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Alleviating Effects of Citrus Polymethoxyflavones on Autoimmune Thyroiditis via IL-17 Signaling Pathway

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ABSTRACT

Autoimmune thyroid diseases (AITDs), characterized by chronic thyroid inflammation and autoantibody production, currently lack targeted therapies, highlighting the need for natural immunomodulators. This study systematically investigated the immunomodulatory effects of citrus flavonoids, particularly polymethoxyflavones (PMFs), on AITDs. Flavonoid extracts from six citrus varieties were screened on Nthy-ori3-1 cells. Extracts from Ougan, Eureka lemon, and Newhall sweet orange significantly reduced thyroid peroxidase antibody (TPO-Ab) levels by 25%–34% ($p < 0.001$), demonstrating potential anti-AITDs activity. High-performance liquid chromatography identified and quantified thirteen flavonoids, among which eight representative compounds were further evaluated. Tangeretin exhibited the strongest activity, lowering TPO-Ab levels by 64% ($p < 0.0001$) and downregulating interleukin 1 beta (IL1B), interleukin 6 (IL6), and tumor necrosis factor-α (TNF) by 1.9-fold–2.5-fold in an AITDs cell model. Mechanistically, PMFs, specifically tangeretin and nobiletin, alleviated AITDs by modulating the IL-17 signaling pathway, regulating key regulators such as TNF receptor-associated factor 3 (TRAF3), TRAF3-interacting protein 2 (Act1), nuclear factor kappa B subunit 1 (NFKB1), interleukin 1 beta (IL-1β), interleukin 6 (IL6), and tumor necrosis factor-α (TNF-α). In a NaI-induced murine AITDs model, oral tangeretin administration (20 and 100 mg/kg/day body weight) for 8 weeks significantly reduced serum TPO-Ab levels ($p < 0.05$), thyroid follicular destruction, and lymphocyte infiltration. Notably, this study is the first to systematically evaluate the immunoregulatory effects of citrus PMFs in AITDs, demonstrating their potential as natural, food-derived therapeutic agents. These findings provide valuable insights into PMFs as novel immunomodulators, paving the way for food-based interventions in autoimmune diseases.

1 | Introduction

Autoimmune thyroiditis (AITDs) are common organ-specific autoimmune disorders, primarily including Graves' disease and Hashimoto's thyroiditis (Antonelli et al. 2015; Pearce et al. 2003). AITDs have a prevalence of approximately 5% in the general population, making them the most prevalent autoimmune diseases and accounting for 30% of all autoimmune disorders (Franco et al. 2013; Hepp et al. 2021). Furthermore, the incidence of AITDs has been rising in recent years, which may be attributed to changes in lifestyle and dietary habits (J. Liu et al. 2020; Tywanek et al. 2024). In addition to their increasing prevalence, AITDs significantly diminish patients' quality of life. This is due to their chronic nature, frequent relapse after treatment, and heightened susceptibility to comorbid conditions (W. Zhang et al. 2024). However, there are currently no specific drugs available for AITDs, which highlights the importance of identifying alternative therapeutic agents, particularly from natural products.

Existing lines of investigation have highlighted the immune-modulating and anti-inflammatory properties of various natural plant compounds (Arce-Reynoso et al. 2023; Gong et al. 2024; Saini et al. 2022; Tomas et al. 2022). Citrus fruits are rich in bioactive substances, especially flavonoids, which exhibit significant antioxidant effects in various disease models (Gandhi et al. 2020; Miles and Calder 2021). Among these, polymethoxyflavones (PMFs) are a distinctive class of flavonoids found in citrus fruits, including compounds such as nobiletin, tangeretin, and 5-demethylnobiletin (Gao et al. 2018). Citrus flavonoids, especially PMFs, have been extensively reported to have anti-inflammatory effects, indicating that they possess the potential for immune regulation. However, their potential regulatory effect on AITDs and related inflammatory pathways remains largely unexplored.

Recent studies have highlighted the critical role of the IL-17/Th17 signaling axis in the pathogenesis of autoimmune thyroid diseases. Polymorphisms in the IL-23 and IL-17RA genes have been shown to be associated with the clinical phenotypes of Graves' disease and Hashimoto's thyroiditis, suggesting a genetic link between the IL-17 pathway and AITDs susceptibility (Cai et al. 2022). In addition, Th17/Treg imbalance correlates with disease subtype and autoantibody levels, while IL-17 expression is elevated in AITD patients and positively associated with inflammatory markers such as NF-κB, IL-6, TNF-α, and thyroid autoantibodies (C. Li et al. 2016; Lu et al. 2022). These findings indicate that IL-17 is a key proinflammatory cytokine involved in thyroid-specific immune responses.

Although the effect of citrus flavonoids on IL-17 signaling in AITDs remains unexplored, flavonoids such as baicalin, icariin, and proanthocyanidins have been reported to suppress IL-17 production and restore Th17/Treg balance in autoimmune models (Kelepouri et al. 2018). Moreover, cyanidin, a natural flavonoid, was shown to block IL-17A binding to its receptor IL-17RA, thereby directly suppressing IL-17 signaling (C. Liu et al. 2017). These findings support the hypothesis that citrus flavonoids may regulate IL-17-mediated inflammatory responses in AITDs through similar mechanisms, making it a promising direction for investigation.

Therefore, this study systematically investigates the effects of citrus peel extracts and their bioactive flavonoids on AITDs. This study seeks to provide valuable insights that could inform medical practice and offer significant benefits to human health. If successful, the findings could pave the way for new and natural preventive options for managing AITDs, offering significant benefits for patient health and addressing a critical gap in current medical knowledge and practice.

2 | Materials and Methods

2.1 | Experimental Materials

Citrus varieties, including "Youliang" mandarin (*Citrus reticulata*), "Newhall" orange (*Citrus sinensis*), "Eureka" lemon (*Citrus limon*), "Cocktail" grapefruit (*Citrus paradisi*), "Yuhuan Wendan" pomelo (*Citrus maxima*), and "Zijin ougan" mandarin (*Citrus reticulata*), were collected from the germplasm resource bank of the Citrus Research Institute, Quzhou Academy of Agricultural and Forestry Sciences, Zhejiang, China, in 2023. The citrus fruits of six varieties were sampled separately based on their tissue location, frozen in liquid nitrogen within 24 h after harvest, and stored at −80°C. The human thyroid follicular epithelial cell line Nthy-ori3-1 was purchased from the BeNa Culture Collection (BNCC340487) and stored in liquid nitrogen. NOD mice (female, 6–8 weeks old, 20 ± 2 g) were purchased from the Zhejiang University Laboratory Animal Center.

2.2 | Chemical Standards and Reagents

Standards of eriocitrin (≥ 98%, CAS: 13463-28-0), neoeriocitrin (≥ 98%, CAS: 13241-32-2), naringin (≥ 98%, CAS: 14259-46-2), narirutin (≥ 98%, CAS: 10236-47-2), hesperidin (≥ 98%, CAS: 520-26-3), neohesperidin (≥ 98%, CAS: 13241-33-3), didymin (≥ 98%, CAS: 14259-47-3), poncirin (≥ 98%, CAS: 14941-08-3), isosinensetin (≥ 98%, CAS: 17290-70-9), sinensetin (≥ 98%, CAS: 2306-27-6), nobiletin (≥ 98%, CAS: 478-01-3), tangeretin (≥ 98%, CAS: 481-53-8), 5-demethylnobiletin (≥ 98%, CAS: 2174-59-6) were purchased from Shanghai yuanye Bio-Technology Co. Ltd., Shanghai, China. Deionized water (ddH_2O) and anhydrous ethanol were purchased from Sinopharm Chemical Reagent Co. Ltd. Roswell Park Memorial Institute (RPMI) 1640 cell culture medium, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) with phenol red, phosphate-buffered saline (PBS, pH 7.4), and fetal bovine serum (FBS) were purchased from Invitrogen (Waltham, USA). 4-(2-Hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES, 1 M, pH 7.3), penicillin-streptomycin solution (100X), cell counting kit-8 (CCK-8), and Tween 80 were purchased from Beyotime (Shanghai, China). Recombinant human interleukin 1 beta (IL-1β) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Dimethyl sulfoxide (DMSO) was purchased from Solarbio (Beijing, China). FastPure Cell/Tissue Total RNA Isolation Kit V2 was purchased from Vazyme (Nanjing, China). PrimeScript RT Master Mix (Perfect Real Time) and TB Green Premix Ex Taq II (Tli RNaseH Plus) were purchased from Takara (Beijing, China). Human thyroid peroxidase antibody (TPO-Ab), IL-1β, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), TNF receptor-associated factor 3 (TRAF3), TRAF3 interacting protein 2 (TRAF3IP2), and nuclear factor kappa B subunit 1

(NFKB1) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Quanzhou Ruixin Biological Technology Co., Ltd., Quanzhou, China. Mouse TPO-Ab and IFN-γ ELISA kits were purchased from FineTest (Wuhan, China).

2.3 | Extraction, Separation, and Purification of Citrus Flavonoids

The peels of the citrus fruits were ground into a fine powder using a grinder under liquid nitrogen. For each variety, 1 g of the powder was mixed with 20 mL of anhydrous ethanol (solid-to-liquid ratio of 1:20*, m/v)* and subjected to ultrasonic-assisted extraction for 30 min (60 kHz, 30 W, 50°C). The mixture was then centrifuged at 3900 rpm for 10 min, and the supernatant was collected. The extraction process was repeated twice, and the combined supernatant was evaporated to dryness using a vacuum rotary evaporator at room temperature (3–4 h).

The residue was dissolved in ddH_2O with the aid of ultrasound, yielding crude citrus flavonoid extracts from six varieties, which were stored at 4°C for further use. Redissolving in ddH_2O serves two purposes: it ensures biocompatibility for subsequent biological assays, and it also allows proper interaction with the hydrophobic stationary phase of the following cartridge, as it typically requires aqueous sample loading to optimize retention and selectivity of flavonoid compounds.

To further purify the crude extracts, the solution was first filtered through a 0.45 μm aqueous membrane to remove insoluble particles. The clarified solution was then subjected to sugar removal and purification using a Sep-Pak C18 solid-phase extraction (SPE) cartridge (2 g, 12 cc; Waters Corp., Milford, MA, USA). The SPE column was preconditioned with 12 mL of methanol, followed by 12 mL of ddH_2O for equilibration. The clarified solution was loaded onto the column, washed with 100 mL of ddH_2O to remove sugars, and sequentially eluted with 24 mL of methanol and 16 mL of chloroform to remove pigments. Subsequently, 8 mL of methanol was used to recondition the SPE column. The methanol eluates were collected, combined, and evaporated under reduced pressure at 37°C. The resulting residue was dissolved in ddH_2O and further dried using a vacuum rotary evaporator, yielding purified citrus flavonoid extract powder.

2.4 | Identification and Quantification of Citrus Flavonoids

A total of 13 flavonoid standards, including eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, poncirin, isosinensetin, sinensetin, nobiletin, tangeretin, and 5-demethylnobiletin, were prepared at equal concentrations. Each standard was dissolved in chromatographic grade methanol to 5 mg/mL and was mixed and diluted stepwise. Mixed standards and extracts were transferred into vials for analysis.

HPLC (2695 pump, 2996 diode array detector; Waters Corp., Milford, MA, USA) coupled with a Waters SunFire C18 analytical column (4.6 × 250 mm) was used for detection. The mobile phase consisted of chromatographic acetonitrile (Eluent A), 0.1% (*v/v*) formic acid in water (Eluent B), and methanol (Eluents C and D). The gradient elution program was as follows: 0–5 min, 20% A; 5–8 min, 20%–34% A; 8–20 min, 34%–60% A; 20–22 min, 60%–100% A; 22–23 min, 100% A. The injection volume was 10 μL, the flow rate was 1 mL/min, the column temperature was 25°C, and the detection wavelength was 200–600 nm.

For HPLC-based quantification, standard curves were established using a mixed standard solution containing 13 flavonoids (Compounds 1–13). Eight concentration gradients were prepared for each standard, and the corresponding peak areas were recorded. Linear regression analysis was performed using concentration as the independent variable (*X*-axis) and peak area as the dependent variable (*Y*-axis). The resulting calibration curves were used to calculate the flavonoid concentrations in citrus peel extracts based on their respective peak areas. All curves showed excellent linearity, with $R^2 > 0.999$.

2.5 | Cell Culture

The Nthy-ori3-1 cell line was cultured in RPMI 1640 cell culture medium supplemented with 10% FBS, 20 mM HEPES, and 1× penicillin-streptomycin solution in a humidified incubator at 37°C with 5% CO_2. Cells were passaged using trypsin-EDTA when they reached 80%–90% confluence.

2.6 | Cell Viability Assay

The cell viability assay was conducted using a CCK-8 assay, based on our previous experimental methods (Y. Chen, Ma, et al. 2024). Nthy-ori3-1 cells were seeded in 96-well plates at a density of 5×10^4 cells per well with 100 μL of RPMI 1640 cell culture medium and cultured overnight. Various concentrations of citrus flavonoid extracts (6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL) and standards (2.5, 5, 10, 20, 40, 80, 160, and 320 μM) dissolved in DMSO were added to the medium and incubated with the cells for 24 h. After incubation, the medium was removed, and the cells were washed twice with PBS. The CCK-8 reagent was diluted in an FBS-free RPMI-1640 cell culture medium and added to the wells. Following a 1-h incubation period, absorbance was measured at 450 and 620 nm using a microplate reader. Cell viability was calculated using the formula: cell viability $(\%) = (OD_{450(\text{treatment})} - OD_{620(\text{treatment})})/(OD_{450(\text{control})} - OD_{620(\text{control})}) \times 100\%$. DMSO was used as a solvent control. Each experiment was independently repeated three times.

2.7 | AITDs Cell Model

The Nthy-ori3-1 cell line was cultured in RPMI 1640 cell culture medium supplemented with 10% FBS, 20 mM HEPES, 1X penicillin-streptomycin solution, and 20 ng/mL IL-1β (dissolved in ddH_2O), in a humidified incubator at 37°C with 5% CO_2. ddH_2O was used as a solvent control.

For the AITDs cell model, Nthy-ori3-1 cells were seeded at a density of 5×10^4 cells per well into 96-well plates and cultured overnight for adhesion. In the treatment groups, the cells were incubated with either flavonoid-enriched extracts (obtained after impurity removal, mainly sugars and pigments, by C18 SPE) or

individual flavonoid monomers for 24 h. IL-1β (20 ng/mL) was added 2 h after the start of treatment. DMSO was used as the solvent control. After incubation, the culture supernatants were collected for further analysis.

2.8 | AITDs Animal Model

Female NOD mice were housed in a specific-pathogen-free environment with ad libitum access to food and water. The temperature was maintained at 22 ± 2°C with a 12-h light-dark cycle. Mice were randomly assigned to five groups, each consisting of five animals: control, model (0.05% NaI solution in drinking water, with 2 mL ddH_2O [0.1% Tween 80, v/v] via oral gavage), low-dose tangeretin treatment (0.05% NaI solution in drinking water, with 4 mg/kg/day body weight (BW) tangeretin via oral gavage), medium-dose tangeretin treatment (0.05% NaI solution in drinking water, with 20 mg/kg/day BW tangeretin via oral gavage), and high-dose tangeretin treatment (0.05% NaI solution in drinking water, with 100 mg/kg/day BW tangeretin via oral gavage). Tangeretin was first prepared as a suspension using Tween 80 (0.1%, v/v) and ddH_2O at the desired concentration and then administered to each mouse via oral gavage according to its specific BW. The experiment lasted 8 weeks.

At the end of the experimental period, blood was collected from the retro-orbital sinus, and euthanasia was performed by cervical dislocation. Blood samples were stored at −80°C for subsequent detection. Thyroid glands were collected and fixed with a 4% paraformaldehyde fix solution (Beyotime Biotechnology) for further histological analysis.

The animal experiment was conducted in compliance with the ethical guidelines of the Animal Experimentation Committee in the College of Medicine, Zhejiang University (animal ethical clearance number: ZJU20240438).

2.9 | Enzyme-Linked Immunosorbent Assay

The ELISA assay was used to detect the protein concentration of TPO-Ab, IL-1β, IL-6, TNF-α, Act1, TRAF3, NFKB1, and IFN-γ. It was conducted in accordance with the manufacturer's instructions. Each experiment was repeated three times independently.

For ELISA quantification, standard curves were generated using six standard concentrations and one blank well provided in each kit. Absorbance values (OD_{450}) were plotted against standard concentrations, and a four-parameter logistic (4PL) model was applied to fit the standard curves using appropriate software. The fitted equation was used to interpolate the sample concentrations from their measured OD values. All curves showed excellent linearity with $R^2 > 0.998$.

2.10 | Quantitative Real-Time Polymerase Chain Reaction

The quantitative real-time polymerase chain reaction (qRT-PCR) assay was used to detect the gene expression level of *IL1B*, *IL6*, *TNF*, *TRAF3IP2*, *TRAF3*, and *NFKB1*. It was carried out as detailed in our previous report, with several modifications (J. Chen, Xu, et al. 2024). Nthy-ori3-1 cells were seeded into six-well plates at a density of 5×10^5 cells per well. Total RNA was extracted from the cells using a commercial kit (Vazyme) according to the manufacturer's protocol. RNA quality and concentration were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription of the RNA was performed using a commercial kit (Takara). Quantitative PCR was conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) with a SYBR qPCR kit (Takara). The primer sequences for qRT-PCR are listed in Table 1. Relative expression levels of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method.

TABLE 1 | The sequences of primers used for qRT-PCR detection.

Genes	Sequences
GAPDH	F: ATC ATC CCT GCC TCT ACT GG R: GTC AGG TCC ACC ACT GAC AC
IL1B	F: AGC TCG CCA GTG AAA TGA TG R: CCT TGC TGT AGT GGT GGT CG
IL6	F: TGA ACT CCT TCT CCA CAA GCG R: GGG CGG CTA CAT CTT TGG AA
TNF	F: TCT TCT CGA ACC CCG AGT GA R: TAT CTC TCA GCT CCA CGC CA
TRAF3IP2	F: CTA GAC CCC CTA GCA ACC CT R: TCA TCA CGG TAT CCC TAA GGT
TRAF3	F: GAC GCA CTT GTC GCT GTT TT R: CGG GCA GAT CCG AAG TAT CC
NFKB	F: AAT GGG CTA CAC CGA AGC AA R: TCT AGA GGT CCT TCC TGC CC

2.11 | Hematoxylin and Eosin Staining

The thyroid glands were fixed in 4% paraformaldehyde fix solution for at least 24 h and then embedded in paraffin for staining. Hematoxylin and eosin (HE) staining were sequentially applied to paraffin-embedded sections. The images were taken by using a microscope set (Zeiss, Germany).

2.12 | Statistics

All data in this study were derived from a minimum of three independent replicates. Data are presented as the means ± standard deviation of at least three independent replicates. Statistical analyses were performed using GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance with Tukey's multiple comparisons test was used for multi-group comparisons. Two-group comparisons were conducted using independent samples Student's *t*-test. A significance level of $p < 0.05$ was considered statistically significant.

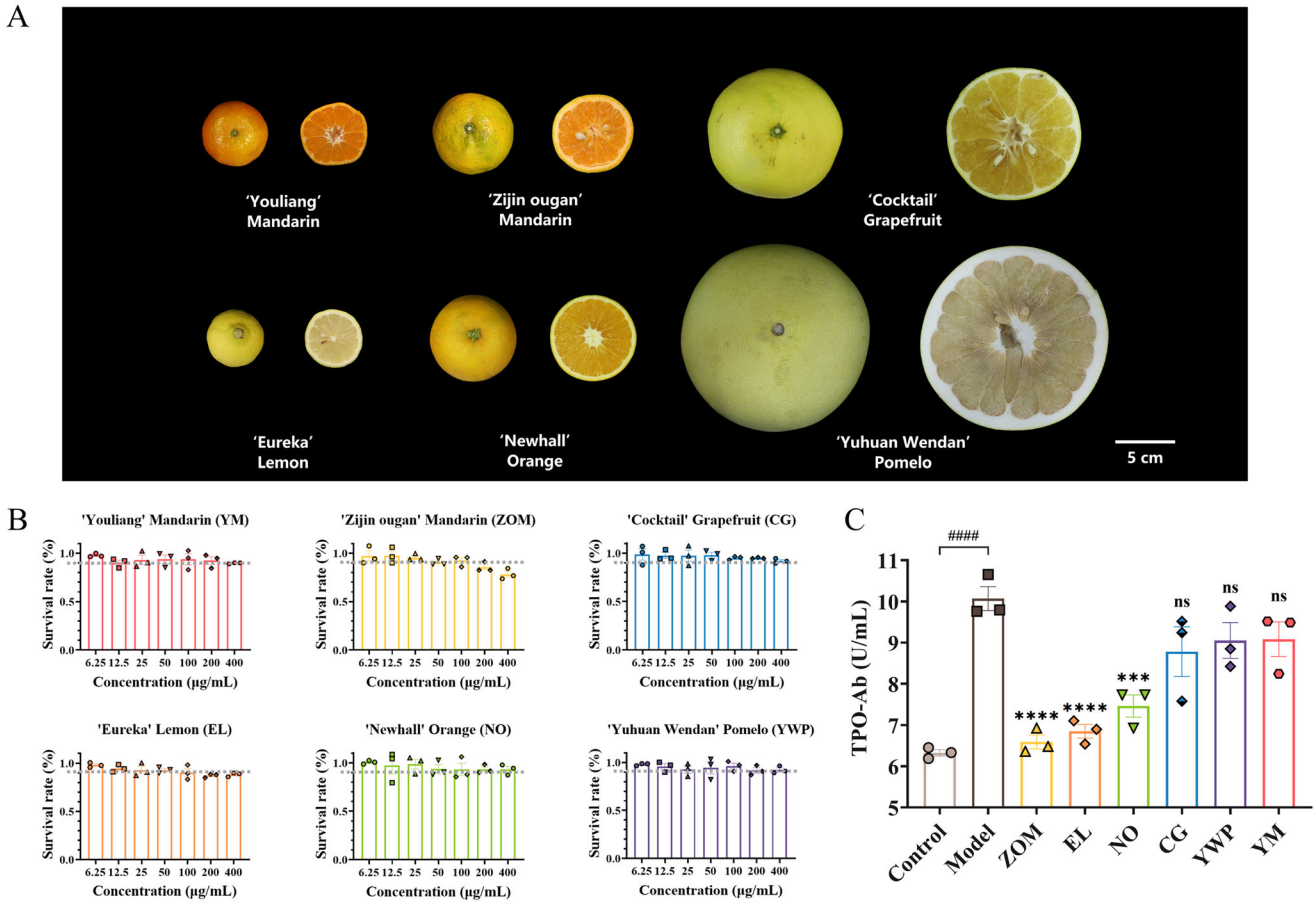

FIGURE 1 | (A) Six citrus varieties, including "Youliang" mandarin (YM), "Newhall" orange (NO), "Eureka" lemon (EL), "Cocktail" grapefruit (CG), "Yuhuan Wendan" pomelo (YWP), and "Zijin ougan" mandarin (ZOM). (B) Effects of citrus flavonoid extracts on the cell viability of Nthy-ori3-1. (C) Effects of citrus flavonoid extracts on the TPO-Ab level in AITDs Nthy-ori3-1 cells. Cell assays were repeated three times independently. One-way ANOVA with Tukey's multiple comparisons was used for multi-group comparisons. Independent samples Student's *t*-test was used for the two groups' comparison. $^{\#}$compared with control group, ns $p > 0.05$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$. *Compared with the model group, ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3 | Results

3.1 | Citrus Flavonoid Extracts Demonstrated the Ability to Alleviate AITDs in a Cell Model

The Nthy-ori3-1 cell line is widely utilized and serves as a convincing in vitro model for studying AITDs. The six selected citrus varieties are shown in Figure 1A. We first extracted, separated, and purified flavonoids from citrus peels using an ultrasound-assisted extraction method, resulting in six distinct citrus flavonoid extracts. We then assessed the potential toxicity of these extracts on Nthy-ori3-1 cells using a CCK-8 assay. Based on the experimental results (Figure 1B), we ultimately selected a concentration of 12.5 μg/mL as the safe dosage for subsequent administration. We further evaluated the anti-AITDs potential of these citrus flavonoid extracts using an AITDs cell model stimulated with 20 ng/mL IL-1β. As shown in Figure 1C, IL-1β significantly increased the level of TPO-Ab in Nthy-ori3-1 cells. Compared to the model group, three citrus flavonoid extracts, Ougan, Eureka lemon, and Newhall sweet orange, significantly reduced the TPO-Ab content in the supernatant of AITDs Nthy-ori3-1 cells. These findings demonstrated that specific citrus flavonoid extracts exhibited significant anti-AITDs effects in vitro.

3.2 | Identification and Quantification of Characteristic Components of Citrus Flavonoid Extracts

Based on previous studies and our preliminary experimental work, flavonoids are the primary active compounds in citrus, including flavanones and PMFs (Zhu et al. 2023). We selected 13 common representative citrus flavonoids, including 8 flavanones and 5 polymethoxyflavones. These are eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, poncirin, isosinensetin, sinensetin, nobiletin, tangeretin, and 5-demethylnobiletin. The HPLC chromatograms of these 13 citrus flavonoids and 6 citrus flavonoid extracts are shown in Figures 2A,B, respectively. They were identified by comparing the retention time of the peaks with authentic standards and quantified with standard curve methods, as shown in Table 2.

TABLE 2 | The content of the 13 flavonoids in each citrus flavonoid extract.

Substance	CAS number	Peak no.	Retention time (min)	Concentration (mg/g FW)					
				YM	NO	EL	CG	YWP	ZOM
Eriocitrin	13463-28-0	1	8.420	n.d.	n.d.	1.092 ± 0.161^{a}	0.355 ± 0.006^{b}	n.d.	n.d.
Neoeriocitrin	13241-32-2	2	9.864	n.d.	n.d.	n.d.	0.638 ± 0.028^{a}	n.d.	n.d.
Narirutin	14259-46-2	3	12.156	3.203 ± 0.172^{a}	1.841 ± 0.368^{b}	0.102 ± 0.002^{d}	0.560 ± 0.008^{c}	n.d.	0.128 ± 0.017^{d}
Naringin	10236-47-2	4	13.077	n.d.	n.d.	0.121 ± 0.005^{d}	5.954 ± 0.353^{b}	6.445 ± 0.395^{a}	0.498 ± 0.070^{c}
Hesperidin	520-26-3	5	13.415	5.313 ± 0.087^{a}	5.151 ± 0.018^{b}	3.124 ± 0.289^{c}	0.178 ± 0.008^{e}	n.d.	0.744 ± 0.094^{d}
Neohesperidin	13241-33-3	6	14.230	n.d.	n.d.	n.d.	3.905 ± 0.265^{b}	n.d.	5.367 ± 0.425^{a}
Didymin	14259-47-3	7	21.641	0.563 ± 0.055^{b}	0.707 ± 0.102^{a}	n.d.	n.d.	n.d.	n.d.
Poncirin	14941-08-3	8	22.714	n.d.	n.d.	n.d.	0.242 ± 0.001^{b}	n.d.	0.608 ± 0.075^{a}
Isosinensetin	17290-70-9	9	29.695	n.d.	n.d.	n.d.	n.d.	n.d.	0.036 ± 0.007^{a}
Sinensetin	2306-27-6	10	32.512	n.d.	0.149 ± 0.056^{a}	n.d.	n.d.	n.d.	0.124 ± 0.013^{a}
Nobiletin	478-01-3	11	34.999	0.034 ± 0.008^{b}	0.199 ± 0.001^{b}	n.d.	0.032 ± 0.003^{b}	n.d.	1.473 ± 0.363^{a}
Tangeretin	481-53-8	12	37.780	n.d.	0.036 ± 0.021^{b}	n.d.	0.030 ± 0.001^{b}	n.d.	0.974 ± 0.209^{a}
5-Demethylnobiletin	2174-59-6	13	39.927	n.d.	n.d.	n.d.	n.d.	n.d.	0.224 ± 0.022^{a}

Note: "n.d." indicates that the substance is not detected. Different superscript letters on the same line indicate a significant difference ($p < 0.05$) and the same superscript letters indicate no significant difference ($p > 0.05$).

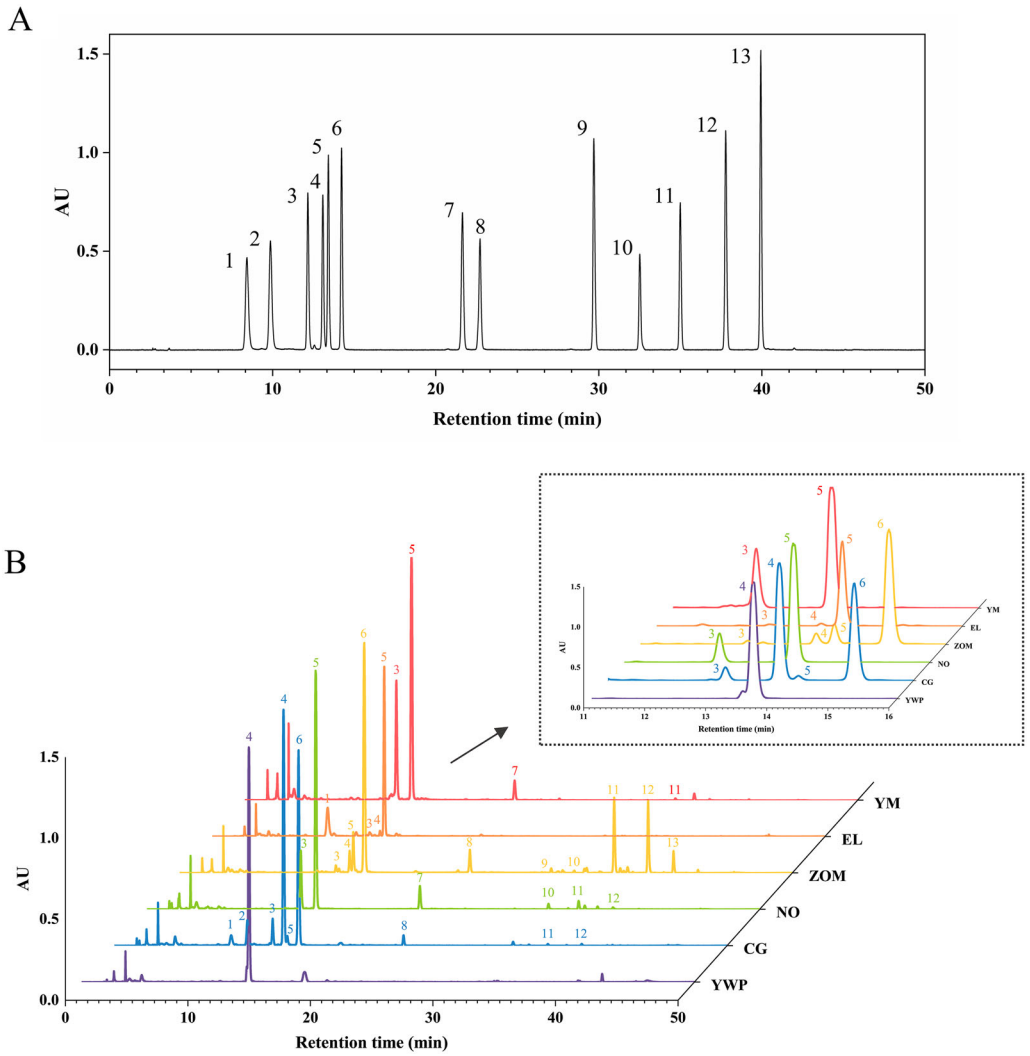

FIGURE 2 | (A) HPLC chromatogram of representative flavonoid standards (1: eriocitrin; 2: neoeriocitrin; 3: narirutin; 4: naringin; 5: hesperidin; 6: neohesperidin; 7: didymin; 8: poncirin; 9: isosinensetin; 10: sinensetin; 11: nobiletin; 12: tangeretin; 13: 5-demethylnobiletin; $\lambda = 280$ nm). (B) HPLC chromatogram of six citrus flavonoid extracts ($\lambda = 280$ nm). The inset shows a zoomed-in view of the 11–16 min region, corresponding to Compounds 3–6, to better distinguish closely eluting peaks and identify principal components in each sample.

3.3 | Citrus Flavonoids Demonstrated the Ability of Alleviating AITDs and Inhibiting the Expression of *IL1B*, *IL6*, and *TNF* in Cell Model

Based on previous experience, we employed the CCK-8 assay to evaluate the safety profiles of eight representative and potentially bioactive citrus flavonoids, including naringin, narirutin, hesperidin, neohesperidin, didymin, sinensetin, nobiletin, and tangeretin (Figure 3A). This allowed us to establish a safe dosage range suitable for subsequent experiments. Using a concentration of 20 μM, which was determined based on previous dose-response studies, we treated the AITDs cell model with these flavonoids and assessed the gene expression levels of key inflammatory cytokines. As shown in Figure 3B, the results demonstrated that all tested flavonoids significantly downregulated the expression of pro-inflammatory factors, including *IL1B*, *IL6*, and *TNF*. Furthermore, we evaluated their preventive potential against AITDs and observed that tangeretin, sinensetin, nobiletin, and neohesperidin notably reduced the secretion of TPO-Ab in the supernatant of AITDs Nthy-ori3-1 cells, with tangeretin exhibiting the most pronounced effect (Figure 3C).

3.4 | PMFs Exerted a Preventive Effect on AITDs Cell Model by Regulating the IL-17 Cell Signaling Pathway

Based on previous findings, we examined the gene expression level (Figure 4A) and protein level (Figure 4B) associated with the two PMFs, tangeretin and nobiletin. Our results indicated that, compared to the model group, PMF treatment significantly inhibited the gene expression levels of *TRAF3IP2*, *NFKBI*, *IL1B*, *IL6*, and *TNF*. Additionally, nobiletin treatment led to a significant upregulation of *TRAF3* gene expression, whereas the upregulation observed with tangeretin was not statistically significant. Furthermore, compared to the model group, the protein levels of Act1, TRAF3, NFKB1, IL-1β, IL-6, and TNF-α were significantly reduced in the groups treated with tangeretin

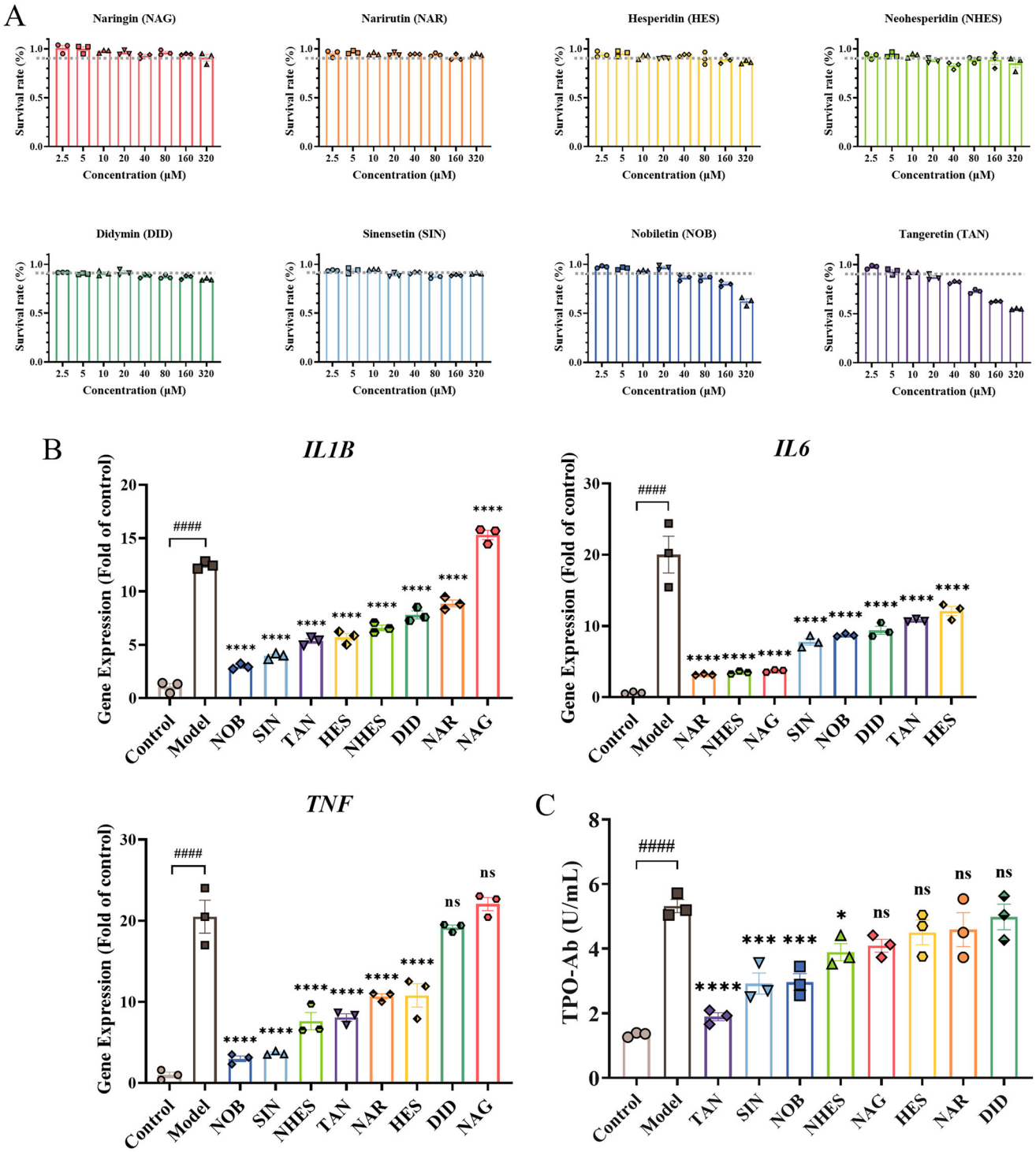

FIGURE 3 | (A) Effects of eight citrus flavonoids on the cell viability of Nthy-ori3-1, including naringin (NAG), narirutin (NAR), hesperidin (HES), neohesperidin (NHES), didymin (DID), sinensetin (SIN), nobiletin (NOB), and tangeretin (TAN). (B) Effects of citrus flavonoids on the *IL1B*, *IL6*, and *TNF* gene expression of Nthy-ori3-1. (C) Effects of citrus flavonoids on the TPO-Ab level in AITDs Nthy-ori3-1 cells. Cell assays were repeated three times independently. One-way ANOVA with Tukey's multiple comparisons was used for multi-group comparisons. Independent samples' Student's *t*-test was used for the two groups' comparison. $^{\#}$Compared with Control group, ns $p > 0.05$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$. *Compared with the model group, ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

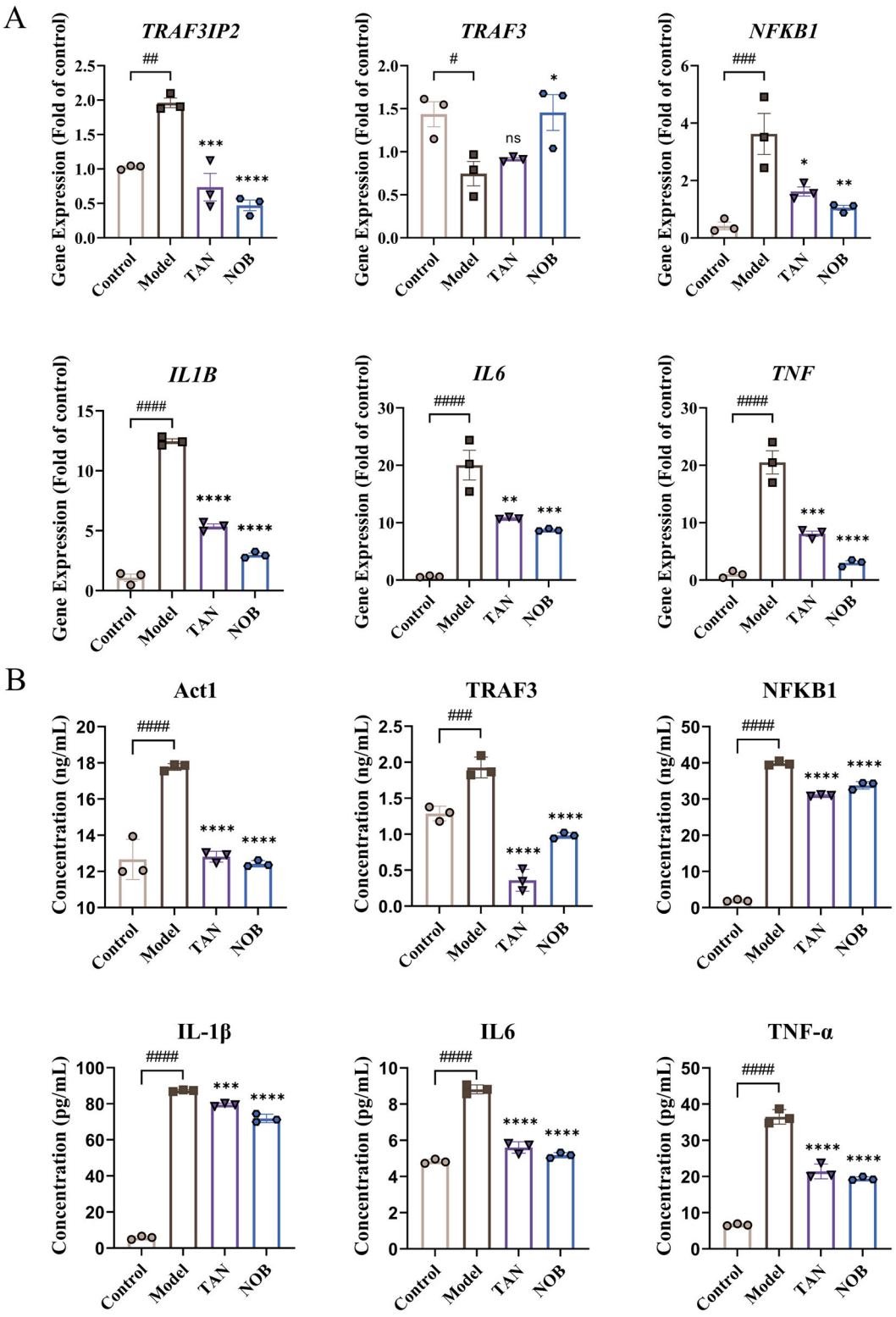

FIGURE 4 | (A) Effects of tangeretin (TAN) and nobiletin (NOB) on the gene expression level of *TRAF3IP2*, *TRAF3*, *TNF, IL1B*, *IL6*, and *TNF*. (B) Effects of tangeretin (TAN) and nobiletin (NOB) on the protein level of Act1, TRAF3, NFKB1, IL-1β, IL-6, and TNF-α. Cell assays were repeated three times independently. One-way ANOVA with Tukey's multiple comparisons was used for multi-group comparisons. Independent samples' Student's t-test was used for the two groups' comparison. #Compared with the control group, ns $p > 0.05$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$. *Compared with the model group, ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

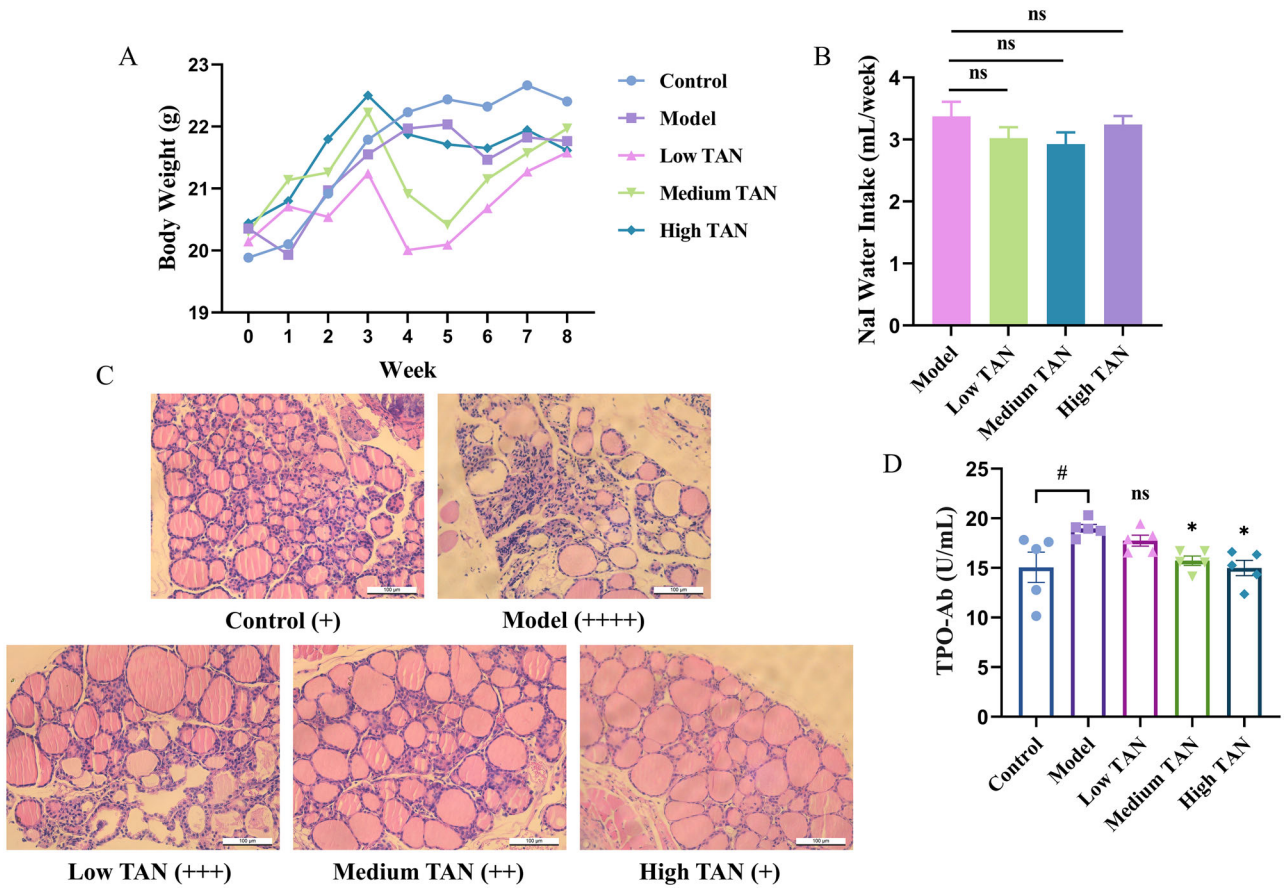

FIGURE 5 | (A) Weight changes of mice in each group during the modeling process. (B) The intake of 0.05% NaI water by mice in the model group and treatment group (low TAN: 4 mg/kg/day BW tangeretin; medium TAN: 20 mg/kg/day BW tangeretin; high TAN: 100 mg/kg/day BW tangeretin). (C) The HE staining of thyroid pathological sections of mice in each group. "+" indicates the degree of thyroid follicular destruction and lymphocyte infiltration (from "+" to "++++," the severity gradually increases). (D) The effects of different dosage tangeretin administration on serum TPO-Ab level in mice. One-way ANOVA with Tukey's multiple comparisons was used for multi-group comparisons. Independent samples Student's *t*-test was used for the two groups' comparison. $^{\#}$Compared with the control group, ns $p > 0.05$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$. *Compared with the model group, ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

and nobiletin. According to these findings and related literature, we hypothesized that PMFs such as tangeretin and nobiletin may enhance *TRAF3* expression and inhibit *TRAF3IP2* expression, thereby reducing Act1 protein binding to IL-17RA. This could weaken NF-κB activation and subsequently suppress the expression of downstream pro-inflammatory genes, leading to a preventive effect on AITDs. However, the efficacy of these compounds in modulating these pathways may differ.

3.5 | Tangeretin Alleviated NaI-Induced AITDs in Mice

In this section, we further validated the effect of tangeretin using a murine AITDs model. The experimental AITDs model was established by administering 0.05% NaI in the drinking water of mice for 8 weeks. Figure 5A presents the natural weight fluctuations observed in each group of mice. These variations were likely attributable to natural variability or potential stress responses associated with the experimental conditions. To exclude water consumption as a confounding factor, we recorded the actual water intake of NaI-treated mice across different groups (Figure 5B) and found no significant differences among them. At the end of the experiment, mice treated with NaI exhibited clear characteristics of AITDs, including a marked increase in serum TPO-Ab levels and significant thyroid follicular destruction and lymphocyte infiltration, confirming the successful induction of AITDs. Tangeretin administration significantly ameliorated NaI-induced AITDs, as evidenced by a substantial reduction in serum TPO-Ab levels (Figure 5D) compared to the model group and a notable decrease in thyroid follicular damage and lymphocyte infiltration (Figure 5C). The high-dose tangeretin (100 mg/kg/day BW) intervention yielded the most pronounced effect, followed by the medium dose (20 mg/kg/day BW). Therefore, tangeretin could greatly improve NaI-induced AITDs in mice, which confirmed its effectiveness at the animal level.

4 | Discussion

As chronic autoimmune diseases targeting thyroid tissue, AITDs, if left untreated, could cause a range of metabolic complications and also result in excessive antibodies continuously attacking

the thyroid gland, leading to hypothyroidism or even complete thyroid failure. Additionally, AITDs may trigger other autoimmune diseases, such as primary Sjögren's syndrome, rheumatoid arthritis, and systemic lupus erythematosus (Parisis et al. 2020). Studies have shown that patients with AITDs have a 3.6-fold higher risk of developing primary Sjögren's syndrome and a 10-fold higher risk of developing systemic lupus erythematosus compared to healthy individuals (Baldini et al. 2018). Worse still, although recent studies have identified potential factors contributing to the development of AITDs, including genetic susceptibility, immune dysregulation, inflammation, stress, and other environmental factors, the exact etiology and treatment of AITDs remain unclear, and thus AITDs are often considered incurable. Therefore, although AITDs are not directly life-threatening, they present significant health risks and merit extensive attention (Saevarsdottir et al. 2020).

Previous studies have investigated the regulatory effects of certain plant-derived bioactive compounds on thyroid function. For instance, curcumin has been shown to mitigate PTU-induced hypothyroidism by reducing thyroid hyperplasia and hypertrophy while enhancing secretory activity (Papież 2023). Myricetin exhibits anticancer effects on human papillary thyroid cancer cells by inducing apoptosis via caspase activation and mitochondrial pathway modulation (Ha et al. 2017). Additionally, naringenin and hesperetin have been reported to modulate pituitary–thyroid function in aged rats through the regulation of Sirt1 expression, thyroid peroxidase activity, and antioxidant enzyme levels (Miler et al. 2020). Among these compounds, flavonoids have garnered attention due to their anti-inflammatory and anticancer properties.

Citrus flavonoids are known for their wide range of biological activities, including antioxidant, anti-inflammatory, and anticancer effects (L. Chen et al. 2020; Y. Wang et al. 2022). Several representative flavonoids, such as hesperidin, naringin, and tangeretin, have been shown to exert immunomodulatory effects by regulating cytokine production and signaling pathways. For instance, naringin has demonstrated beneficial effects in inflammatory models of metabolic syndrome and autoimmune diseases through modulation of cytokine release and T-cell activation (Alam et al. 2014). Tangeretin has been reported to reduce IL-6, IL-1β, and TNF-α levels in a sepsis-induced acute lung injury model, indicating its potential in suppressing excessive proinflammatory responses (H. Zhang, Wang, et al. 2025). Moreover, PMFs, such as nobiletin and tangeretin, have shown promising effects on immune homeostasis via the regulation of transcription factors and proinflammatory mediators (Y. Wu et al. 2021). However, the immunoregulatory potential of these compounds in organ-specific autoimmune diseases, such as AITDs, remains largely underexplored (Musumeci et al. 2023). Given the increasing prevalence of AITDs, especially in the post-COVID-19 era, and the unclear pathogenesis and treatment strategies, this study aimed to investigate the effects of citrus flavonoids on AITDs with a specific focus on the IL-17 signaling pathway in both cell and animal models (Chaachouay and Zidane 2024; Lee et al. 2015; Mogensen et al. 2023).

TPO-Ab are key markers of AITDs, and elevated levels are often associated with disease progression (Attard et al. 2022). Our study first demonstrated that several citrus flavonoid extracts significantly reduced TPO-Ab levels in an AITDs cell model, indicating their potential immunomodulatory effects. HPLC analysis identified key flavonoids, including tangeretin, nobiletin, sinensetin, and neohesperidin, which exhibited pronounced effects in lowering TPO-Ab levels. These findings suggest that these flavonoids may play critical roles in the prevention and treatment of AITDs. Among these, tangeretin was particularly effective, highlighting its potential therapeutic application for AITDs. Interestingly, although some flavonoids like hesperidin were highly abundant in extracts such as EL and YM, their inhibitory effects on TPOAb levels differed significantly. This inconsistency suggests that the overall bioactivity of citrus flavonoid extracts cannot be simply inferred from the concentration of a single compound. Instead, it may be influenced by the presence of other minor constituents, interactions among flavonoids, and variations in the overall phytochemical composition of each extract. In particular, PMFs such as tangeretin and nobiletin have been shown to exert potent biological activity at much lower concentrations than other flavonoids, due to their unique structural features. Previous studies have also reported that flavonoids can interact synergistically or antagonistically, leading to unpredictable effects on biological outcomes (Hajimehdipoor et al. 2014; Hidalgo et al. 2010). These findings highlight the importance of considering both compound potency and extract complexity when interpreting the activity of plant-derived mixtures. To the best of our knowledge, this study is the first to systematically investigate the role of PMFs, particularly tangeretin and nobiletin, in AITDs. We demonstrate that these compounds not only exert immunomodulatory effects by suppressing inflammation and autoimmune responses but also specifically target the IL-17 signaling pathway, a key driver of AITDs pathogenesis.

IL-17 is a pivotal cytokine in the pathogenesis of autoimmune diseases, including AITDs (Majumder and McGeachy 2021). Previous studies have implicated IL-17 in chronic inflammatory responses in diseases such as rheumatoid arthritis and multiple sclerosis (Kuwabara et al. 2017). Notably, increasing evidence suggests that IL-17 plays a central role in the immune dysregulation observed in AITDs (Lu et al. 2022). Elevated IL-17 levels have been detected in both serum and thyroid tissues of patients with Graves' disease and Hashimoto's thyroiditis, correlating with disease severity and autoantibody titers (Zake et al. 2018). Mechanistically, IL-17 contributes to thyroid inflammation by activating NF-κB signaling, leading to the secretion of TNF-α, IL-6, and IFN-γ, while suppressing anti-inflammatory cytokines such as IL-10 (Huangfu et al. 2023). This cytokine imbalance exacerbates autoimmune-mediated tissue damage and promotes a chronic inflammatory environment. Furthermore, IL-17 enhances the recruitment of immune cells by upregulating CXCL1 and CXCL2, thereby amplifying local inflammation and fibrosis within the thyroid gland (X. Zhang, Li, et al. 2025). Its involvement in Th17/Treg imbalance, a key feature of AITDs, further underscores its pathogenic role (S. Zhang et al. 2021). Given these findings, targeting IL-17 signaling represents a promising therapeutic avenue for AITDs management. And our findings further confirm its role in AITDs. TRAF proteins act as important mediators in the IL-17 signaling pathway, with TRAF3 binding to IL-17RA to prevent the formation of the IL-17R-Act1-TRAF6 complex, thereby inhibiting excessive activation of the IL-17 pathway (Amatya et al. 2017). We found that treatment with tangeretin and nobiletin significantly upregulated *TRAF3* gene expression, consistent with TRAF3's

role as a negative regulator of the IL-17 pathway (Swaidani et al. 2019).

Interestingly, despite increased gene expression, TRAF3 protein levels were reduced, possibly due to increased binding of TRAF3 to IL-17RA. This suggests a complex regulation between gene expression and protein levels that warrants further investigation. The discrepancy between TRAF3 mRNA and protein levels in the model group may be attributed to post-transcriptional modifications and protein stability regulation, mechanisms widely recognized in immune signaling pathways. Extensive studies have demonstrated that post-transcriptional modifications, such as ubiquitination, play a critical role in regulating protein degradation and stability, which can result in increased protein levels despite decreased mRNA expression (Makita et al. 2021; Zhou et al. 2022). Specifically, members of the TRAF protein family undergo distinct ubiquitination modifications that modulate their functions. For instance, TRAF3 is subject to K63-linked polyubiquitination, which regulates downstream immune responses such as IFN-β induction in antiviral signaling (Kim et al. 2021). Additionally, in the TLR signaling pathway, TRAF3 undergoes K48-linked polyubiquitination, catalyzed by cIAP1/2, which controls its turnover rate and functional activity (Xie 2013). Similarly, in the NLR signaling pathway, TRAF6 degradation is mediated by RNF19A-catalyzed K48-linked polyubiquitination, further underscoring the role of protein degradation in immune regulation (C. Wu et al. 2017). These findings suggest that, in the model group, TRAF3 may undergo reduced degradation due to altered ubiquitination patterns, leading to its accumulation at the protein level despite decreased mRNA expression. This observation implies that TRAF3 protein levels may be regulated at the post-transcriptional level rather than being solely dictated by transcriptional activity (F. Li et al. 2018). We hypothesize that the observed reduction in Act1 binding to IL-17RA upon *TRAF3* upregulation and *TRAF3IP2* downregulation may be due to a competitive binding mechanism involving TRAF3 and TRAF6. Previous studies have suggested that TRAF3 and TRAF6 serve distinct roles in IL-17RA-mediated signaling, and their competition for receptor binding could influence downstream signaling cascades (Swaidani et al. 2019). When *TRAF3* expression increases, it may outcompete TRAF6 for IL-17RA binding, thereby preventing Act1 from forming a signaling complex with TRAF6 and IL-17RA. This competitive interaction may account for the observed reduction in complex formation and subsequent alterations in downstream signaling. Moreover, additional factors may also modulate TRAF3 interactions at the IL-17 receptor. For example, FXYD3 has been found to competitively bind to TRAF3 at the IL-17 receptor, thereby promoting the formation of the IL-17R-Act1 complex and enhancing IL-17A signaling (Yang et al. 2023). These findings indicate the potential involvement of other molecules in modulating TRAF3 and TRAF6 binding dynamics. However, further experimental validation is required to determine whether TRAF3 and TRAF6 or other proteins directly compete for IL-17RA binding in our model.

While previous studies have primarily focused on the anti-inflammatory and antioxidant properties of citrus flavonoids, our research is the first to systematically evaluate their immunomodulatory effects in AITDs. Through preliminary screening, we identified tangeretin and nobiletin as the most potent PMFs, exhibiting significantly stronger effects than other citrus flavonoids. Notably, the 'Zijin Ougan' mandarin—previously identified as the most effective variety—contained markedly higher concentrations of tangeretin and nobiletin than other PMFs like sinensetin, further supporting their selection. Our findings reveal that PMFs, particularly tangeretin and nobiletin, modulate immune responses through the IL-17 pathway, aligning with research in other immune-related diseases. Given its superior efficacy in reducing TPO-Ab levels in vitro, subsequent experiments focused on tangeretin, which also demonstrated notable thyroid-protective effects. These findings highlight tangeretin's promising therapeutic potential for AITDs, expanding current knowledge on flavonoid-based interventions.

The animal experiments confirmed tangeretin's ability to alleviate AITDs, particularly at high doses, where it reduced thyroid follicle destruction and lymphocyte infiltration. This widely recognized evaluation model validated tangeretin's preventive potential. However, we lacked the precise quantitative assessment of the pathological situation of the thyroid due to constraints in sample size. Additionally, the choice of the NaI-induced murine model has inherent limitations that should be considered when interpreting the results. AITDs result from a combination of genetic and environmental factors, among which excessive iodine intake is a well-established trigger. In this study, we employed a NaI-induced murine model to investigate the autoimmune response associated with thyroid dysfunction. NaI-induced models are widely used in AITDs research due to their physiological relevance, as iodine overload has been shown to promote lymphocytic infiltration and thyroidal damage in genetically susceptible strains (Braley-Mullen et al. 1999). In contrast to Tg-adjuvant models that require multiple subcutaneous immunizations, NaI-induced models offer a non-invasive and reproducible alternative through dietary supplementation. Despite these advantages, the NaI-induced model has certain limitations. First, its success is highly dependent on genetic susceptibility, with NOD mice exhibiting a stronger autoimmune response compared to other strains (Burek and Rose 2008). Given the constraints of our study, we selected female NOD mice due to their well-documented autoimmune predisposition, which aligns with the higher prevalence of AITDs in females. Although alternative approaches, such as using NOD.H-2h4 mice or direct antibody injection, could establish models more rapidly and specifically, resource limitations necessitated the use of NaI-induced models (Braley-Mullen and Yu 2015). Second, NaI-induced thyroiditis typically requires a prolonged induction period (6–12 weeks), whereas Tg-adjuvant models can elicit autoimmunity more rapidly (Zaccone et al. 2002). Additionally, while Tg-adjuvant models induce a robust T-cell-mediated immune response, NaI-induced models primarily lead to localized thyroidal changes with comparatively lower systemic immune activation. Nevertheless, the NaI-induced model remains a valuable tool for studying iodine-related mechanisms of thyroid autoimmunity. Future studies could integrate additional experimental approaches, such as antibody injection or genetically modified mouse models, to better mimic the complex pathophysiology of AITDs. By combining different models, it may be possible to achieve a more comprehensive understanding of AITDs pathogenesis and identify potential therapeutic targets. Additionally, in this study, we only utilized tangeretin for animal experiments, while other PMFs remain to be explored in future research to further elucidate their potential immunomodulatory effects.

To date, no study has directly investigated the role of PMFs in modulating the IL-17 signaling pathway in murine models, including the present study. However, accumulating evidence suggests that PMFs may influence IL-17-mediated inflammation through multiple mechanisms. First, PMFs have been reported to mitigate IL-17-driven inflammation by suppressing Th17 differentiation and IL-17 production. For instance, nobiletin significantly attenuated disease severity in experimental autoimmune encephalomyelitis (EAE) mice by inhibiting Th17 differentiation and IL-17A production, potentially through histone acetylation regulation and RORγt suppression (Nakamoto et al. 2021). Similarly, tangeretin reduced Th17 responses in a murine model of LPS-induced acute lung injury by inhibiting Notch signaling, decreasing IL-17+CD4+T cells, and downregulating RORγt and IL-23 receptor expression (M. Li et al. 2020). Additionally, a systematic evaluation of five PMFs found that nobiletin and heptamethoxyflavone significantly suppressed Th17 proliferation and IL-17A production, while sudachitin, demethoxysudachitin, and natsudaidain had no effect on Th17 proliferation but all reduced IL-17A levels (Nakamoto et al. 2023). Second, IL-17 exerts its pro-inflammatory effects primarily through NF-κB and MAPK activation. While direct evidence linking PMFs to the IL-17 signaling pathway remains limited, multiple studies have demonstrated that PMFs can modulate NF-κB and MAPK activity. For instance, in a murine colitis model, oral administration of tangeretin attenuated inflammation by inhibiting IL-12 and TNF-α expression and blocking NF-κB activation via suppression of lipopolysaccharide binding on dendritic cells (Eun et al. 2017). Similarly, our Supplementary Information (Figure S1) indicated that tangeretin treatment downregulates serum IFN-γ levels in NaI-induced AITDs mice, as shown in Figure S1, suggesting a comparable inhibitory effect on inflammatory signaling. Furthermore, emerging research highlights a strong link between IL-17 signaling and TRAF6/NF-κB activation. A study on Alzheimer's disease found that IL-17 exacerbates Aβ-induced neurotoxicity by activating the TRAF6/NF-κB axis, whereas IL-17 blockade alleviates synaptic dysfunction and cognitive impairment (Y. Liu et al. 2023). Given that TRAF family proteins are key mediators of IL-17 signaling, and considering our in vitro findings suggesting that tangeretin and nobiletin may regulate IL-17 signaling via TRAF proteins, it is plausible that PMFs exert similar effects in vivo. Building on these insights, we hypothesize that PMFs may regulate the IL-17 signaling pathway through three potential mechanisms: (1) suppression of Th17 differentiation and IL-17 production, (2) modulation of NF-κB and MAPK activation, and (3) potential interaction with TRAF family proteins. However, direct in vivo evidence remains limited, and further research is needed to elucidate the precise molecular mechanisms. Future investigations utilizing IL-17 reporter mice, pathway-specific inhibition assays, or genetic manipulation of TRAF proteins will be essential to clarify the role of PMFs in IL-17 signaling within murine models of autoimmune thyroid diseases.

This study demonstrates that citrus flavonoids, particularly PMFs, possess significant immunomodulatory potential in the prevention and treatment of AITDs. Given the rising prevalence of AITDs and the limited availability of effective treatment options, dietary intervention with bioactive flavonoids represents a promising therapeutic strategy. As public awareness of functional foods continues to grow, citrus flavonoids show promise as future immunomodulators and dietary supplements (Alzaabi et al. 2022; Cornara et al. 2020). Recent reviews have highlighted the increasing interest in food-based immunotherapies for autoimmune diseases, including the use of flavonoids and other bioactive compounds from plant-based sources (Zebeaman et al. 2023). These compounds are known to exert broad immunoregulatory effects, potentially modulating inflammatory responses and immune cell activity, making them suitable candidates for managing autoimmune conditions. However, several challenges must be addressed before PMFs can be effectively translated into clinical applications. First, their bioavailability and metabolic stability in humans remain largely unexplored. To date, no clinical trials have characterized the primary metabolites of PMFs in circulation, their distribution across tissues and biological fluids, or their respective concentrations (Toledo et al. 2024). A deeper understanding of these pharmacokinetic properties is essential for evaluating their therapeutic potential and optimizing their clinical application. Moreover, recent studies on flavonoids from various dietary sources have emphasized the need for advanced research on their bioavailability and the development of more effective delivery methods to enhance their clinical applicability in autoimmune disease treatment (Tang et al. 2025). Additionally, while our findings establish a clear link between PMF treatment and improvements in AITDs biomarkers, the long-term effects and optimal dosage require further investigation. Future clinical studies should focus on assessing the pharmacokinetics and immunomodulatory potential of PMFs in AITDs patients to determine their efficacy and safety profiles. Moreover, functional foods, including those enriched with flavonoids, have been increasingly recognized as adjunctive therapeutic options for autoimmune diseases (Vieira et al. 2024). These foods can complement conventional treatments by promoting immune balance and reducing inflammation (Golonko et al. 2024). Furthermore, novel formulation strategies, such as nanoparticle-based delivery systems, warrant exploration to enhance the stability, bioavailability, and therapeutic efficacy of PMFs. Advanced drug delivery systems may improve their absorption and prolong their half-life, ultimately increasing their clinical utility. Advanced drug delivery systems may improve their absorption and prolong their half-life, ultimately increasing their clinical utility. The application of nanotechnology in functional food formulations has shown promise in improving the targeted delivery of bioactive compounds, ensuring sustained release, and enhancing therapeutic effects in autoimmune diseases (J. Wang et al. 2023). Further research is warranted to expand the scope of PMFs investigated, assess their long-term effects, and explore their interactions with key immune regulatory pathways. A deeper understanding of their mechanisms could facilitate the development of novel functional dietary components for AITDs management and potentially broader applications in autoimmune disease therapy. Addressing these challenges will be critical in bridging the gap between experimental findings and clinical translation, ensuring that PMFs can be effectively utilized in AITDs treatment.

5 | Conclusion

This study is the first to systematically evaluate the immunomodulatory effects of citrus PMFs in AITDs, demonstrating their ability to regulate IL-17 signaling and reduce TPO-Ab levels. Our results provide new insights into the immunomodulatory potential of citrus flavonoids, particularly

PMFs, in the context of AITDs. Specifically, we found that citrus flavonoids, especially tangeretin, significantly reduced TPO-Ab levels, an important marker of AITDs, and suppressed the expression of pro-inflammatory cytokines through the regulation of the IL-17 signaling pathway. These results highlight the potential of citrus flavonoids, particularly tangeretin and nobiletin, as promising therapeutic agents for AITDs.

In addition to the cellular models, the animal experiments further validated the protective effects of tangeretin on thyroid cells, where high doses reduced both thyroid follicle destruction and lymphocyte infiltration, commonly seen in AITDs pathology. These findings underscore the potential of PMFs in preventing and treating autoimmune conditions by targeting key inflammatory pathways such as IL-17.

Despite the promising results, this study also revealed several areas requiring further investigation. The observed reduction in TRAF3 protein levels, despite its upregulated gene expression, suggests a complex regulatory mechanism that warrants deeper exploration. Moreover, the study's limitations in quantifying the exact degree of alleviation due to sample size constraints highlight the need for larger-scale studies.

In conclusion, citrus flavonoids, particularly PMFs, hold significant promise as natural bioactive compounds for the management of AITDs. Future research should focus on elucidating the long-term effects of these compounds, exploring their dose-dependency, and investigating other immune regulatory pathways beyond IL-17. Such studies will provide valuable insights for the development of novel, food-based therapeutic strategies for autoimmune diseases.

Author Contributions

Manxi Wu: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing–original draft, writing–review and editing. **Han Yang**: data curation, software, writing–review and editing. **Yuhao Wu**: formal analysis, investigation, writing–original draft. **Yuanxiao Yin**: conceptualization, methodology, resources. **Junhao Li**: project administration, supervision. **Yongfu Ge**: software, visualization. **Cui Sun**: funding acquisition, supervision. **Jinping Cao**: validation, funding acquisition. **Dengliang Wang**: resources. **Yixiong Zheng**: resources, supervision. **Yue Wang**: funding acquisition, project administration, writing–review and editing. **Chongde Sun**: funding acquisition, supervision, writing–review and editing.

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Ethics Statement

The animal experiment was conducted in compliance with the ethical guidelines of the Animal Experimentation Committee in the College of Medicine, Zhejiang University (animal ethical clearance number: ZJU20240438).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the article or from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Figure S1. The effects of different dosage tangeretin administration on serum IFN-γ level in mice. One-way ANOVA with Tukey's multiple comparisons was used for multi-group comparisons. Independent samples' Student's *t*-test was used for the two groups' comparison. # compared with the control group, ns $p > 0.05$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$. *Compared with the model group, ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.