were detected using an ECL kit, followed by film exposure, development and fixation in a dark room. Protein band intensities were quantified using ImageJ software (Version 1.50).

2.9 Statistical analysis

Except for the metabolomics analysis, all experiments were performed with five biological replicates. Results are presented as mean $\pm$ standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for post hoc multiple comparisons in SPSS software (version 27, IBM Corp., NY, USA), with differences considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Antioxidant capacities of TCH and nine other honey varieties

Among ten characteristic Chinese commercial honeys, TCH ranked second in both TPC and TFC, surpassed only by LSH (Figure 1A, B). TCH also showed strong antioxidant activity. Notably, TCH exhibited the highest DPPH radical scavenging capacity and FRAP assays among the ten commercial honeys and ranked second in ABTS⁺ scavenging activity (Figure 1C-E). The strong antioxidant activity of TCH was closely associated with its high content of phenolic acids and flavonoids, which are recognized as the major antioxidant constituents in honey [24]. These compounds primarily originate from plant nectar collected by foragers and their concentrations are strongly influenced by botanical origin and geographical location [25]. These compounds demonstrate diverse bioactivities, have been associated with potential benefits in cardiovascular disease prevention, diabetes management and cancer chemoprevention [26, 27].

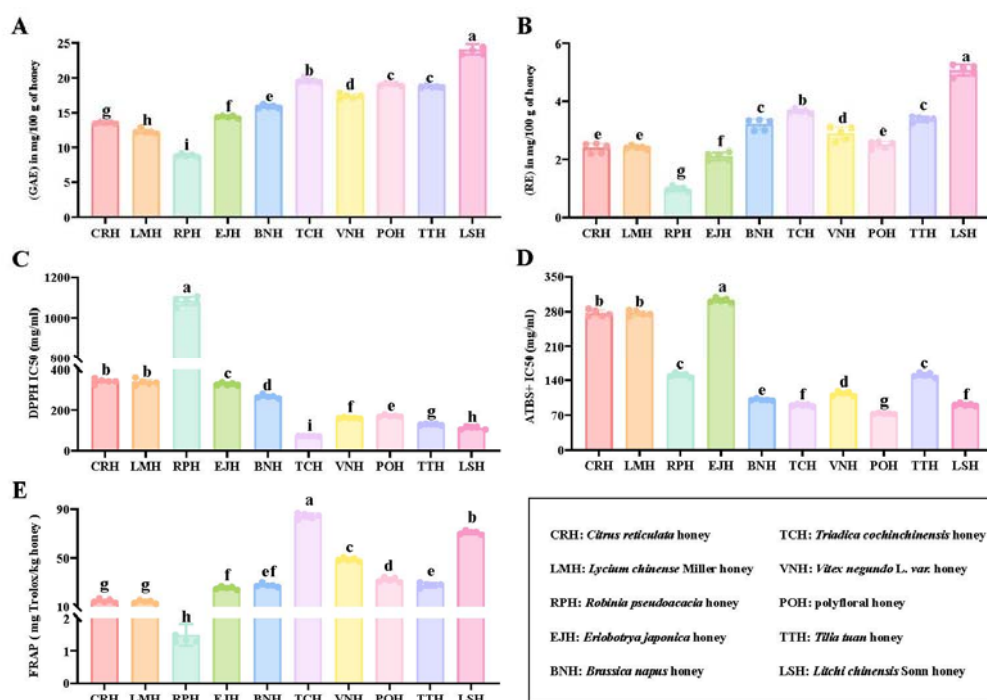


Figure 1. Total phenolics, flavonoids content and *in vitro* antioxidant capacity of *Triadica cochinchinensis* honey (TCH) and nine other common Chinese commercial honey varieties. (A) Total phenolics content (mg gallic acid equivalents (GAE)/100 g). (B) Total flavonoids content (mg rutin equivalents (RE)/100 g). (C) The 2,2-diphenyl-1-picrylhydrazyl (DPPH) 50% inhibitory concentration (IC₅₀, mg/mL). (D) The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS+) IC₅₀ (mg/mL). (E) The ferric ion reducing antioxidant power (FRAP; mg Trolox/kg honey). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test to adjust for multiple pairwise comparisons. The same lowercase letter in the same figure indicated no significant difference ($P > 0.05$), different lowercase letters indicated significant difference ($P < 0.05$).

Significant regional variations exist in total phenolic and flavonoids content of different honeys [28]. Zhang et al., in a study of ten monofloral honeys, further confirmed that these levels depend not only on geographical origin but also, critically, on plant species, consistent with the findings of the present study [29]. Moreover, honey color correlates with polyphenolic content. A study by Estevinho et al. demonstrated that dark honeys contain significantly higher phenolic levels and exhibit stronger DPPH radical scavenging capacity than lighter varieties [30]. In this study, amber-colored TCH exhibited strong antioxidant activity, reflected by significantly lower DPPH IC₅₀ values than those of the other nine commercial honeys.

3.2. Untargeted metabolomics analysis of TCH and nine other honey varieties

To gain a better understanding of the reasons for the strong *in vitro* antioxidant activity of TCH, untargeted metabolomics analysis was performed on TCH and nine other honey varieties. A total of 207 secondary metabolites, including 152 compounds detected in the ion positive mode and 55 compounds in the negative ion mode, were identified across all honey samples. Detailed information on these metabolites is provided in Table S4.

The three-dimensional partial least squares-discriminant analysis (3D PLS-DA) score plot (Figure 2A) demonstrated that quality control (QC) samples clustered tightly, indicating good repeatability and stability of the acquired MS data [31]. A clear distinction was observed among different honey types, with TCH exhibiting distinct metabolic features. Subsequently, samples were divided into TCH group and non-TCH groups for

comparative analysis to elucidate metabolic differences and characterize the specific features of TCH. The orthogonal partial least squares-discriminant analysis (OPLS-DA) score plot (Figure S1 A-I) showed excellent model performance, with R^2 and Q^2 values exceeding 0.5 for all comparisons, indicating a significant difference between TCH and the other nine honey varieties.

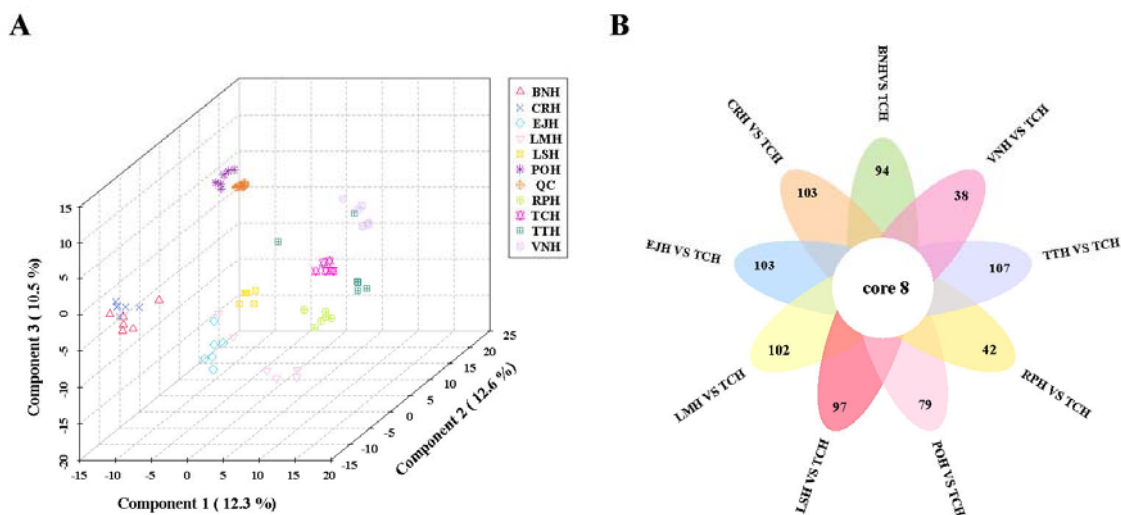


Figure 2. Metabolomics analysis of TCH and nine other common Chinese commercial honey varieties. (A) The three-dimensional partial least squares-discriminant analysis (3D PLS-DA) score plot of metabolites. (B) Common differential metabolites between TCH and nine other honey varieties.

The up-regulated differential metabolites were identified in TCH compared with the other nine honey varieties using threshold of $P < 0.05$, variable importance in projection (VIP) > 1 and $\log_2FC > 1$. As shown in Figure 2B, eight commonly up-regulated differential metabolites were detected in TCH compared with the other nine honey varieties. These compounds are listed in Table 1. Among them, D-chiro-inositol (DCI) exhibited significant antioxidant properties, including the ability to scavenge ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radicals [32], and to reduce intracellular oxidative stress [33]. DCI, one of the nine stereoisomers of inositol, exhibits optical activity. It shares structural similarities with glucose but has lower sweetness and does not contribute to energy metabolism [34]. Under natural conditions, DCI occurs either in a free form or as a derivative of galactose and is widely distributed in legumes, buckwheat, members of Cucurbitaceae family and other plants [35].

Table 1. 8 common up-regulated differential metabolites in *Triadica cochinchinensis* honey (TCH) compared to nine other honey varieties.

Up-regulated differential metabolites	m/z	RT (s)	Formula
Adipic acid	129.0525	241.8	$C_6H_{10}O_4$
D-chiro-inositol	163.0578	694.0	$C_6H_{12}O_6$
Sorbitol	182.9852	32.8	$C_6H_{14}O_6$
3,4-Methylenedioxyamphetamine	194.1158	378.0	$C_{11}H_{15}NO_2$
Dodecanoic acid	199.9733	32.5	$C_{12}H_{24}O_2$
Procaine	236.1468	103.8	$C_{13}H_{20}N_2O_2$
Uridine diphosphategalactose	565.0472	77	$C_{15}H_{24}N_2O_{17}P_2$
Uridine diphosphate glucuronic acid	579.0273	68.6	$C_{15}H_{22}N_2O_{18}P_2$

3.3. Quantification of DCI in TCH

To elucidate the contribution of DCI to the total antioxidant activity of TCH and to provide a quantitative basis for subsequent evaluation of its antioxidant activity in cellular models, absolute quantification of DCI in TCH was performed. As a cyclitol, DCI lacks strong ultraviolet-visible (UV-Vis) absorption chromophores, making routine UV detection difficult [36]. Therefore, an ELSD, a semi-universal detector capable of detecting nonvolatile analyte, was employed for HPLC analysis.

A calibration curve was constructed using DCI standard solutions. As shown in Table S5, a strong linear correlation between peak area and DCI concentration was observed over the tested range, with $R^2 > 0.99$. Based on this calibration curve, the DCI content in TCH was determined to be 6.27 ± 0.04 mg/g. Furthermore, the relative standard deviation (RSD) of TCH measurements was 0.98%, indicating good repeatability and analytical precision.

Previous studies have reported DCI in various natural products. The DCI content in 38 types of monofloral honey from Poland, Australia, Romania, Greece, Cameroon, France, the Czech Republic, Russia, and Brazil have been reported to range from as 0.1-1.38 mg/mL [37]. In comparison, reported DCI contents include 1.1-1.9 mg/g in Tartary buckwheat, 0.43 to 5.79 mg/g in legumes (*Vigna radiata* L.), and approximately 3.3 mg/g in the extract of Cucurbitaceae (*Cucurbita ficifolia*) [38-40]. Notably, TCH in the present study exhibited relatively higher DCI content compared with the reported values for these honey and plant sources, suggesting that it may serve as a rich natural source of DCI. Moreover, the concentration of the high-dose TCH group used in cellular experiments was 10 mg/mL, corresponding to an estimated DCI content of 62.7 μ g. This level exceeded the DCI concentration used in the cellular experiments (15.67 μ g/mL), suggesting that the selected dose is biologically relevant and supporting a potential role of DCI to the antioxidant activity of TCH.

3.4. Protective effect of TCH and TCMs on H₂O₂-induced oxidative damage in LO2 cells

3.4.1. Concentration screening of H₂O₂, TCH and TCMs

In this study, H₂O₂ was used to induce oxidative damage in LO2 cells. As a widely used *in vitro* inducer of oxidative stress, H₂O₂ is commonly employed to simulate the pathological process of oxidative injury conditions observed *in vivo* [41]. As shown in Figure 3A, cell activity decreased in a concentration-dependent manner. At 500 μ mol/L, the cell viability was approximately 50%. Therefore, 500 μ mol/L was selected as the optimal concentration for establishing the oxidative damage model in LO2 cells.

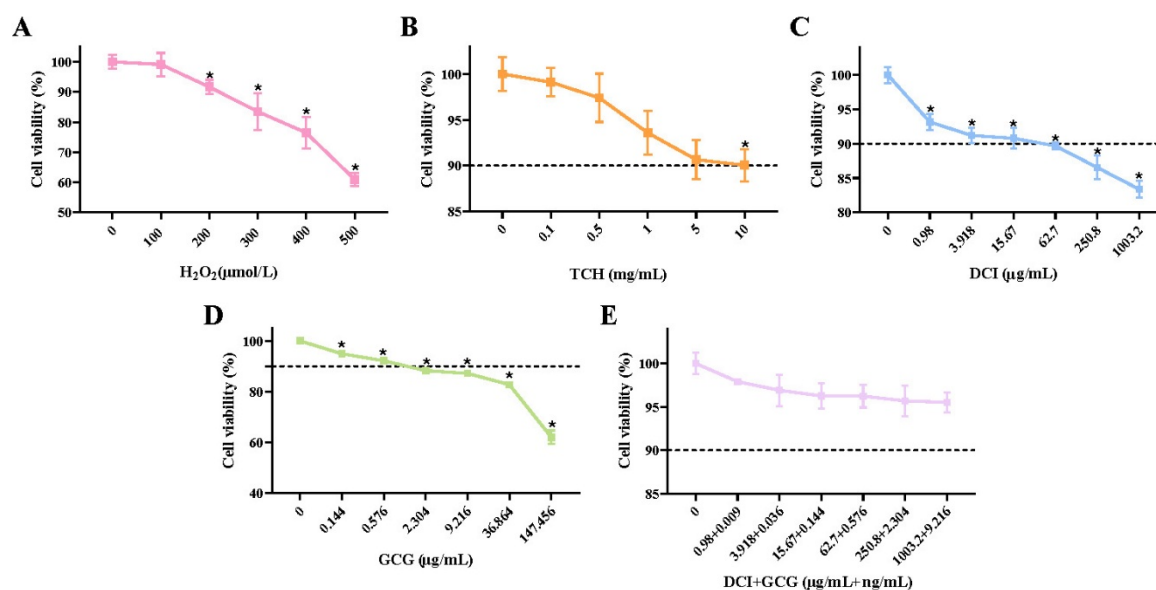


Figure 3. Effects of treatments with different concentrations of hydrogen peroxide (H₂O₂), TCH, D-chiro-inositol (DCI) and (-)-gallicocatechin gallate (GCG) on the viability of LO2 cells. (A) H₂O₂. (B) TCH. (C) DCI. (D) GCG. (E) DCI+GCG. Data were analyzed using one-way ANOVA followed by Tukey's HSD test to adjust for multiple pairwise comparisons. * Indicated significant difference ($P < 0.05$) versus the control group, respectively.

The cytotoxicity of TCH and TCMs on LO2 cells was evaluated. As shown in Figure 3B-E, TCH at concentrations ranged from 0.1 to 5 mg/mL did not significantly affect cell viability compared with the control group ($P > 0.05$). At 10 mg/mL, a statistically significant difference was observed. However, cell viability remained at 90.03%, exceeding the commonly accepted 90% viability threshold, indicating no evident cytotoxicity at this concentration^[42]. Therefore, within the concentration range of 0.1-10 mg/mL, TCH did not exhibit significant inhibitory effects on cellular viability. Therefore, TCH concentrations ≤ 10 mg/mL were considered non-toxic and selected for subsequent experiments. Similarly, DCI (≤ 15.67 $\mu\text{g/mL}$), GCG (≤ 0.576 $\mu\text{g/mL}$), and DCI+GCG (≤ 1003.2 $\mu\text{g/mL} + 9.216$ ng/mL) maintained LO2 cell viability above 90%, indicating no inhibitory effects on cellular viability. Accordingly, these concentration ranges were selected for further experiments.

3.4.2 Effect of TCH and TCMs on the viability of LO2 cells subjected to H₂O₂-induced oxidative damage

To evaluate the protective effects of TCH and TCMs against H₂O₂-induced oxidative damage, LO2 cells were pretreated with various concentrations of TCH, TCMs or VC for 24 h, followed by exposure to H₂O₂ for 6 h. VC, a well-established antioxidant, was used as a positive control because of its protective effects against H₂O₂-induced cell death.^[43] As illustrated in Figure 4A, H₂O₂ treatment significantly reduced cell viability to 54.59% with control group (cell viability = 100%) ($P < 0.05$), indicating successful establishment of the oxidative damage model. Pretreatment with VC (15 $\mu\text{g/mL}$) significantly alleviated the reduction in cell viability. Similarly, pretreatment with TCH (2.5-10 mg/mL) significantly protected LO2 cells against H₂O₂-induced cell death in a dose-dependent manner. At 10 mg/mL, TCH exhibited a protective effect comparable to that of VC (15 $\mu\text{g/mL}$), restoring the cell viability to 68.83% and 70.78%, respectively. Therefore, TCH concentrations of 2.5, 5, and 10 mg/mL were selected as the low-, medium-, and high-dose levels, respectively, for subsequent experiments.

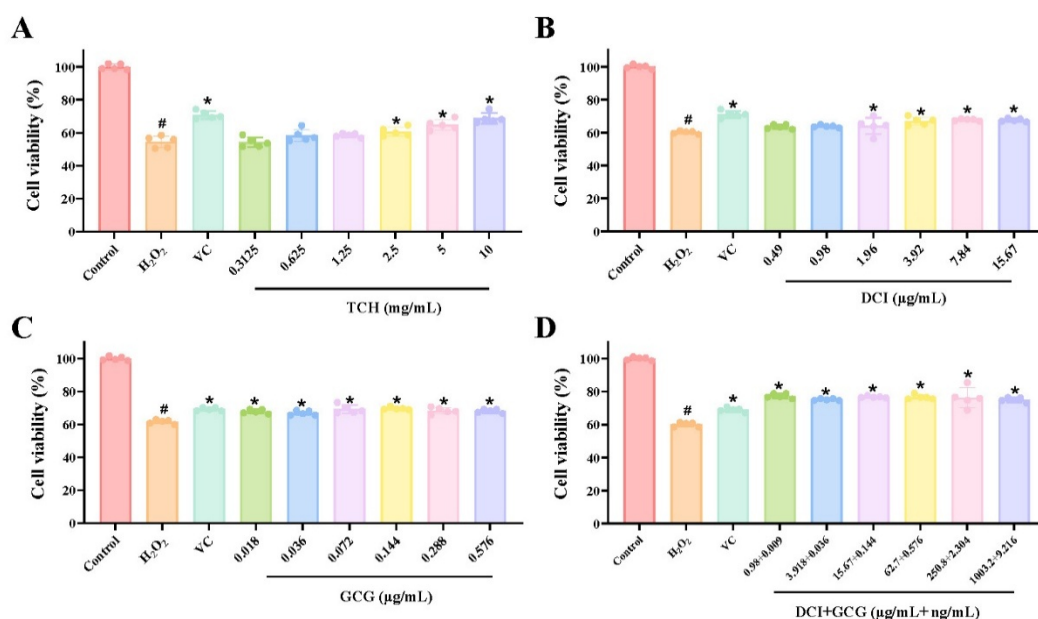


Figure 4. Protective effects of treatments of Vitamin C (VC), TCH and *Triadica cochinchinensis* honey monomers (TCMs) against H₂O₂-induced viability damage in LO2 cells. (A) VC and TCH. (B) DCI. (C) GCG. (D) DCI+GCG. Data were analyzed using one-way ANOVA followed by Tukey's HSD test to adjust for multiple pairwise comparisons. # indicated significant difference ($P < 0.05$) versus the control group, * indicated significant difference ($P < 0.05$) versus the H₂O₂ group.

As shown in Figure 4B-D, DCI treatment at concentrations ranging from 1.96 to 15.67 $\mu\text{g/mL}$ significantly increased the viability of LO2 cells subjected to H₂O₂-induced oxidative damage ($P < 0.05$). GCG and the DCI+GCG combination treatments also improved cell viability across all tested concentrations. These results suggested that TCMs can alleviate H₂O₂-induced oxidative damage, with the combination treatment exhibiting the strongest protective effect. Based on these results, 15.67 $\mu\text{g/mL}$ DCI, 0.576 $\mu\text{g/mL}$ GCG, and 15.67 $\mu\text{g/mL}$ +0.144 ng/mL DCI+GCG were selected for further mechanistic investigations.

3.4.3 Effects of TCH and TCMs on intracellular oxidative stress parameters in H₂O₂-induced oxidative damage in LO2 cells

MDA, a lipid peroxidation product formed when ROS attack membrane phospholipids and polyunsaturated fatty acids, is widely used as a biomarker of oxidative stress and cellular membrane damage [44]. T-AOC is a comprehensive indicator of overall antioxidant status [45]. Compared with control group, H₂O₂ treatment significantly increased MDA levels ($P < 0.05$), while decreasing T-AOC levels. In contrast, treatment with TCH at different concentrations and with TCMs significantly inhibited MDA production and increased T-AOC level, except in the low-dose TCH treatment group (Figure 5 A-D).

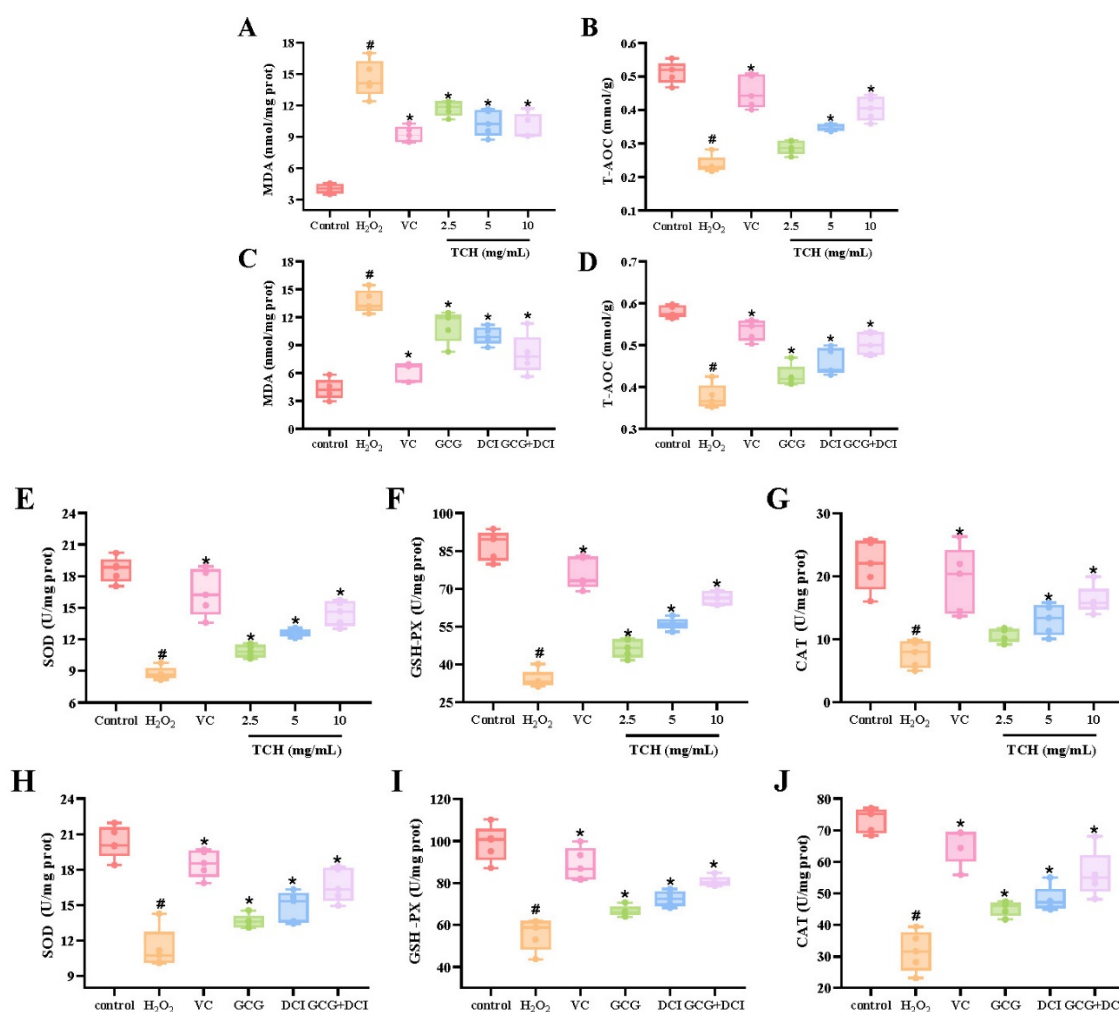


Figure 5. Effects of treatments of TCH and TCMs on the malondialdehyde (MDA), total antioxidant capacity (T-AOC) level and antioxidant enzyme activities in H_2O_2 -injured LO2 cells. (A, B) MDA level. (C, D) T-AOC level. (E, F) Superoxide Dismutase (SOD) activity. (G, H) Glutathione Peroxidase (GSH-Px) activity. (I, J) Catalase (CAT) activity. Data were analyzed using one-way ANOVA followed by Tukey's HSD test to adjust for multiple pairwise comparisons. # indicated significant difference ($P < 0.05$) versus the control group, * indicated significant difference ($P < 0.05$) versus the H_2O_2 group.

Excessive ROS generated by H_2O_2 can induce oxidative damage and ultimately lead to cell death. Intracellular antioxidant enzymes play a critical role in maintaining redox homeostasis by scavenging free radicals [46]. SOD rapidly catalyzes the dismutation of superoxide anion radicals, thereby reducing oxidative stress and limiting lipid peroxidation [47]. GSH-Px, an important antioxidant enzyme, reduces hydrogen peroxide and other peroxides to less harmful hydroxy compounds, thereby protecting cellular structure from oxidative damage [48]. CAT is the primary enzyme responsible for the decomposition of H_2O_2 into water and oxygen and exhibits one of the highest turnover rates among antioxidant enzymes [49]. Within biological systems, SOD catalyzes the dismutation of superoxide anions to form H_2O_2 , which is subsequently decomposed into water and molecular oxygen by GSH-Px and CAT [50]. As shown in Figure 5 E-J, all TCMs treatment groups exhibited significantly increased activities of the antioxidant enzymes SOD, GSH-Px and CAT compared with the model group. Notably, TCH treatment at all tested doses significantly increased SOD and GSH-Px activities compared with the model group. Furthermore, CAT activity was significantly higher in the medium- and high-dose TCH treatment groups than in the model group.

Collectively, the effects of TCH exhibited a clear dose-dependent trend. Moreover, among the TCMs treatments, the DCI+GCG combination showed the strongest protective effects. These findings demonstrated that both TCH and TCMs possess potent antioxidant activity in the cellular oxidative stress model. They effectively alleviated oxidative damage in LO2 cells by reducing the formation of the MDA levels, enhancing total antioxidant activity, and increasing the activities of intracellular antioxidant enzymes.

Previous studies have demonstrated that DCI protects HepG2 cells against palmitic acid-induced oxidative damage by enhancing SOD and glutathione reductase activities, restoring mitochondrial membrane potential, and reducing intracellular ROS and MDA levels [51]. It has also been reported to alleviate oxidative stress in 3T3-L1 adipocytes by decreasing H₂O₂ levels, increasing GSH-Px activity, and regulating the ratio of reduced to oxidized glutathione [40]. Similarly, the neuroprotective effects of GCG against glutamate-induced oxidative stress in HT22 cells have been attributed to reduced intracellular free radicals' accumulation and Ca²⁺ influx, as well as the inhibition of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK) phosphorylation [52].

3.4.4. Effects of TCH and TCMs on ROS production, apoptosis, and cell cycle in H₂O₂-induced oxidative damage in LO2 cells

ROS act as key signaling molecules involved in cell growth, differentiation, proliferation and death [53]. Under physiological conditions, ROS participate in normal hepatocyte function. However, excessive ROS production induces oxidative stress, disrupts cellular membrane integrity, and impairs liver cell function [54]. To investigate the effects of TCH and TCMs on H₂O₂-induced ROS generation, the DCFH-DA fluorescent probe was employed. As shown in Figure 6A, B, the model group exhibited significantly higher fluorescence intensity than the control group, with increases of 1.3- and 1.8-fold, respectively ($P < 0.05$). In contrast, treatments with TCH and TCMs significantly reduced fluorescence intensity. Notably, the 10 mg/ml TCH and TCMs combination treatment groups showing decreases of 37.5% and 11.3%, respectively, which were comparable to those observed in the VC treatment group (39.7% and 15.0%, respectively) ($P < 0.05$). These findings indicate that TCH and TCMs mitigate oxidative damage by suppressing H₂O₂-induced ROS overproduction.

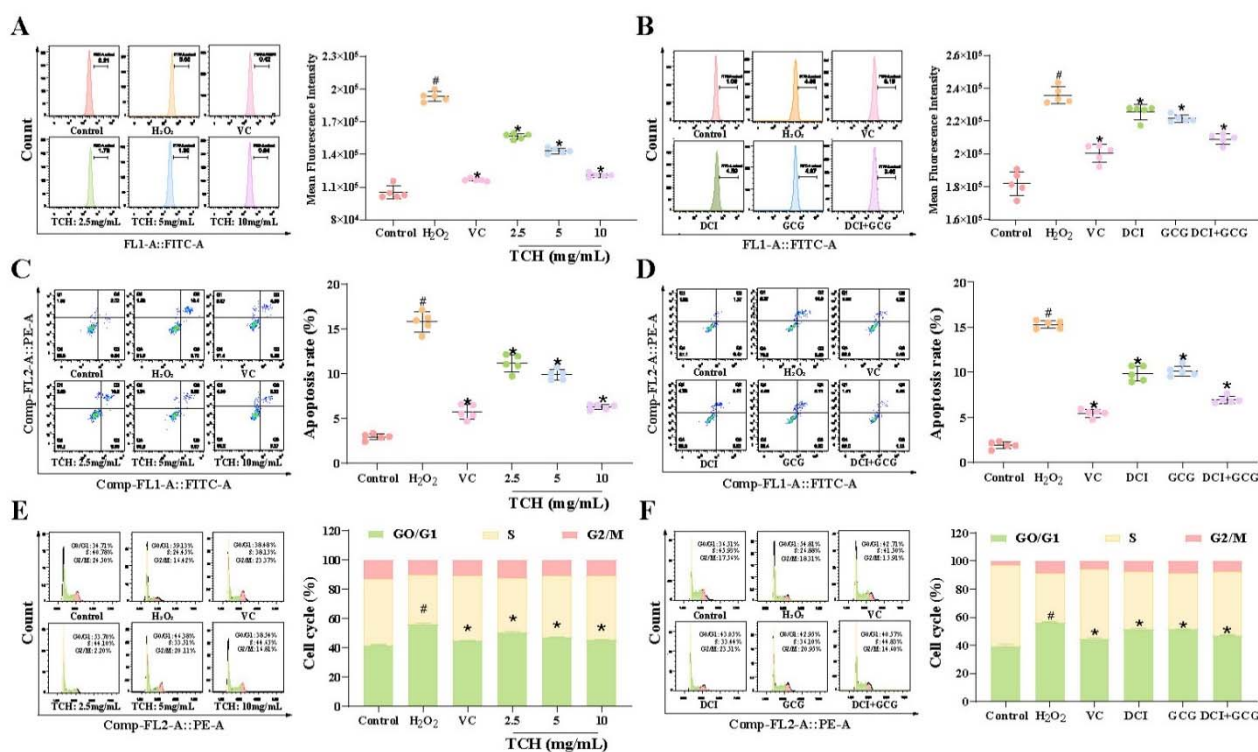


Figure 6. Effects of treatments of TCH and TCMs on reactive oxygen species (ROS), apoptosis and cycle in H₂O₂-injured LO2 cells. (A, B) Representative ROS fluorescence histogram and quantified analysis of ROS production percentages in cells. (C, D) Representative apoptosis scatter plots and quantified analysis of apoptosis percentages in cells. Representative cell cycle histogram and quantified analysis of cycle percentages in cells (E, F). Data were analyzed using one-way ANOVA followed by Tukey's HSD test to adjust for multiple pairwise comparisons. # indicated significant difference ($P < 0.05$) versus the control group, * indicated significant difference ($P < 0.05$) versus the H₂O₂ group.

ROS are also key regulators of hepatocyte apoptosis [55]. H₂O₂ serves as a potent inducer of oxidative stress, triggering excessive ROS accumulation, disrupting intracellular redox homeostasis, and thereby leading to cell apoptosis [56, 57]. Compared with the control group, the apoptosis rate of LO2 cell was significantly increased in the model, TCHs-treated and TCMs-treated groups ($P < 0.05$) (Figure 6C, D). However, compared with the model group, all TCH and TCMs treatment groups significantly reduced apoptosis rates, with all reductions exceeding 30.1%. The 10 mg/mL TCH and combination treatment groups showed the most pronounced effects. These findings demonstrate that TCH and TCMs can effectively inhibit H₂O₂-induced apoptosis of LO2 cells.

The cell cycle refers to the series of events through which a cell progresses from one division to the next. It consists of the interphase and mitotic phase (M phase). The interphase consists of three stages: gap 1 phase (G1 phase), DNA synthesis phase (S phase), and gap 2 phase (G2 phase) [58]. Cell cycle arrest refers to the interruption of cell cycle progression under stress or damaging conditions, preventing cells from progressing to the next phase. This mechanism maintains genomic integrity by halting cell division in response to DNA damage or insufficient cellular resources [59]. The G1 and G2 checkpoint surveillance systems play a crucial role in monitoring genomic integrity during the cell cycle. Upon detection of DNA damage or replication stress, these checkpoints induce cell cycle arrest, with progression resuming only after repairing or resolution of cellular abnormalities [60].

The present study demonstrated that the proportion of cells in the G0/G1 phase was significantly higher in the model group compared with the control group (Figure 6E, F). In contrast, all three TCH-treated and TCMs-treated groups exhibited a significant decrease in the proportion of cells in the G0/G1 phases compared with the model group ($P < 0.05$), with the 10 mg/ mL TCH and combination treatment groups showing the most pronounced effects. Furthermore, the combination treatment exhibited a synergistic effect, with the inhibition percentage exceeding the sum of the effects observed for the individual treatments. Overall, these results indicated that TCH and TCMs reversed G0/G1 phase arrest in LO2 cells, thereby contributing to cellular protection against oxidative damage.

3.5. Protective mechanisms of TCMs against oxidatively damaged LO2 cells

3.5.1. Integrated analysis of transcriptomic and network pharmacology

As illustrated in the volcano plots (Figure 7A-D), the comparison between the Con and H₂O₂ groups identified 3,953 significantly upregulated and 3,959 significantly downregulated DEGs. GCG vs H₂O₂ yielded 678 significantly upregulated and 758 significantly downregulated DEGs. DCI vs H₂O₂ showed 2,144 significantly upregulated and 3,619 significantly downregulated DEGs. GCG+DCI vs H₂O₂ exhibited 1,858 significantly upregulated and 1,826 significantly downregulated DEGs.

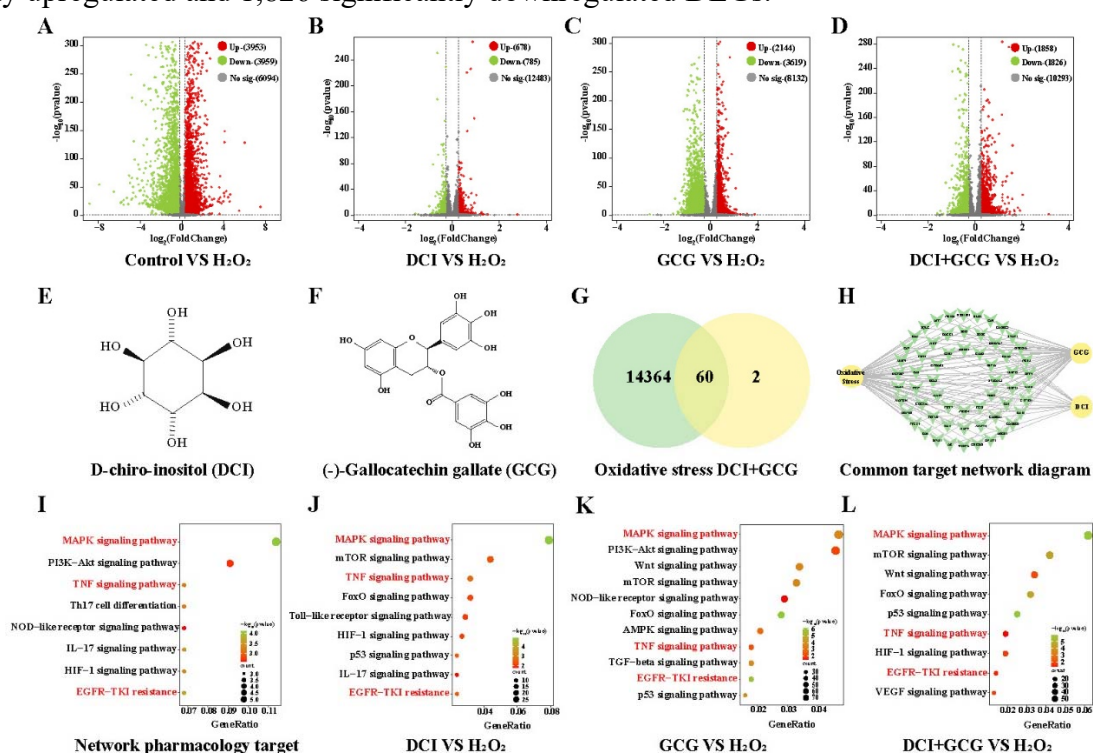


Figure 7. Integrated analysis of transcriptomic and network pharmacology for protective mechanisms of TCMs against oxidative damage-induced LO2 cells. (A-D) Volcano plots of differentially expression genes (DEGs) between the control group and H₂O₂ group, the DCI group and H₂O₂ group, the GCG group and H₂O₂ group, and the DCI+GCG group and H₂O₂ group. (E, F) Structural formula of DCI and GCG. (G) Intersection targets of Venn diagram of oxidative stress and TCMs. (H) Common target network of oxidative stress and TCMs. (I) Kyoto Encyclopedia of Genes and Genomes (KEGG) significantly enriched antioxidant-related pathways of targets from oxidative stress and TCMs. (J-L) KEGG significantly enriched antioxidant-related pathways of DEGs between the DCI group and H₂O₂ group; the GCG group and H₂O₂ group; DCI+GCG group and H₂O₂ group.

Figure 7E, F shows the structural formulas of the TCMs. DCI is a cyclitol with a hydroxyl group on each carbon atom of the ring. It may exert antioxidant effects directly by scavenging hydroxyl radicals or indirectly

by enhancing antioxidative enzymes activity ^[61]. GCG, a flavan-3-ol, contains two benzene rings and a pyranose ring, along with multiple hydroxyl groups and a galloyl group. As a flavonoid, it exhibits strong antioxidant activities ^[62]. Initial target screening of the monomers using the SwissTargetPrediction database identified 63 potential targets, of which 62 were retained after deduplication. Concurrently, 14,424 oxidative stress-associated targets were retrieved from the GeneCards database. Intersection analysis identified 60 overlapping targets (Figure 7G, H), suggesting potential molecular targets through which TCMs may alleviate oxidative stress.

KEGG enrichment analysis was conducted separately for the DEGs from the GCG vs H₂O₂, DCI vs H₂O₂ and GCG+DCI vs H₂O₂ groups, as well as for the 60 potential targets identified through network pharmacology. As shown in Figure 7I-L, all significantly enriched antioxidant-related pathways were identified across the four groups. Among them, the mitogen-activated protein kinase (MAPK) signaling pathway, tumor necrosis factor (TNF) signaling pathway and epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) resistance were consistently enriched across all comparisons. These three signaling pathways may collectively contribute to the antioxidant effects of TCMs in H₂O₂-induced LO2 cells.

The MAPK signaling pathway is a crucial signal transduction cascade involved in cellular processes, such as cell proliferation, differentiation, and stress responses ^[63]. Its major branches, including the ERK, JNK, and p38 MAPK pathways, are closely associated with antioxidant defense mechanisms ^[64]. Activation of these pathways can induce the expression of antioxidant enzymes, thereby enhancing cellular antioxidant activity. The association between the TNF signaling pathway and antioxidant responses is primarily mediated through NF- κ B. TNF activates the I κ B kinase (IKK) complex, which promotes phosphorylation and degradation of I κ B α , thereby activating NF- κ B signaling ^[65]. This pathway regulates the expression of genes involved in oxidative stress responses and modulates cellular antioxidant capacity ^[66]. EGFR is a transmembrane receptor tyrosine kinase receptor whose autophosphorylation initiates multiple downstream signaling cascades. The activation of the janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is crucial in normal cell growth by promoting cell proliferation and apoptosis inhibiting apoptosis ^[67].

3.5.2. Effect of TCMs treatment on antioxidant-related genes and protein expression in H₂O₂-induced oxidatively damaged LO2 cells

To investigate the transcriptomic results and evaluate the effects of TCMs on antioxidant-related genes expression in oxidatively damaged LO2 cells, five significantly down-regulated oxidative stress-related genes (*CASP3*, *FAS*, *CHUK*, *CYP1B1*, and *HSPA6*) shared among the MAPK, TNF and EGFR-TKI resistance signaling pathways, were selected for further analysis. The mRNA and protein expression levels of these genes were subsequently examined. As illustrated in Figure 8, compared with the model group, both mRNA and protein expression levels of all five genes were significantly decreased in the three TCMs treatment groups ($P < 0.05$).

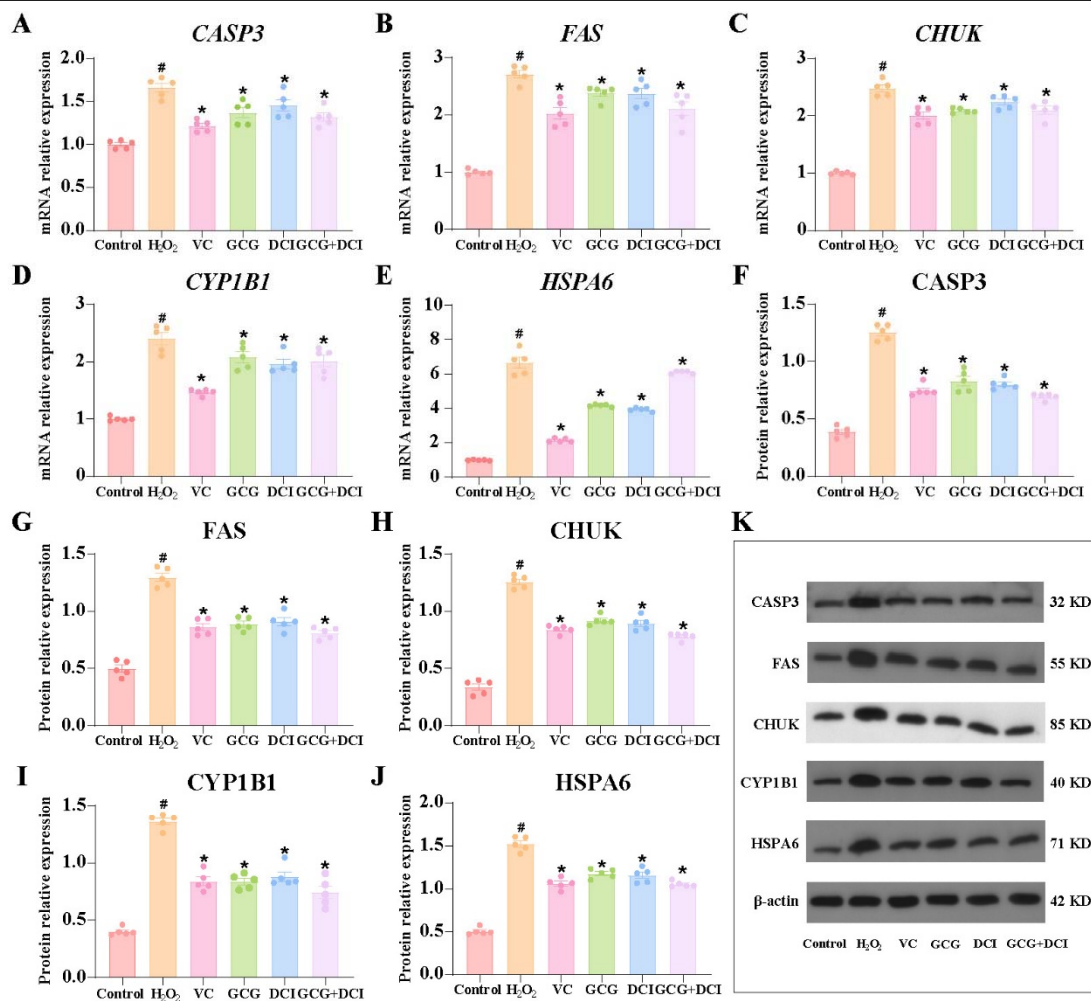


Figure 8. Effects of TCMs on gene and protein levels of mitogen-activated protein kinase (MAPK) signaling pathway, tumor necrosis factor (TNF) signaling pathway and epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) resistance in LO2 cells. (A-E) Relative quantification of caspase 3 (*CASP3*), Fas cell surface death receptor (*FAS*), conserved helix-loop-helix ubiquitous kinase (*CHUK*), cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) and heat shock protein family A member 6 (*HSPA6*) gene expressions. (F-J) Relative quantification of *CASP3*, *FAS*, *CHUK*, *CYP1B1* and *HSPA6* protein expressions. (K) Western blot images of *CASP3*, *FAS*, *CHUK*, *CYP1B1* and *HSPA6* proteins. Data were analyzed using one-way ANOVA followed by Tukey's HSD test to adjust for multiple pairwise comparisons. # indicated significant difference ($P < 0.05$) versus the control group, * indicated significant difference ($P < 0.05$) versus the H₂O₂ group.

Studies have indicated that under oxidative stress, mitochondrial damage in ovarian granulosa cells triggers the B-cell lymphoma-2 (BCL-2) family-regulated apoptotic pathway, leading to *CASP3* activation [68]. As a key executioner caspase, activated *CASP3* initiates DNA fragmentation and cell membrane disintegration by cleaving downstream substrates such as poly (ADP-ribose) polymerase 1 (PARP1) [69]. In studies on ovarian function decline, aberrant *CASP3* expression has been closely associated with oxidative stress-induced granulosa cell apoptosis, indicating its role in senescence-associated oxidative stress responses [70]. The *FAS*/*FASL* signaling pathway is a key regulator of apoptosis, and is activated under oxidative stress, thereby promoting the caspase cascade and ultimately leading to apoptosis [71]. Transcriptional expression of the *CHUK* gene can influence cellular antioxidant activity [72]. Flavonoids (e.g., quercetin and catechins) can enhance cellular antioxidant defenses by inhibiting *CHUK* transcription [72, 73]. *CYP1B1*, a member of the cytochrome P450 enzyme family, catalyzes various monooxygenase reactions. Its upregulation induces cellular oxidative stress, whereas its inhibition reduces oxidative stress levels [74]. Under normal physiological

conditions, HSPA6 exhibits low expression and is primarily localized in tissues such as the kidneys and digestive tract [75]. However, its expression is rapidly induced under pathological conditions, including heat shock, oxidative stress, inflammation, and ischemia-hypoxia, where it exerts cytoprotective effects [76]. These findings suggested that TCMs alleviate H_2O_2 -induced oxidative damage in LO2 cells by regulating the transcription and protein expression of oxidative stress-related gene, indicating potent antioxidant efficacy. Based on these findings, a schematic model was constructed to integrate the multi-target network underlying the potential cellular protective mechanism of TCMs with cellular phenotypes (Figure 9).

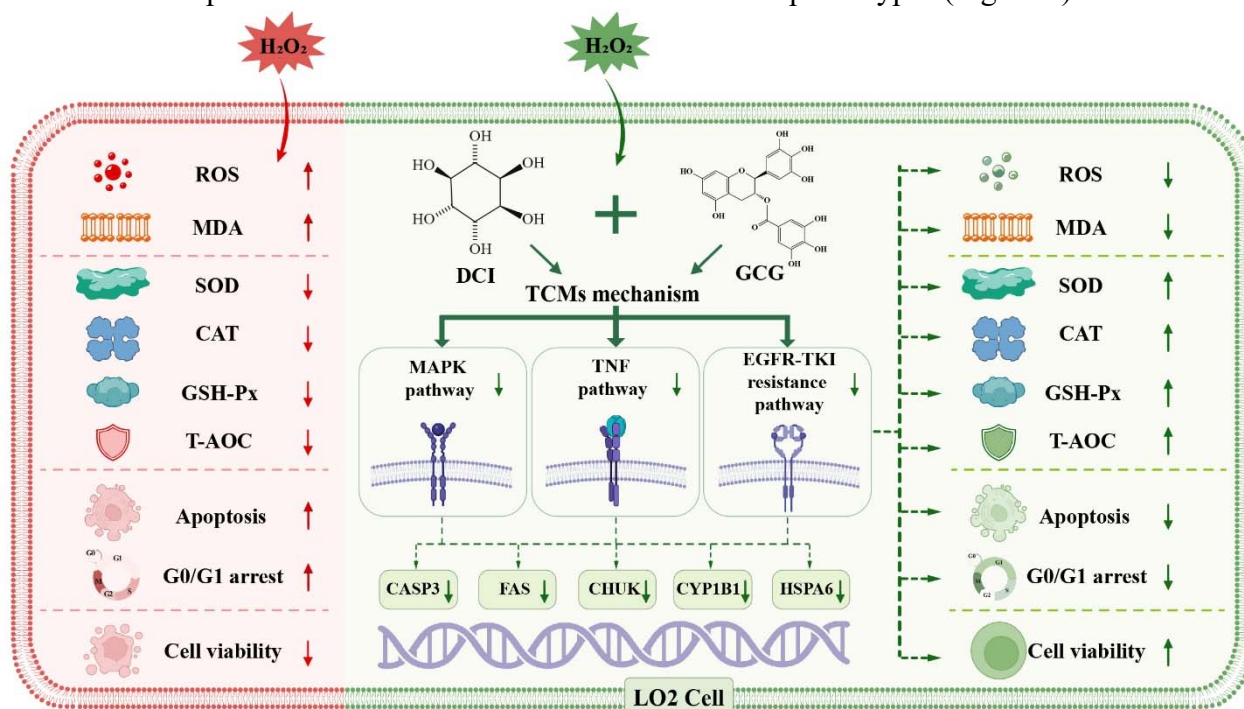


Figure 9. The schematic model integrating the multi-target network and observed cellular phenotypes underlying the antioxidant protective effects of TCMs against H_2O_2 -induced oxidative damage in LO2 cells.

4. Conclusions

This study revealed the antioxidant activity and underlying mechanisms of TCH based on a “composition-activity-mechanism” cascade regulatory framework. Chemical assays showed that TCH was rich in phenolic acids and flavonoids and exhibited significantly higher DPPH radical scavenging activity and FRAP reducing power than nine other common commercial honeys. Further metabolomic analysis of these ten honey varieties identified DCI as a characteristic antioxidant monomer enriched in TCH. DCI levels were significantly higher in TCH compared with the other nine honey varieties. Absolute quantification showed that the DCI content in TCH reached 6.27 mg/g, which was significantly higher than that reported for other honey sources. DCI and GCG, with GCG identified as a unique constituent of TCH in our previous study, collectively represent the characteristic antioxidant monomers of TCH (TCMs).

At the cellular level, both TCH and TCMs improved the viability of LO2 cells under H_2O_2 -induced oxidative damage, reduced intracellular MDA and ROS levels, enhanced the activities of antioxidant enzyme (SOD, GSH-Px, and CAT), increased T-AOC, inhibited apoptosis, and alleviated G0/G1 phase cell cycle arrest, thereby exerting protective effects. Moreover, TCH exhibited a dose-dependent antioxidant effect, and

the DCI+GCG combination showed a significantly stronger effect than either component alone. Transcriptomics and network pharmacology analyses indicated that the potential cellular protective mechanisms of TCMs may be associated with the downregulation of oxidative stress-related genes and signaling pathways, including the MAPK, TNF and EGFR-TKI resistance pathways, in H₂O₂-damaged cells. Collectively, this study provides a molecular basis for the strong antioxidant properties of TCH and supports its potential development and application as a natural functional food with antioxidant activity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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