

## Tangeretin maintains antioxidant activity by reducing CUL3 mediated NRF2 ubiquitination

Yue Wang <sup>a</sup>, Rong Jin <sup>a</sup>, Jiebiao Chen <sup>a</sup>, Jinping Cao <sup>a</sup>, Jianbo Xiao <sup>b</sup>, Xian Li <sup>a</sup>, Chongde Sun <sup>a,\*</sup>

<sup>a</sup> Laboratory of Fruit Quality Biology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Fruit Science Institute, Zhejiang University, Zijingang Campus, Hangzhou 310058, China

<sup>b</sup> Nutrition and Bromatology Group, Department of Analytical Chemistry and Food Science, Faculty of Food Science and Technology, University of Vigo - Ourense Campus, E-32004 Ourense, Spain

### ARTICLE INFO

#### Keywords:

Flavonoids  
Oxidative stress  
NRF2  
CUL3  
Ubiquitin-mediated degradation

### ABSTRACT

To explore the antioxidant capacity of citrus flavonoids under different evaluation systems, chemical and biological methods were engaged to determine the antioxidant abilities of flavanones and polymethoxyflavones. Results showed that flavanones exhibited good antioxidant activity, while polymethoxyflavones had a weak ability to scavenge free radicals. Both flavanones and polymethoxyflavones exerted the ability to inhibit H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, but the effective concentration of polymethoxyflavones was lower. Further exploration showed that neohesperidin and tangeretin selectively regulated antioxidant enzyme activity, both *in vitro* and *in vivo*. Tangeretin also maintained the expression of antioxidant enzymes in L02 cells and in ICR mice liver. The mechanism exploration showed that both neohesperidin and tangeretin promoted the expression of NRF2 and inhibit the expression of KEAP1, but tangeretin could inhibit the ubiquitination of NRF2 by inhibiting CUL3. The mechanism was verified by *CUL3* gene silencing. This study demonstrates a novel antioxidant mechanism of natural products.

### 1. Introduction

Oxidative stress is a state of redox imbalance in organisms, which is caused by excessive accumulation of free radicals and can further cause toxic effects through the production of peroxides and free radicals. When oxidative stress intensifies, the antioxidant system of the organism cannot coordinate the recovery of redox balance, exogenous antioxidants need to be supplemented to assist the recovery of health (Poprac et al., 2017). Research reports, clinical trials and epidemiological studies have shown that the supplementation of bioactive substances from fruit sources helps to promote and maintain oxidative balance (Sun et al., 2020). Flavonoids and other natural products can scavenge free radicals and regulate the expression of antioxidant pathways to play a protective role in oxidative stress, thereby reducing the incidence of diabetes, obesity, nervous system diseases and other chronic diseases (Wang et al., 2021).

Citrus is an important fruit in the world, which is rich in flavonoids and other natural active ingredients. The basic skeleton structure of flavonoids consists of 15 carbon atoms, consisting of two benzene rings (A and B) and a heterocyclic ring (C). The substitution of different sites

and quantities of hydroxyl, methoxy, glycosides and other functional groups on the basic skeleton of C6-C3-C6 contributed to the diversity of flavonoids (Wang et al., 2021). Flavanones and polymethoxyflavones (PMFs) are the most abundant flavonoids in citrus fruits. Structurally, flavanones are usually characterized by glycosides and the double bond hydrogenation between the two and three positions of the heterocyclic ring. On the other hand, PMFs have four or more methoxy substituents, making them less polar and be able to penetrate the cell membrane easily (Wen & Walle, 2006). There have been many reports on the antioxidant effects of citrus flavonoids, especially the scavenging effect of free radicals (Wang et al., 2021). However, the regulatory effects of fruit flavonoids on endogenous antioxidant pathways and their structure-activity relationship remain unclear and need to be further studied.

Nuclear factor (erythroid-derived 2)-like 2, also known as NRF2, is a basic leucine zipper (bZIP) protein. NRF2 is a major regulator of endogenous antioxidant pathways, which activates and promotes the expression of antioxidant enzymes by entering the nucleus and binding to antioxidant response element (ARE) (Chikara et al., 2018). Under silence state, NRF2 is localized in the cytoplasm and is periodically ubiquitinated by Cullin 3 (CUL3)-Kelch-like ECH-associated protein 1

\* Corresponding author.

E-mail address: [adesun2006@zju.edu.cn](mailto:adesun2006@zju.edu.cn) (C. Sun).

(KEAP1)-E3 ubiquitin ligase and targeted for degradation by 26S proteasome. Maintaining low NRF2 expression can prevent excessive inhibition of physiological ROS signal and ensure normal physiological state of cells (Silva-Palacios, Ostolga-Chavarria, Zazueta, & Königsberg, 2018). Under oxidative stress, NRF2 can be activated in two ways. One is the interaction of electrophilic reagents or reactive oxygen species with cysteine residues in KEAP1, which leads to conformational changes and interferes with the ubiquitination degradation of NRF2. The other is that mercaptan modification leads to CUL3 dissociation from KEAP1, resulting in inhibition of NRF2 ubiquitination (Chikara et al., 2018). When the degradation of NRF2 ubiquitination is inhibited, NRF2 remains stable and moves to the nucleus, where it combines with other bZIP proteins to form a heterodimer, which binds to ARE and then activates the expression of antioxidant enzymes. Antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD), nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), and heme oxygenase 1 (HO-1) (Wang et al., 2021). These enzymes coordinate with each other and play a role in redox regulation. It has been reported that fruit flavonoids can activate NRF2 (Yang et al., 2020), but the effect of flavonoids on the periodic degradation of NRF2 and the effect of flavonoids on the maintenance of endogenous antioxidant function remain to be studied.

In this research, the antioxidant capacities of citrus flavanone and PMF components were evaluated using chemical and biological antioxidant methods. PMF fractions and monomers showed considerable differences in antioxidant capacity between the two different evaluation systems. In addition, tangeretin maintained the antioxidant ability of L02 cells; therefore, L02 cells still showed the ability to resist oxidative stress even following the removal of tangeretin. Real-time polymerase chain reaction (RT-PCR) and western blot (WB) assay showed that tangeretin treatment up-regulated NRF2 expression and inhibited the ubiquitination and degradation of NRF2 by inhibiting CUL3 expression. Our hypothesis was verified by *CUL3* gene silencing experiments. *In vivo* experiments showed that tangeretin could induce and maintain the activity of antioxidant enzymes in the liver of mice by inhibiting the ubiquitination degradation of NRF2. Our study illustrates a new mechanism of antioxidant activity of PMFs and demonstrates the diversity of the biological activities of natural citrus products.

## 2. Materials and methods

### 2.1. Materials and reagents

Ougan (*Citrus reticulata* cv. *Suavissima*) extracts (OG), flavanone components (FC), polymethoxyflavone components (PMFC), nobiletin (NOB), tangeretin (TAN) and 5-demethylnobiletin (5DN) were isolated and purified in our laboratory according to our previous report (Wang, Zang, Ji, Cao, & Sun, 2019). Neohesperidin (NHP) and *N*-acetyl-l-cysteine (NAC) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Human normal liver cells (L02), human umbilical vein endothelial cells (HUVEC), human embryonic lung diploid fibroblast (WI38), and human gastric mucosal epithelial cells (GES) were obtained from the Department of Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University. Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Gibco (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Shanghai, China). ROS Assay Kit, CAT Assay Kit, Total SOD Activity Assay Kit, *N*-acetyl-l-cysteine (NAC), and Enhanced Bicinchoninic Acid (BCA) Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Hangzhou, China).  $\gamma$ -Glutamylcysteine Ligase (GCL) Assay Kit was purchased from Solarbio (Beijing, China). Cell fraction kit and NQO1 Activity Assay Kit was purchased from Abcam (Cambridge, MA, USA). MG-132, Anti-

Ubiquitin (P4D1) was purchased from Cell Signaling Technology (Boston, MA, USA). NRF2 antibody, KEAP1 antibody, CUL3 antibody, GCLC antibody, GCLM antibody, CAT antibody, NQO1 antibody, SOD antibody, Lamin B and  $\beta$ -ACTIN antibody were purchased from Proteintech (Rosemont, IL, USA).

### 2.2. Ultra performance liquid chromatography (UPLC) analysis

UPLC analysis was performed according to our previous report (Wang, Zang et al., 2019). Briefly, flavonoids were analyzed by a UPLC system (2695 pump, 2996 diode array detector, Waters) coupled with an Ethylene Bridged Hybrid (BEH) C18 analytical column (ACQUITY UPLC 2.1  $\times$  150 mm, Waters). The injection volume was 2  $\mu$ L and the flow rate was 0.3 mL/min. The detection wavelength was 280 nm for flavanones and 330 nm for PMFs. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The elution gradient procedure was as follows: 0–5 min, 80% of A; 5–8 min, 80 to 66% of A; 8–20 min, 66 to 40% of A; 20–22 min, 40 to 0% of A; 22–23 min, 0 to 80% of A; and 24–25 min, 80% of A.

### 2.3. Chemical antioxidant capacity evaluation

Chemical antioxidant capacities were measured according to (Wang, Ji et al., 2019). Trolox was used as the control; the results were calculated using the standard curve method. The experiment was repeated three times, and the result was expressed as mg TE/g DW.

#### 2.3.1. DPPH free radical scavenging activity

For DPPH free radical scavenging activity, test solution with 2  $\mu$ L volume was added to 198  $\mu$ L of freshly prepared 60  $\mu$ M DPPH solution and reacted for 2 h in the dark at 25 °C. The absorbance at 517 nm was measured using a microplate reader (Synergy H1, BioTek, VT, USA).

#### 2.3.2. Ferric reducing antioxidant power (FRAP)

For ferric reducing antioxidant power (FRAP), 180  $\mu$ L FRAP working solution was mixed with 20  $\mu$ L diluted sample and reacted in the dark for 5 min at 25 °C. The absorbance at 593 nm was measured with a microplate reader.

#### 2.3.3. Oxygen radical absorbance capacity (ORAC)

For oxygen radical absorbance capacity (ORAC), 150  $\mu$ L of 40 nM sodium fluorescein solution and 25  $\mu$ L diluted test samples were added to the 96-well plate. The mixture was reacted in the dark for 10 min at 37 °C before 25  $\mu$ L of 750 mM AAPH solution was added to the well. The fluorescence intensity was measured using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The data reading was performed at 2 min intervals for a total duration of 2 h. PBS was used as a blank control.

#### 2.3.4. ABTS radical scavenging activity

For ABTS radical scavenging activity, 200  $\mu$ L ABTS working solution was mixed with 10  $\mu$ L appropriately diluted test solution, then reacted for 5 min in the dark at 25 °C. The absorbance was measured at 734 nm with a microplate reader.

### 2.4. Cellular antioxidant capacity evaluation

#### 2.4.1. Cell culture

The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 20 mM HEPES, and 100 U/mL penicillin and streptomycin. The cells were grown at 37 °C in a 5% CO<sub>2</sub> humidified incubator (ThermoFisher Scientific 3111, ThermoFisher Scientific, Waltham, MA, USA).

#### 2.4.2. H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in L02 cell line

The cells were pretreated in serum-free RPMI 1640 medium for 24 h.

Different concentrations of flavonoid components and monomers were dissolved in DMSO for a final concentration of 0.1%, and then incubated with cells for 6 h. DMSO was used as the solvent control, while NAC was used as a positive control. The reagent treatments were separated into retained or removed groups. For the removing pretreatment, cells were washed twice with PBS; then new serum-free RPMI 1640 medium was added and incubated for 30 min. Cells were incubated with H<sub>2</sub>O<sub>2</sub> at a concentration of 0.78 mM. Cell viability and ROS content were determined as described below.

#### 2.4.3. Cell viability assay

The cell viability assay was performed using a CCK-8 assay. Briefly, the cell culture medium was removed; the cells were washed twice with PBS. The CCK-8 solution was diluted in serum-free RPMI 1640 medium and added to the wells. After incubation for 1 h, the absorbance was measured at 620 nm and 450 nm using a microplate reader. Each experiment was performed in triplicate and repeated at least three times independently.

#### 2.4.4. ROS detection

After reagent pretreatments, cells were washed twice with PBS. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), diluted in serum-free medium to a final concentration of 10 µM was added to the well. After incubating at 37 °C for 20 min, the DCFH-DA was removed; the cells were washed three times with serum-free medium. Fluorescence microscope was used for observation; the fluorescence intensity was detected using a microplate reader, with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Three independent replicates were performed for each experiment.

#### 2.4.5. Antioxidant enzyme activity assay

Antioxidant enzyme activities were determined using commercial kits. CAT activity was measured via Catalase Assay Kit (Beyotime, S0051); SOD activity was measured using a Total Superoxide Dismutase Assay Kit (Beyotime, S0101); GCL activity was measured with a γ-Glutamylcysteine Ligase Assay Kit (Solarbio, BC1210); NQO1 activity was measured using an NQO1 Activity Assay Kit (Abcam, ab184867). The experimental operations were carried out according to the respective instructions.

#### 2.4.6. Animal experiment

Six-week-old Institute of Cancer Research (ICR) mouse was used for in vivo exploration of tangeretin and neohesperidin. The mice were maintained at 23 – 25 °C and 50 – 60% humidity in the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The mice were randomly divided into three groups with 60 mice in each group: a control group (water), tangeretin treatment group (1 mg·kg<sup>-1</sup>·bw<sup>-1</sup>) and neohesperidin treatment group (500 mg·kg<sup>-1</sup>·bw<sup>-1</sup>). Mice were subjected to a single intragastric administration of the corresponding dose of reagents. Five mice were sacrificed by cervical dislocation after anesthesia at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24 h after intragastric administration. The content of flavonoids and antioxidant indexes were determined in the liver of mice. Our animal experiment ethics approval code is ZJU20190122.

#### 2.5. Real-Time PCR (RT-PCR)

Total RNA was extracted from L02 cells using Trizol (ThermoFisher, 15596–018) according to the protocol from the manufacturer. The contaminating genomic RNA was removed; complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, RR047A). QRT-PCR was carried out in the CFX96 instrument (Bio-Rad) using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, 1725202). β-ACTIN was used as an internal control. The primer sequences used in the RT-PCR are listed in *Supplemental Table 1*. Each treatment was performed in triplicate and repeated at least three times

independently. The relative gene expression was calculated using the 2<sup>-ΔΔCt</sup> method.

#### 2.6. Western blot (WB) assay

Cells or shredded tissues were lysed using NP40 Lysis Buffer (ThermoFisher Scientific, FNN0021) containing 1 × Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, 78440), followed by vortex on ice using a cell crusher (JY98-IIIDN, HUXI). The supernatant was collected after centrifuging at 12,000 rpm for 15 min at 4 °C. Cell fractions were obtained using a Cell Fraction Kit from Abcam (Ab109719) following their instructions. The protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime, P0009); the same amount of proteins was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a PVDF membrane (0.45 µm, ThermoFisher Scientific, 88518). The antibodies used in this study are listed in *Supplemental Table 2*; β-Actin was used as the control. The blot complex was detected with an ECL kit (Service bio, G2014) using a ChemiDoc™ XRS + System (Bio-rad). The amount of protein relative to the control was quantified using Image Lab (Bio-rad, Version 3.0).

#### 2.7. Immunoprecipitation

Cells were freshly lysed using NP40 Lysis Buffer (ThermoFisher Scientific, FNN0021) containing 1 × Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, 78440). The lysate was pre-cleaned with protein G magnetic beads (Bio-rad, 1614023) at 4 °C. Protein G magnetic beads were co-incubated with Nrf2 Antibody (Proteintech, 16396–1AP); IgG control of the same species was added into protein lysates; the mixture was shaken overnight at 4 °C on a rotating wheel. Magnetic beads bound with the immunoprecipitates were collected and washed with PBS-T (Phosphate Buffered Saline with 0.05% Tween-20) five times. The loading buffer was added to the mixture and boiled for 10 min at 100 °C. The supernatants were collected after centrifugation and analyzed by WB assay. MG-132 (Cell Signaling Technology, 2194) was added to the cells as a positive control.

#### 2.8. RNA interference (RNAi) assay

RNAi was performed according to a previous report (Mercer, Snijder, Sacher, Burkard, Bleck, Stahlberg, Pelkmans, & Helenius, 2012). For small interfering RNA (siRNA) transfection, 5 × 10<sup>4</sup> L02 cells were seeded in a 6-well dish for 24 h before transfection. A 20 nM final concentration of siRNA was used to transfet cells and incubated for 72 h. The transfection mix for one well contained 0.5 µL Lipofectamine 2000. siRNAs were purchased from QIAGEN: AACAACTTTCTTCAAACGCTA. The expression of CUL3 was confirmed by RT-PCR and WB analysis.

#### 2.9. Statistics

Results were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were carried out using SPSS version 19.0 (IBM). Data were analyzed by one-way ANOVA. Multiple comparison between the groups was performed using the Tukey method. Graphical representations were generated using OriginPro (version 2019, OriginLab).

### 3. Results

#### 3.1. The chemical antioxidant capacity of PMFs was significantly lower than that of flavanones

Chemical antioxidant assessments are usually used for preliminary screening of antioxidants due to their advantages of simplicity and

rapidity. In this research, four chemical methods were used to evaluate the antioxidant capacity of citrus flavonoids. They were the DPPH, ABTS, ORAC method for evaluating free radical scavenging ability and the FRAP method for evaluating metal ion reduction ability. To compare the chemical antioxidant capacity of citrus flavonoids, we selected FC, PMFC, NOB, TAN and 5DN extracted and purified in our laboratory and purchased chemical standard NHP as materials (Fig. S1). The results showed that the chemical antioxidant capacities of PMFC were significantly lower than FC (Table 1), and all the four antioxidant methods showed similar results. The chemical antioxidant capacities of the three PMFs monomers (NOB, TAN and 5DN) were also lower than that of the flavanone monomer NHP, and there was an order of magnitude difference. The DPPH free radical scavenging ability of 5DN was higher than that of TAN. In the other three antioxidant evaluation methods, there was no significant difference in the chemical antioxidant capacity of PMFs monomers. Few previous studies have shown that neohesperidin or neohesperidin-enriched citrus extracts have strong antioxidant capacities under chemical antioxidant evaluations (Ye et al., 2011; Yu et al., 2005). For tangeretin, there was no previous report on its strong chemical antioxidant capacities. These reports were consistent with our results. Therefore, we concluded that PMFs had a weak ability to directly scavenging free radicals, and their chemical antioxidant capacity was significantly lower than that of flavanones.

### 3.2. Tangeretin showed distinct antioxidant abilities in biological antioxidant evaluation systems

To compare and explore the difference between the biological antioxidant ability and chemical antioxidant capacity of citrus flavonoids, we established cellular antioxidant system and animal antioxidant evaluation system.

#### 3.2.1. TAN inhibited ROS accumulation and maintained the antioxidant enzyme activities in L02 cells

Four cell lines including HUVEC, L02, GES and WI38 were engaged in cellular oxidative stress model (Fig. S2). Cells pretreated with flavonoids were treated with H<sub>2</sub>O<sub>2</sub> to induce oxidative stress, and the antioxidant capacity of citrus flavonoids was evaluated by cell viability and ROS content. The results showed that both flavanones and PMFs could inhibit H<sub>2</sub>O<sub>2</sub>-induced ROS accumulation and cell viability decline, but their effective concentration ranges were different (Figure S3, Fig. 1). The cell viability was the highest when the treatment concentration of FC was 400 mg/L, while the optimal concentration of PMFC was only 0.78 mg/L, showing a significant order of magnitude difference between the two treatments (Figure S3, Fig. 1A). Similar results were also shown in comparison of monomer treatments. The optimal concentration of

NHP was 500 μM, while the optimal concentration of NOB, TAN and 5DN was only 2 μM. PMFs treated with more than 4 μM showed cytotoxicity and inhibited cell viability (Figure S3, Fig. 1A). Among the four cell lines, L02 showed the highest cell viability after flavonoid treatment, while in PMFs monomers, TAN exhibited the strongest cell viability protection effect (Figure S3). Therefore, TAN was used as the representative of PMFs monomer and L02 cells were used as the carrier for subsequent experiments.

The DCFH-DA probe was used to detect the ROS content of cells. The results showed that H<sub>2</sub>O<sub>2</sub> significantly induced the accumulation of ROS, while the two citrus flavonoid monomers TAN (2 μM) and NHP (500 μM) could inhibit the ROS content (Fig. 1B & C). Chemical antioxidant evaluation found that the ability of TAN to eliminate free radicals was weak, so we speculated that TAN exerted its ROS inhibiting effect by inducing cell antioxidant enzymes. The activity and expression of NQO1, SOD, CAT and GCL were analyzed. It was found that both NHP and TAN could selectively induce the expression of antioxidant enzymes (Fig. 1D to G). TAN induced the expression of CAT and NQO1, while NHP induced the expression of GCL and SOD. At the same time, it was also found that TAN and NHP had concentration gradient effect on the induction of antioxidant enzymes. TAN and NHP showed high antioxidant enzyme induction ability at 2 μM and 500 μM, respectively.

In order to verify whether PMFs such as TAN could maintain the antioxidant system, after pretreatment for a period of time, the flavonoid pretreatment in the cell evaluation model was removed, and UPLC detection was used to verify that the flavonoids in the cell and culture medium were removed (Table S3). Then, the antioxidant capacity of cells was evaluated by cell viability, ROS content and the expression of antioxidant enzymes. The results showed that after removal of pretreatment, PMFC, NOB, TAN and 5DN could still maintain cell viability, while FC and NHP had no maintain effect on cell viability (Figure S4, Fig. 1A). At the same time, it was also found that the cell ROS content in the TAN pretreatment group remained low, while the ROS content in the NHP pretreatment group was significantly higher than that in the pretreatment retention group (Fig. 1B & 1C). It was speculated that TAN inhibited the content of ROS by maintaining the activity of antioxidant enzymes. The antioxidant enzyme activity test showed that the gene expression, protein expression and enzyme activity of CAT and NQO1 were still significantly higher than those of the control group after the pretreatment of TAN, while NHP had no such effect (Fig. 1D to 1G).

#### 3.2.2. TAN maintained antioxidant activity in the liver of ICR mice

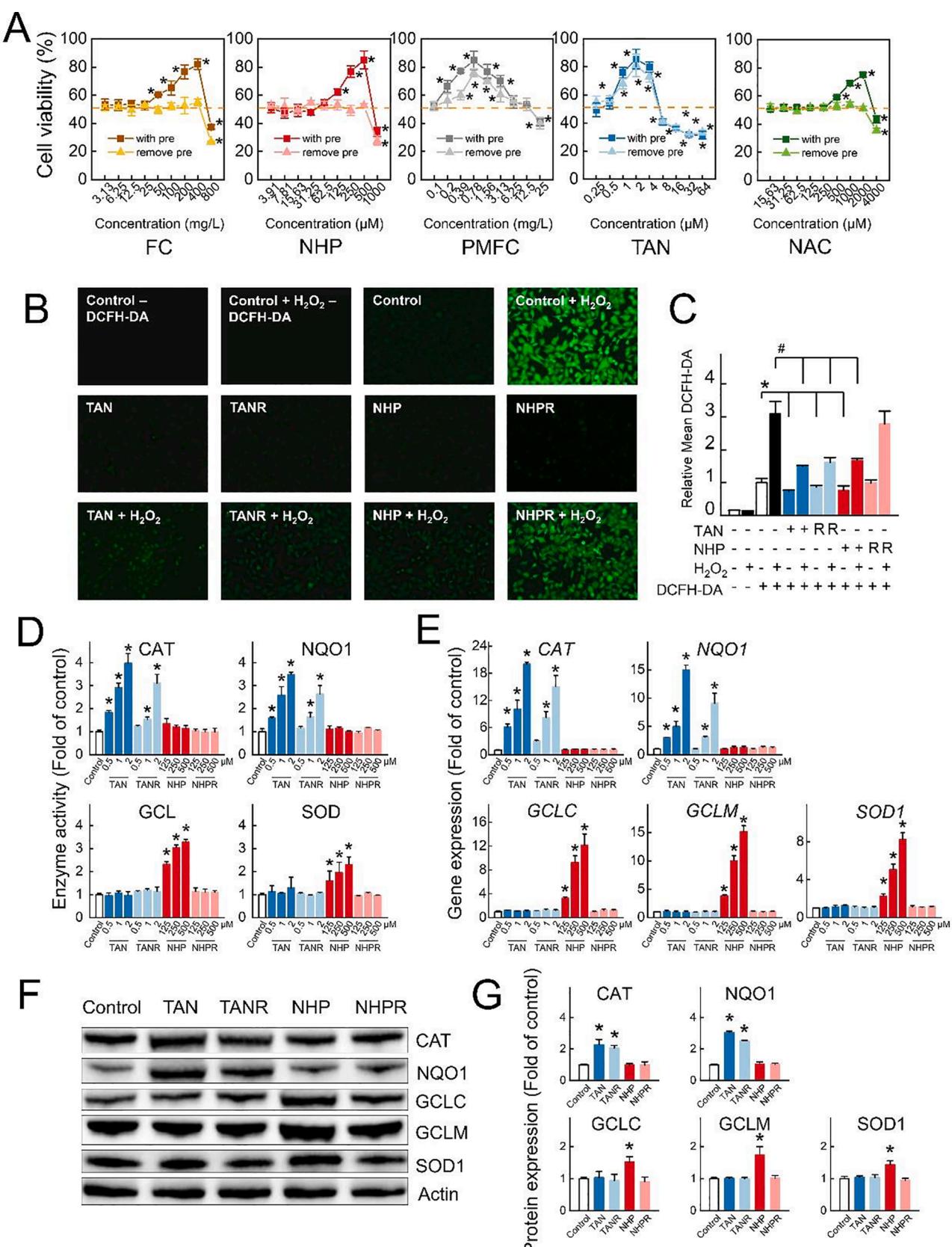
In order to explore the antioxidant capacity of TAN *in vivo*, ICR mice were used to evaluate the effect of TAN on liver antioxidant enzymes. The results showed that the contents of TAN and NHP in mouse liver reached the highest level at 2 h after oral administration of TAN and NHP, while the contents of citrus flavonoids in mouse liver reached the undetected level about 5 h after administration (Fig. 2A). The activity and expression of antioxidant enzymes in mouse liver showed that TAN and NHP showed selective regulation similar to that of antioxidant enzymes in L02 cells (Fig. 2B to 2E). It is worth noting that the intragastric concentrations of TAN and NHP were 1 mg/kg and 500 mg/kg, respectively, indicating that both TAN and NHP could activate antioxidant enzymes in the liver of mice, but the effective concentration of TAN was lower.

The difference of effective dose between TAN and NHP may be caused by different cell absorption rates. In L02 cells, the absorption rate of NHP was only 4.87%, while TAN reached 28.04%, which was 5.8 times higher than that of NHP (Table S3). In mouse liver, the highest accumulation concentration of TAN was 1.97%, while that of NHP was only 0.05, which was 39.4 times of that of NHP (Fig. 2A). However, in cell and animal models, the treatment doses of TAN and NHP differed by 250-fold and 500-fold, and the difference in absorption rates could not fully explain the difference in effective doses. Considering that the antioxidant effect is the cumulative effect of antioxidant enzyme activity, we hypothesized that the low-dose effect of TAN may be related to

**Table 1**  
Chemical antioxidant capacities of the citrus components and monomers.

	DPPH (mg TE/g DW)	FRAP (mg TE/g DW)	ABTS (mg TE/g DW)	ORAC (mg TE/g DW)
FC	91.32 ± 0.34 <sup>d</sup>	95.10 ± 7.77 <sup>b</sup>	203.96 ± 9.81 <sup>c</sup>	4949.16 ± 110.15 <sup>b</sup>
PMFC	4.85 ± 0.30 <sup>b</sup>	6.53 ± 0.38 <sup>a</sup>	4.66 ± 0.34 <sup>a</sup>	89.77 ± 7.20 <sup>a</sup>
NHP	100.77 ± 0.52 <sup>e</sup>	102.97 ± 14.88 <sup>b</sup>	253.58 ± 6.75 <sup>d</sup>	6011.65 ± 192.39 <sup>c</sup>
NOB	3.94 ± 0.03 <sup>ab</sup>	5.42 ± 0.21 <sup>a</sup>	4.34 ± 0.25 <sup>a</sup>	72.66 ± 3.04 <sup>a</sup>
TAN	3.38 ± 0.22 <sup>a</sup>	4.64 ± 0.11 <sup>a</sup>	3.75 ± 0.28 <sup>a</sup>	63.50 ± 2.63 <sup>a</sup>
5DN	4.47 ± 0.28 <sup>b</sup>	6.24 ± 0.24 <sup>a</sup>	4.85 ± 0.28 <sup>a</sup>	87.52 ± 1.61 <sup>a</sup>

DPPH: DPPH free radical scavenging activity; FRAP: ferric reducing antioxidant power; ABTS: ABTS radical scavenging activity; ORAC: oxygen radical absorbance capacity; FC: flavanone components; PMFC: polymethoxyflavone components, NHP: neohesperidin, NOB: nobiletin, TAN: tangeretin, 5DN: 5-demethylnobiletin. The antioxidant capacities (DPPH, FRAP, ABTS, and ORAC) were calculated as mg Trolox equivalent (TE)/g dry weight (DW). Values within each column followed by different superscript letters were significantly different according to Student *t* test (*p* < 0.05).

**Fig. 1.** Cellular antioxidant abilities of the citrus fractions and monomers.

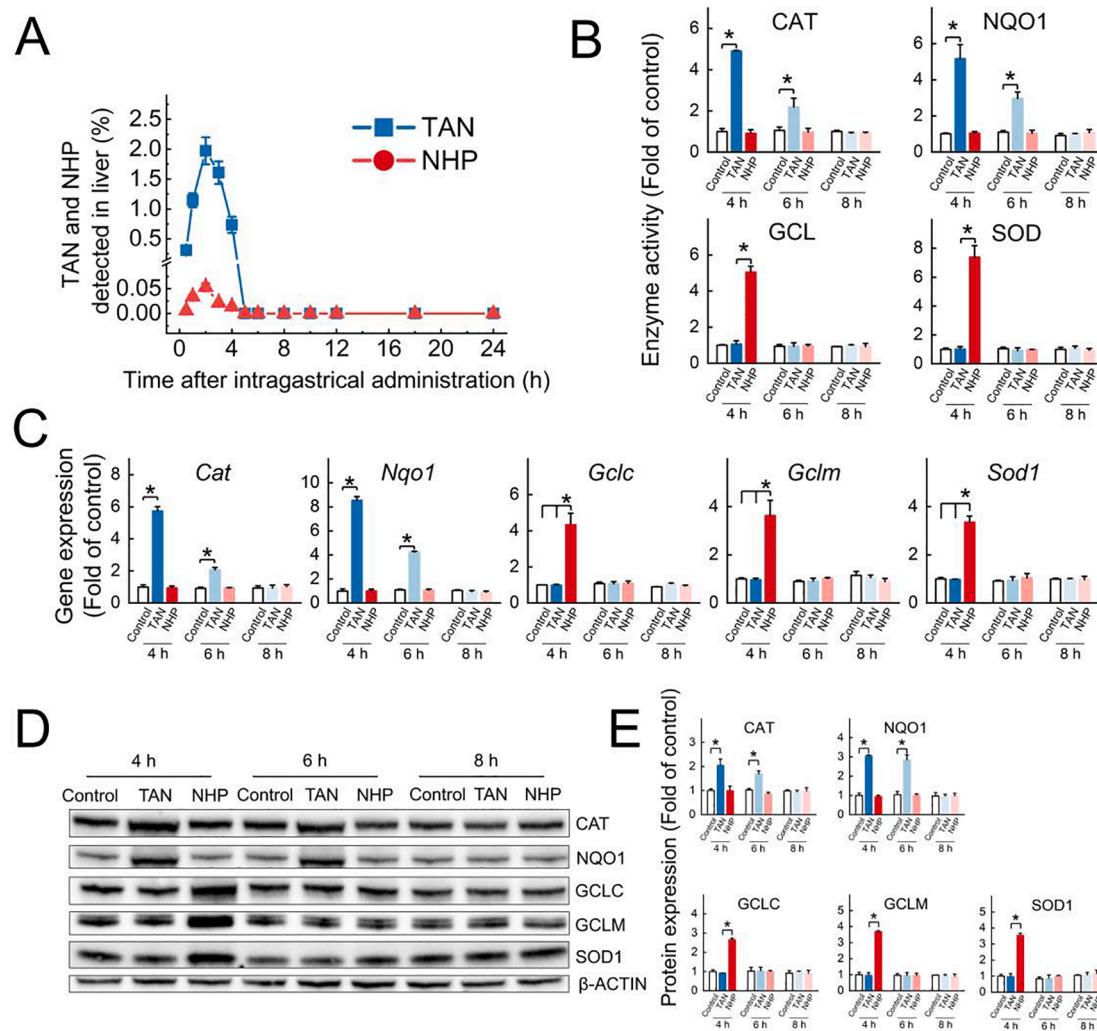


Fig. 2. Effects of TAN and NHP on oxidative stress in the liver of ICR mouse.

the maintaining effect of antioxidant enzymes.

In vivo, we selected 6 h and 8 h time points after intragastric administration of citrus flavonoids (1 h and 3 h after undetectable flavonoids, respectively) to detect the expression and activity of antioxidant enzymes in mice. The results showed that TAN could maintain the expression and activity of antioxidant enzymes at 6 h point, but the effect disappeared at 8 h point, which indicated that TAN could maintain the activity of antioxidant enzymes *in vivo* for a certain period (Fig. 2). There was no difference in antioxidant enzyme activity between the control group and NHP group at 6 h and 8 h points. The above results indicated that TAN could maintain the antioxidant effect *in vitro* and *in vivo*, which partially confirmed our hypothesis, and indicated that the low-dose effect of TAN may be related to the antioxidant enzyme maintenance effect.

### 3.3. Tangeretin reduced ubiquitin-mediated degradation of NRF2 through inhibition of CUL3 expression

#### 3.3.1. Both TAN and NHP promoted the expression and nuclear transfer of NRF2

NRF2 is a major regulator of the activity of antioxidant enzymes. The regulation of citrus flavonoids on the NRF2 was studied by RT-PCR and WB assay. Results showed that both TAN and NHP up-regulated NRF2 gene (Fig. 3A). After the removal of TAN, the changes in antioxidant pathway gene NRF2 expression also disappeared. These results suggest that the maintenance of the antioxidant capacity of L02 cells may not be

caused by the continuous expression of the NRF2 gene caused by TAN pretreatment. The protein expression was determined by WB. Results showed that both TAN and NHP induced NRF2 protein expression (Fig. 3B & 3C). After removing TAN pretreatment, the protein expression of NRF2 was still higher than that of the control group. In vivo, both TAN and NHP up-regulated the expression of *Nrf2* gene at 4 h point after intragastric administration, and the up-regulating effect disappeared at 6 h and 8 h point (Fig. 4A). For protein, TAN and NHP up-regulated the expression of NRF2 protein at 4 h (Fig. 4B & 4C). One hour after citrus flavonoids could not be detected, TAN, but not NHP, continued to regulate NRF2. These results indicated that both TAN and NHP could promote NRF2 gene and protein expression *in vivo* and *in vitro*, but TAN had a protein-level NRF2 maintenance effect. In previous reports, neohesperidin attenuated cerebral ischemia-reperfusion injury by activating the Akt/Nrf2/HO-1 pathway and relieving oxidative stress damage (Wang & Cui, 2013). Tangeretin protected HepG2 cells from tert-butyl hydroperoxide-induced oxidative damage by regulating Nrf2 and mitogen-activated protein kinase (MAPK) signaling pathways (Liang et al., 2018). In addition, as an intrinsic mechanism for exerting biological activity, antioxidation has been a bridge between tangeretin and its anti-inflammatory activity (Arab, Mohamed, Barakat, & Arafa el, 2016) as well as neuroprotective (Braidy et al., 2017) effects.

The transport of NRF2 from cytosol to nucleus is a necessary step in activating antioxidant enzymes (Ge et al., 2017). By isolating nuclear protein and cytoplasmic protein and detecting the expression of NRF2, we found that both TAN and NHP promoted the transport of NRF2 from

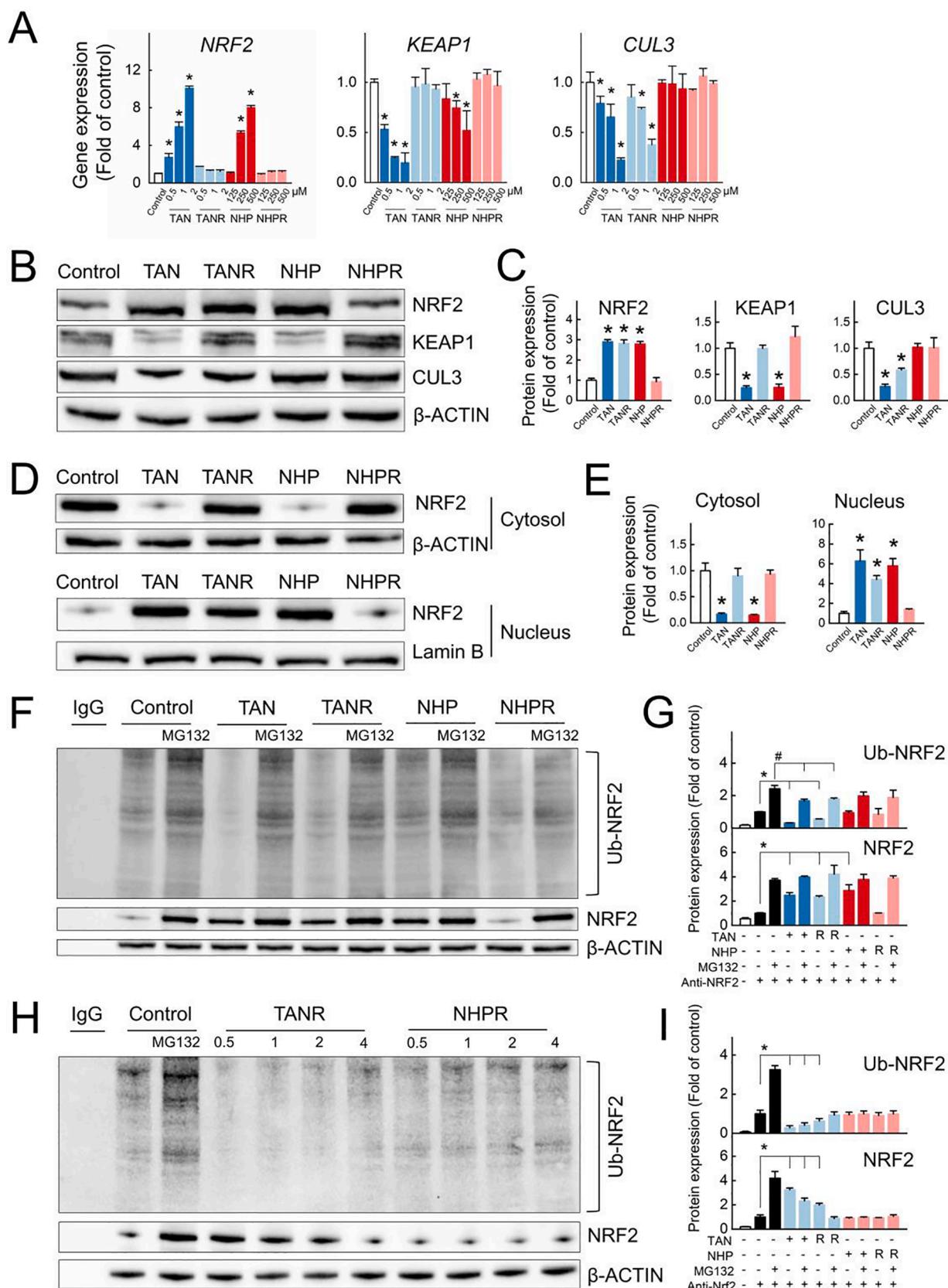


Fig. 3. Regulation of the NRF2-KEAP1-CUL3 pathway in L02 cells by citrus flavonoids.

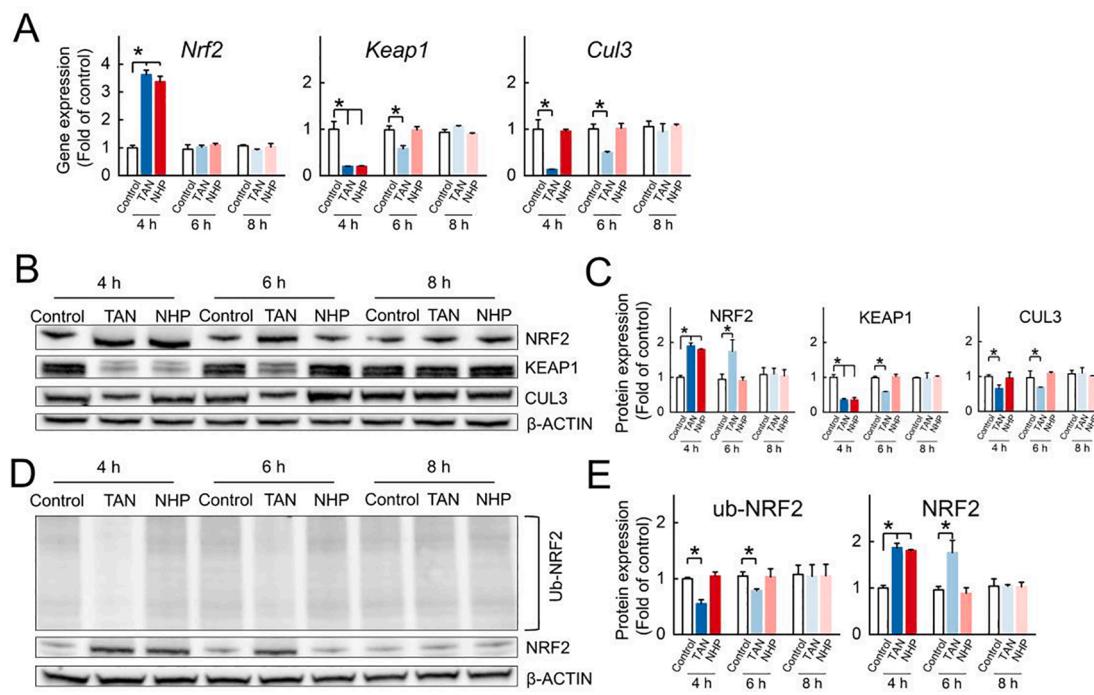


Fig. 4. Regulation of the NRF2-KEAP1-CUL3 pathway in ICR mice liver by citrus flavonoids.

cytosol to nucleus (Fig. 3D and E). When the pretreatment was removed, the protein distribution in the NHP removal group returned to the original level. However, the nuclear NRF2 protein in the TAN removal group was still significantly higher than that in control group. These results suggest that the maintaining effect of TAN on the protein-level of NRF2 may be caused by increasing the residence time of NRF2 in the nucleus, which could be explained by the inhibition of NRF2 ubiquitination degradation.

### 3.3.2. TAN inhibited CUL3 expression and NRF2 ubiquitination

Ubiquitin-mediated degradation is the main factor for the decrease of NRF2 expression. We further studied whether TAN maintained the protein expression of NRF2 through ubiquitin inhibition. Ubiquitin-NRF2 (Ub-NRF2) was determined by immunoprecipitation (IP) of NRF2 followed by western blot assay with anti-ubiquitin antibody. Results showed that TAN, but not NHP, inhibited ubiquitination level (Fig. 3F & G). Through the detection of NRF2 expression and ubiquitin level at different times after removal and pretreatment, we found that within 2 h of removal of TAN, NRF2 expression remained significantly high compared to the control group, and ubiquitin-mediated degradation was also inhibited within 2 h (Fig. 3H and I). These results suggest that TAN could maintain the ability of L02 cells to resist oxidative stress for a certain period of time (2 h) by inhibiting the ubiquitin degradation of NRF2. *In vivo*, TAN intragastric administration treatment inhibited the ubiquitin-mediated degradation, but the NHP did not show this effect (Fig. 4D & E). The ubiquitination inhibiting effect of TAN lasted for 1 h after the TAN metabolism in mouse liver. We concluded that TAN inhibited the ubiquitination degradation of NRF2, both *in vitro* and *in vivo*.

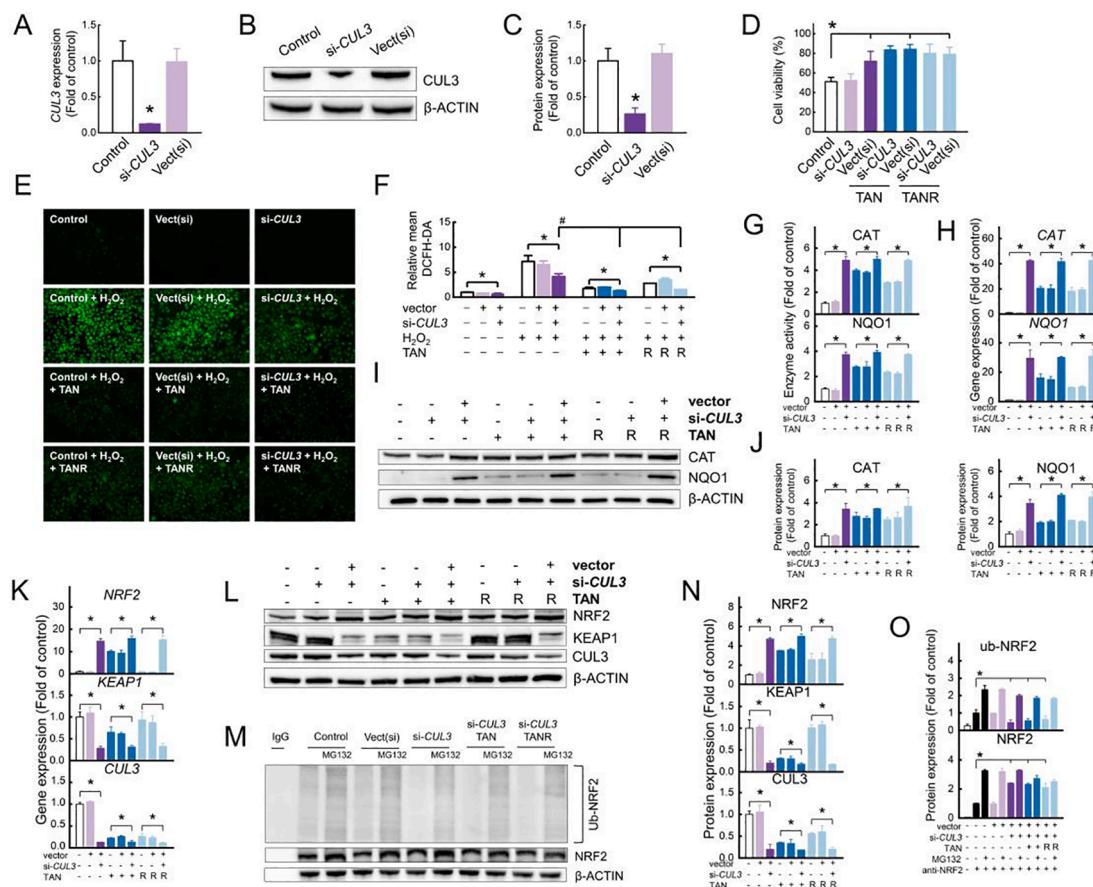
KEAP1 and CUL3 are key proteins that mediate the ubiquitination of NRF2. The KEAP1-CUL3 complex recruits NRF2 to transfer from the nucleus to the cytoplasm and catalyzes the ubiquitination of NRF2. The effects of citrus flavonoids on the expression of KEAP1 and CUL3 were tested by RT-PCR and WB. Results showed that both TAN and NHP inhibited the KEAP1 expression in L02 cells and mice liver (Fig. 3A to C, Fig. 4A to C). In L02 cells, TAN treatment did not show retention of the inhibitory effect of KEAP1 (Fig. 3A to C). While in the mice liver, at 6 h point after TAN treatment, the KEAP1 gene and protein in the TAN

treated group was still significantly lower than that in the control group, which was different from the cell phenotype (Fig. 4A to C). For CUL3, only TAN had an inhibitory effect on the CUL3 gene and protein, NHP treatment had no significant effect on the expression of CUL3 (Fig. 3A to C, Fig. 4A to C). Not only that, TAN also showed a maintenance effect on CUL3 inhibition, and showed a correlation with NRF2 ubiquitination inhibition maintenance (Fig. 3A to C, Fig. 4A to C). The above results indicated that the inhibitory effect of TAN on NRF2 ubiquitination may be mediated by the inhibition of CUL3.

### 3.3.3. CUL3 gene silencing verification

The *CUL3* gene silencing (*si-CUL3*) system was established to verify the inhibition of NRF2 ubiquitination by TAN was mediated by suppressing the expression of CUL3. The establishment of *CUL3* gene silencing was confirmed using RT-PCR and WB. The expression of the *CUL3* gene and CUL3 protein in *si-CUL3* L02 cells was significantly lower than that of the control group and vector group (Fig. 5A to C). The CCK-8 assay was used to examine the effect of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress on cell viability in *si-CUL3* cells after pretreatment. It was found that after *CUL3* was silenced, L02 cells gained an enhanced ability to resist oxidative stress (Fig. 5D), showing the same trend as the TAN treatment. The DCFH-DA probe was used to detect ROS content in L02 cells and the results showed that ROS content decreased after *CUL3* gene silencing (Fig. 5E & F). *CUL3* silencing also led to an increase in antioxidant enzyme activity (Fig. 5G), the gene expression (Fig. 5H) and protein expression (Fig. 5I & J). The changes in CAT and NQO1 activity and expression after gene silencing were consistent with the trend of TAN treatment. Moreover, *CUL3* gene silencing and TAN exhibited synergistic effects on CAT and NQO1 regulation (Fig. 5G to J). These results further indicated that the regulation of antioxidant enzymes and antioxidant capacity of TAN was caused by inhibition of *CUL3* gene.

After *CUL3* silencing, the ubiquitin-mediated degradation of NRF2 protein was inhibited (Fig. 5M and O). This result was in line with the retention or removal of TAN treatment, indicating that the ubiquitination inhibition of NRF2 by TAN treatment was indeed associated with a decreased expression of *CUL3*. The above results further confirmed that TAN could inhibit the ubiquitin process of NRF2 by suppressing *CUL3*, thus enabling L02 cells to maintain antioxidant capacity.

Fig. 5. Mechanism verification by *CUL3* gene silencing.

#### 4. Conclusions

In this study, the antioxidant capacities of two main flavonoids in citrus fruits, flavanones and polymethoxyflavones, were compared. The two flavonoids exhibited different antioxidant effects in the chemical antioxidant evaluation system and the biological antioxidant evaluation system. Flavanones exerted better antioxidant activity under chemical systems, while PMFs showed weak abilities to directly scavenge free radicals. In cellular and animal antioxidant evaluation model, both TAN and NHP exhibited antioxidant effects, but the effective concentration of TAN was lower, NHP exhibited an optimal concentration range of more than 200 times that of TAN. TAN had a maintaining effect on cellular antioxidant abilities, in which the L02 cells exerted resistance against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress even after TAN pretreatment was removed. We tentatively explored the mechanism by which TAN reduced the ubiquitin-mediated degradation of NRF2 via inhibiting CUL3 expression. The mechanism was verified by silencing the key gene, *CUL3*. In vivo experiments showed that TAN could maintain the activity of antioxidant enzymes in mouse liver by inhibiting the ubiquitination degradation of NRF2 after 1 h that TAN could not be detected in mice liver. Our study demonstrates the diverse biological activities of natural products and reveals a new mechanism by which flavonoids exert antioxidant effects.

#### 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.

#### Acknowledgements

This research was supported by the National Natural Science Foundation of China (32072132), the Key Research and Development Program of Zhejiang province (2021C02018), the Fundamental Research Funds for the Central Universities (2020XZX003-03), the Agricultural Outstanding Talents and Innovation Team of the State Agricultural Ministry on Health and Nutrition of Fruit and the 111 project (B17039).

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.130470>.

#### References

- Arab, H. H., Mohamed, W. R., Barakat, B. M., & Arafa el, S. A. (2016). Tangeretin attenuates cisplatin-induced renal injury in rats: Impact on the inflammatory cascade and oxidative perturbations. *Chemico-Biological Interactions*, 258, 205–213. DOI: 10.1016/j.cbi.2016.09.008.
- Braidy, N., Behzad, S., Habtemariam, S., Ahmed, T., Daghia, M., Nabavi, S. M., ... Nabavi, S. F. (2017). Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin and tangeretin in Alzheimer's and Parkinson's Disease. *Cns & Neurological Disorders-Drug Targets*, 16(4), 387–397. <https://doi.org/10.2174/187152731666170328113309>.
- Chikara, S., Nagaprashantha, L. D., Singhal, J., Horne, D., Awasthi, S., & Singhal, S. S. (2018). Oxidative stress and dietary phytochemicals: Role in cancer chemoprevention and treatment. *Cancer Letters*, 413, 122–134. <https://doi.org/10.1016/j.canlet.2017.11.002>.
- Ge, W., Zhao, K., Wang, X., Li, H., Yu, M., ... Hu, Y. (2017). iASPP is an antioxidative factor and drives cancer growth and drug resistance by competing with Nrf2 for Keap1 Binding. *Cancer Cell*, 32(5), 561–573.e6. <https://doi.org/10.1016/j.ccr.2017.09.008>.
- Liang, F., Fang, Y., Cao, W., Zhang, Z., Pan, S., & Xu, X. (2018). Attenuation of *tert*-butyl hydroperoxide (t-BHP)-induced oxidative damage in HepG2 cells by tangeretin:

- Relevance of the Nrf2-ARE and MAPK signaling pathways. *Journal of Agricultural and Food Chemistry*, 66(25), 6317–6325. <https://doi.org/10.1021/acs.jafc.8b01875>.
- Mercer, J., Snijder, B., Sacher, R., Burkard, C., Bleck, C. K., Stahlberg, H., Pelkmans, L., & Helenius, A. (2012). RNAi screening reveals proteasome- and Cullin3-dependent stages in vaccinia virus infection. *Cell Reports*, 2(4), 1036–1047. DOI:10.1016/j.celrep.2012.09.003.
- Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C. J., & Valko, M. (2017). Targeting free radicals in oxidative stress-related human diseases. *Trends in Pharmacological Sciences*, 38(7), 592–607. <https://doi.org/10.1016/j.tips.2017.04.005>.
- Silva-Palacios, A., Ostolaga-Chavarria, M., Zazueta, C., & Königsberg, M. (2018). Nrf2: Molecular and epigenetic regulation during aging. *Ageing Research Reviews*, 47, 31–40. <https://doi.org/10.1016/j.arr.2018.06.003>.
- Sun, C., Liu, Y., Zhan, L., Rayat, G. R., Xiao, J., Jiang, H., ... Chen, K. (2020). Anti-diabetic effects of natural antioxidants from fruits. *Trends in Food Science & Technology*. <https://doi.org/10.1016/j.tifs.2020.07.024>.
- Wang, J. J., & Cui, P. (2013). Neohesperidin attenuates cerebral ischemia-reperfusion injury via inhibiting the apoptotic pathway and activating the Akt/Nrf2/HO-1 pathway. *Journal of Asian Natural Products Research*, 15(9), 1023–1037. <https://doi.org/10.1080/10286020.2013.827176>.
- Wang, Y., Ji, S., Zang, W., Wang, N., Cao, J., Li, X., & Sun, C. (2019). Identification of phenolic compounds from a unique citrus species, finger lime (*Citrus australasica*) and their inhibition of LPS-induced NO-releasing in BV-2 cell line. *Food and Chemical Toxicology*, 129, 54–63. <https://doi.org/10.1016/j.fct.2019.04.006>.
- Wang, Y., Liu, X., Chen, J., Cao, J., Li, X., & Sun, C. (2021). Citrus flavonoids and their antioxidant evaluation. *Critical Reviews in Food Science and Nutrition*, 1–22. <https://doi.org/10.1080/10408398.2020.1870035>.
- Wang, Y., Zang, W., Ji, S., Cao, J., & Sun, C. (2019). Three Polymethoxyflavones Purified from Ougan (*Citrus reticulata* cv. *Suavissima*) Inhibited LPS-Induced NO Elevation in the Neuroglia BV-2 Cell Line via the JAK2/STAT3 Pathway. *Nutrients*, 11(4), 791. <https://doi.org/10.3390/nu11040791>.
- Wen, X., & Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition*, 34(10), 1786–1792. <https://doi.org/10.1124/dmd.106.011122>.
- Yang, C., Hsiao, L., Lin, H., Tseng, H., Situmorang, J. H., Leu, Y., & Yang, C. (2020). Induction of HO-1 by 5,8-dihydroxy-4',7-dimethoxyflavone via activation of ROS/p38 MAPK/Nrf2 attenuates thrombin-induced connective tissue growth factor expression in human cardiac fibroblasts. *Oxidative Medicine and Cellular Longevity*, 2020, 1–18. <https://doi.org/10.1155/2020/1080168>.
- Ye, X.-Q., Chen, J.-C., Liu, D.-H., Jiang, P., Shi, J., Xue, S., ... Kakuda, Y. (2011). Identification of bioactive composition and antioxidant activity in young mandarin fruits. *Food Chemistry*, 124(4), 1561–1566. <https://doi.org/10.1016/j.foodchem.2010.08.013>.
- Yu, J., Wang, L., Walzem, R. L., Miller, E. G., Pike, L. M., & Patil, B. S. (2005). Antioxidant activity of citrus limonoids, flavonoids, and coumarins. *Journal of Agricultural and Food Chemistry*, 53(6), 2009–2014. <https://doi.org/10.1021/jf0484632>.