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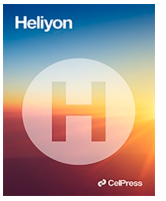

Research article

Citrus polymethoxyflavones degrade estrogen receptor-alpha (ERα) and combine with tamoxifen for the treatment of estrogen receptor-positive breast cancer

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ABSTRACT

Estrogen receptor-positive (ER^+) breast cancer seriously endangers the women's physical and mental health worldwide and ER targeting therapy is vital. Here, we found that a citrus polymethoxyflavones (PMFs)-rich hydrolysate (C–H) and its major components (nobiletin and 3-methoxynobiletin) potently degrade ERα protein *via* the ubiquitin-proteasome pathway, thereby impairing the proliferation of ER^+ breast cancer cells. Moreover, our study exhibited that C–H combined with tamoxifen (TAM) inhibited the cell proliferation of ER^+ breast cancer in *vitro*. It was further confirmed that C–H decreased tumor growth of ER^+ breast cancer in tumor-bearing 129 mice in *vivo* and improved the efficacy of tamoxifen. Our study revealed that the citrus PMFs have potential applications as pharmaceutical and healthcare products in breast cancer treatment by targeting ERα protein degradation.

1. Introduction

As one of the most prevalent malignancies, breast cancer is the leading killer of women's health worldwide [1]. Of all breast cancers, ER^+ breast cancer takes for 80 % [2]. The early occurrence and late development of ER^+ breast cancer is strongly correlated with activation of the estrogen/estrogen receptor signaling pathway. Clinically, targeting this pathway to block estrogen production and inhibit its receptor activation has become a major therapeutic approach in ER^+ breast cancer treatment, and is also a primary strategy within the endocrine therapy [3]. Selective estrogen receptor degraders (SERDs), selective estrogen receptor modulators (SERMs), and aromatase inhibitors (AIs) are the three major endocrine therapies in female patients with ER^+ breast cancer.

As a SERM drug, tamoxifen is first choice for systemic treatment to high-risk patients with the ER^+ breast cancer, which has been shown to significantly increase patient disease-free and overall survival [4]. The mechanism of tamoxifen has been clarified that its

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metabolite, 4-hydroxytamoxifen (4OHT), inhibits the estrogen signaling pathway by competitively binding to ER [5]. However, drug resistance of tamoxifen resurfaced as it was extensively utilized in clinical situations [6]. ER degradation has been proposed as a potential strategy to overcome resistance to endocrine therapy [7]. Though fulvestrant as the only clinical use of ERα-targeted degradation agent has achieved good therapeutic benefits, the application is severely hampered by its poor bioavailability, drug resistance, and other flaws [8]. Thus, it is urgently needed to develop new drugs for targeted ER degradation in clinical treatments.

Citrus fruits are one of the most popular fruits and widely used in traditional medicine for disease treatment and health improvement. Polymethoxyflavones (PMFs) exclusively exist in the citrus fruits and have been demonstrated to be a class of bioactive ingredients with antioxidant, anti-inflammatory, anticancer and anti-obesity properties [9]. Our previous study has shown that both methanol extract of precipitation (JCCD-EXT) from citrus brewing vinegar during ageing and its PMF-ingredients exhibited potently anti-proliferative effect on MCF7 cells [10]. Although PMFs have been extensively studied in anti-breast cancer, there are still some new treatment targets and strategies that have not been revealed.

As a continuing work to further uncover the mechanism, this study exhibited significant ERα degradation effect of JCCD-EXT *via* a ubiquitin-proteasome pathway. Moreover, we found that a citrus PMFs-rich hydrolysate (C–H) with the same major components (nobiletin and 3-methoxynobiletin) as JCCD-EXT similarly showed ERα degradation activity to inhibit the cell proliferation of ER^+ breast cancer. Moreover, in *vitro* and in *vivo* evaluation of its combination with tamoxifen in the treatment of ER^+ breast cancer demonstrated that the synergetic strategy may improve the sensitivity and efficacy of tamoxifen. In summary, this work suggests that citrus-derived C–H may be an effectively natural agent in the treatment of ER^+ breast cancer.

2. Experimental procedures

2.1. Cell lines and reagents

MCF7 cell line was kindly provided by Prof. Hao Wu (Nanjing Medical University, Jiangsu, China). T47D cell line was purchased from Cell Resource Center of Institute of Basic Medical Sciences (IBMS), Chinese Academy of Medical Sciences (CAMS). SSM2 cell line was kindly gifted from Prof. Dr. Robert D. Schreiber (University of Washington, Seattle, USA). MCF7 and T47D were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10 % superior grade of fetal bovine serum (FBS, ExCell Bio, Cat. NO. FSP500, USA), and 100 U/mL penicillin and 100 g/mL streptomycin with 5 % CO_2 at 37 °C. According to the literature [11], SSM2 were cultured in the mixture (1:1 mixture, Gibco, USA) of DMEM media and Nutrient Mixture F-12 (F12) media containing 10 % FBS, 1 % P/S, 2 % L-glucose and supplemented with 0.05 mM β-mercaptoethanol (Solarbio, Beijing, China), 5 μg/mL recombinant human insulin (Pricella, Wuhan, China), 0.3 μM Hydrocortisone (Yuanye Bio-Technology, Shanghai, China), and 10 ng/mL Bovine Transferrin (Yuanye Bio-Technology, Shanghai, China). Nobiletin was purchased from Macklin (Shanghai, China), while 3-methoxynobiletin, isosinensetin, sinensetin and tangeretin from Yuanye Bio-Technology (Shanghai, China). 4-Hydroxytamoxifen (4-OHT) was obtained from Sigma-Aldrich (St. Louis, USA) and tamoxifen (TAM) was purchased from MedChemExpress (MCE, Monmouth Junction, USA).

2.2. RNA isolation and quantitative real-time PCR

The mRNA levels of ESR1 in MCF7 cells treated with JCCD-EXT for 24h were detected by quantitative real-time PCR (qPCR) assay. Briefly, the extraction of total RNA was performed using the TRIzol kit (Invitrogen, California, USA). The reverse transcription of equal amounts of total RNA (1 μg) was carried out using the PrimeScript RT Master Mix (Takara, Beijing, China). In accordance with the operation manual, qPCR was carried out by qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The 2^(−ΔΔCT) method was used to calculate the relative fold changes between the expression of target genes. Gene utilized as references included GAPDH. Relative expression mRNA levels of *ESR1* (gene symbol of ERα) were normalized to the mean of the control tumor samples. Gene expression analysis was carried out for human *ESR1* gene (forward primer: 5′-AAGCGC CAGAGA GATGAT GG-3′, reverse primer: 5′-CTCAGC ATCCAA CAAGGC AC-3′) and GAPDH (forward primer: 5′-GGTGGT CTCCTCT GACTTC AACA-3′, reverse primer: 5′-GTTGCT GTAGCC AAATTCG TTGT-3′).

2.3. Western blotting

According to our previous study [12], western blotting was performed. Briefly, MCF7, SSM2 and T47D cells (2×10^5 cells per well) were respectively seeded in a 6-well plate and incubated for 24 h. Then cells were treated with JCCD-EXT at the concentration of 7.5, 15, 30 μg/mL or C–H at the concentration of 30, 60, 120 μg/mL for 24, 48, 72 h. The cells were cleaned with ice-cold PBS to prepare them for western blotting, and all samples were then scraped in RIPA buffer. The whole-cell lysates were collected following 15 min of centrifugation at 4 °C. After separation using 10 % SDS-PAGE, the proteins were transferred and deposited onto nitrocellulose membranes (Bio-Rad, Hercules, USA). The membranes were blocked with 5 % skim milk for 1 h at room temperature (RT) and then incubated with rabbit ERα (1: 2500, Millipore, USA) primary antibodies or rabbit β-actin (1:3000, Servicebio, Wuhan, China) at 4 °C overnight. Subsequently, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibodies (Servicebio, Wuhan, China) and immunoreactive protein bands were quantified and digitized.

2.4. Protein stability assays

MCF7 cells (2×10^5 cells per well) were seeded in a 6-well plate and incubated for 24 h, followed by the addition of 10 μg/mL cycloheximide (CHX, Medchemexpress, Monmouth Junction, USA) and 15 μg/mL JCCD-EXT, and incubation for indicated different time points. Meanwhile, the group treated with 10 μg/mL cycloheximide separately was regarded as control group with same indicated time points. For the protein degradation assay, cells (2×10^5 cells per well) were seeded in 6-well plate for 24 h, then treated with indicated extracts and 5 μM MG132 (Selleck, USA) or 5 μM BafA1 (Selleck, USA) for another 24 h as inhibitor of the proteasome or lysosome pathway, respectively. All samples were subject to Western blot for ERα stability assessment.

2.5. Ubiquitination assay

For ubiquitination assay in MCF7 cells, cells (2×10^5 cells per well) were seeded in 6-well plate for 24 h until the cells were completely adherent. And the cells were incubated with MG132 (5 μM) or indicated samples for another 24 h. The cell lysis lysates were prepared by RIPA buffer and spinning at 12000 rpm for 20 min at 4 °C. Then the supernatant was collected and co-incubated with protein A/G magnetic beads (Bimake, Houston, USA), which was pre-cleared by ubiquitinated antibody (1:150, Beyotime, Shanghai, China), at 4 °C under constant rotation overnight. The immunoprecipitated samples were analyzed by western blotting with ERα antibody to identify the ubiquitination of ERα.

2.6. Cell proliferation assay

MCF7, SSM2 and T47D cells (5000 cells per well) were respectively seeded in a 96-well plate with 100 μL medium and incubated until the cells were completely adherent. Then the indicated samples were administrated and cultured for 72 h. Following discard supernatant, cells were stained with 50 μL of 0.5 % crystal violet solution (0.5 g powder in 100 mL 40 % methanol) for 30 min at RT. Cells were then washed with gentle running buffer for 5 min and dried at RT overnight. Then the stained cells were resuspended in 200 μL of sodium citrate dihydrate solution (0.1 M in 50 % ethanol) with shaking for 1 h at RT, and then the optical density was recorded at 570 nm on a microplate reader (Infinite F200 pro, Tecan, Swiss). The relative cellular activity was calculated according to the following equation: The cell survival (%) = $(A_{test} - A_{blank})/(A_{control} - A_{blank}) \times 100$, in which A represents the absorbance at 570 nm. In the control group, the cells were treated with DMSO. The blank groups were plates without cells and also stained with crystal violet. The combination indexes (CI) were calculated by the CompuSyn software according to the Chou-Talalay method [13,14].

2.7. Preparation of C–H

Fresh and ripe *Citrus sinensis* 'Valencia orange' (produced in Zigui, Hubei Province) weighing 178.8 g was cleaned, dried, and then ground in 20.0 mL of water. After centrifugation at 10000 rpm for 10 min at RT, 200.0 mL of 1.0 mmol/L hydrochloric acid solution was added to the supernatant prior to heating for 14 h at 80 °C. When the reaction temperature naturally dropped to RT, the precipitate was collected after centrifugation and washed with deionized water for 3 times. Then the dried precipitate was extracted with 5.0 mL of ethanol for 3 times. After concentration of extracts under *vacuum*, the citrus polymethoxyflavones-rich hydrolysate (C–H) was prepared.

2.8. High-performance liquid chromatography (HPLC) analysis

A qualitative analysis of C–H was performed using an InertSustainAQ-C18 column (250 × 4.6 mm, 5 μm) with the column temperature of 30 °C on the Shimadzu LC-2030C 3D Plus HPLC system. The mobile phase was a mixture of water (A) and methanol (B) and eluted using a gradient program as follows: 0–10 min, 10–60 % B, 10–30min, 60–80 % B, 30–32 min, 80–100 % B, 32–35 min, 100 % B, 35–45 min, 100-10 % B. The injection volume, flow rate and detection wavelength were 10.0 μL, 1.0 mL/min and 254 nm, respectively.

2.9. Animal studies

All experimental procedures involving animals were conducted in accordance with the ARRIVE Guidelines, and the protocols approved by the Three Gorges University Laboratory Animal Center Committee on Use and Care of Animals (approval number: 2020B010C). 129 mice (female, 6–8 weeks old, 18–20 g) were purchased from Beijing Vitalstar Biotechnology Co., Ltd. (Beijing, China) and maintained under pathogen-free conditions during the experiments. For the ER^+ breast cancer syngeneic model, SSM2 cells (1×10^6 cells in 100 μL PBS) were injected into the 4th mammary fat pad of 129 mice. When tumor became detectable, the tumor volume was assessed by caliper measurements according to the formula as follows: Volume (mm^3) = (length × $width^2$)/2. When the tumor volume reached to 50 mm^3, the mice were randomized into different experimental groups. C–H or TAM was dissolved in a 100 μL solvent of DMSO and core oil ($v/v = 1:5$), which was used as vehicle in control group, and injected intraperitoneally every two days with indicated dosages. The body weight of mice and tumor volume were assessed every two days. When the tumor volume reached to 2 cm^3, the mice were euthanized and the solid tumors were excised and weighted. Mice organs and tumors were prepared for Western blot or processed into paraffin sections for hematoxylin-eosin (H&E) staining.

2.10. Statistical analysis

To compare the quantitative variables between the two groups, a two-tailed Student's t-test was used and a value of $p < 0.05$ was deemed significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). The combination index (CI) value was calculated based on the cell survival inhibition rate using CompuSyn Software (version 1.0, CompuSyn, USA).

3. Results and discussion

3.1. JCCD-EXT degrades ERα proteins via ubiquitin-proteasome system

In the previous studies, the methanolic extract of precipitation from citrus brewing vinegar during ageing exhibited potential

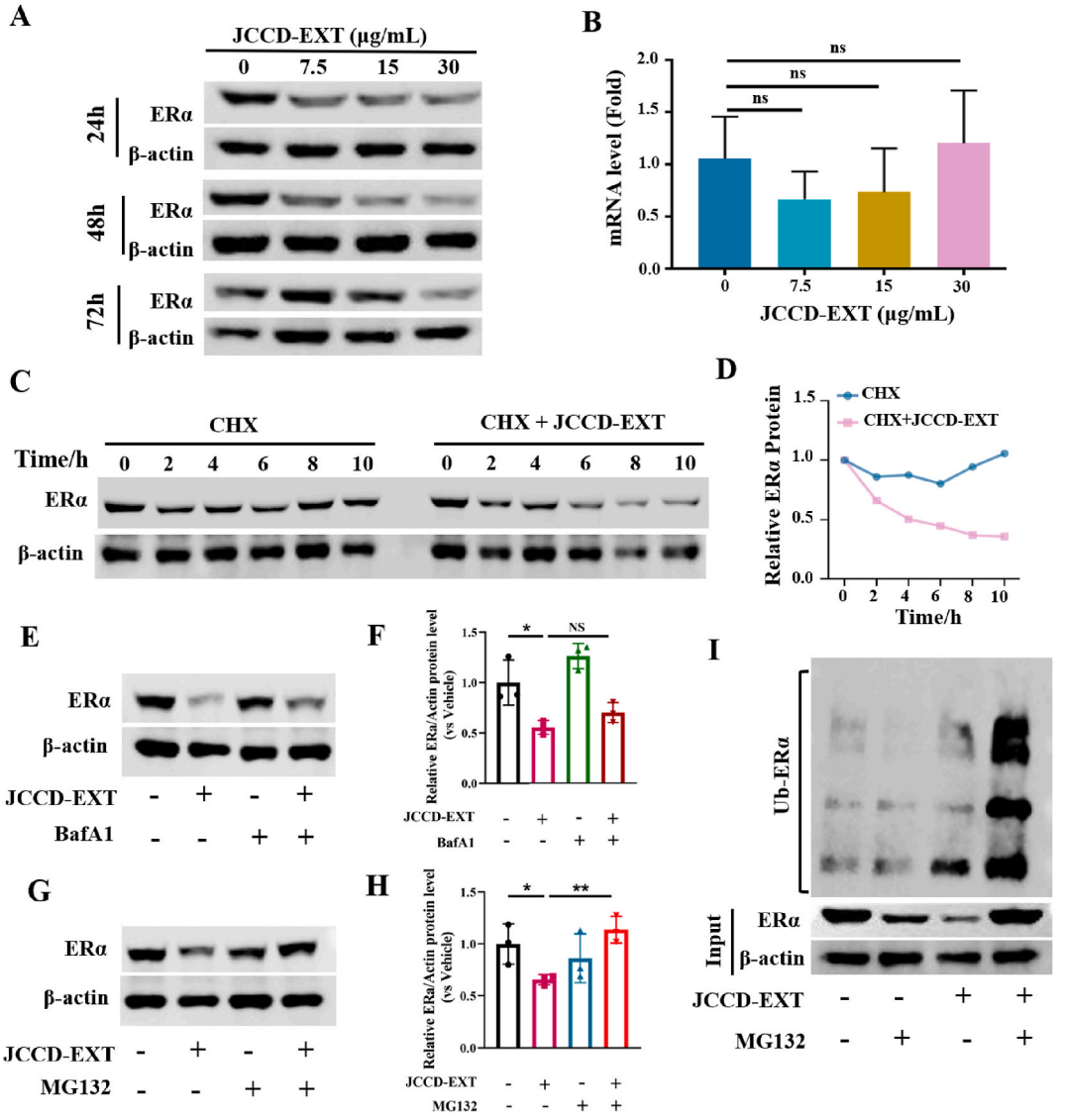

Fig. 1. JCCD-EXT degrades ERα proteins *via* the ubiquitin-proteasome system in MCF7 cells. (A) The decrease of ERα levels in JCCD-EXT-treated MCF7 cells in a dose- and time-dependent manner. (B) The mRNA levels of *ESR1* in MCF7 cells treated with JCCD-EXT for 24h. (C) Half-life of ERα in MCF7 cells treated with JCCD-EXT (15 μg/mL) or/and CHX (10 μg/mL) for indicated time. (D) Densitometric analysis of ERα expression in MCF7 cells treated with JCCD-EXT (15 μg/mL) or/and CHX (10 μg/mL) for indicated time. (E, F) The protein levels and quantitative analysis of ERα in MCF7 cells treated with JCCD-EXT (30 μg/mL) for 24 h in the absence or presence of lysosomal inhibitor BafA1 (1 mM). (G, H) The protein levels and quantitative analysis of ERα in MCF7 cells treated with JCCD-EXT (30 μg/mL) for 24 h in the absence or presence of proteasomal inhibitor MG132 (5 μM). (I) The ubiquitination status of ERα in MCF7 cells treated with JCCD-EXT (30 μg/mL) in the absence or presence of proteasomal inhibitor MG132 (5 μM) .

cytotoxicity to the ER$^+$ breast cancer cell MCF7 [10]. In further investigation, we found that the protein levels of ERα were dramatically decreased in a dose-dependent manner following treatment with the indicated JCCD-EXT concentration of 7.5, 15 and 30 μg/mL for 24, 48 and 72 h in MCF7 cells, respectively (Fig. 1A, Fig. S3).

To elucidate the mechanism of JCCD-EXT-induced decrease in ERα protein level, the mRNA level of *ESR1* (gene symbol of ERα) was analyzed and the results showed that the transcriptional level of *ESR1* was not significantly influenced by JCCD-EXT (Fig. 1B). Then cycloheximide (CHX), a protein synthesis inhibitor, was administered to MCF7 cells to determine the influence of JCCD-EXT on ERα protein synthesis. Compared with only using CHX (10 μg/mL), the additional given JCCD-EXT (15 μg/mL) dramatically reduced the half-life of ERα (Fig. 1C–D, Fig. S3). The above results revealed that JCCD-EXT induced down-regulation of ERα in MCF7 cells through boosting ERα degradation, rather than reducing the transcriptional level of *ESR1* or the production of ERα.

The autophagy-lysosome system and the ubiquitin-proteasome system (UPS) are involved in protein quality control mechanism for maintaining protein homeostasis in eukaryotic cells [15]. To elucidate the roles of two pathways possibly involved in ERα degradation, the lysosomal inhibitor BafA1 (1 μM) and proteasome inhibitor MG132 (5 μM) were used to investigate their reversal effects on ERα degradation in MCF7 cells treated with JCCD-EXT (30 μg/mL) for 24 h. In contrast to BafA1 (Fig. 1E–F, Fig. S3), MG132 prevented the ERα deterioration caused by JCCD-EXT (Fig. 1G–H, Fig. S3). To further clarify whether JCCD-EXT regulates ERα stability *via* the ubiquitin-proteasome pathway, the co-immunoprecipitation tests were performed and the results confirmed the raised ubiquitination level of ERα was induced by JCCD-EXT (Fig. 1I, Fig. S3). In a word, these findings proved that JCCD-EXT regulated ERα protein stability through ubiquitin-proteasome pathway.

3.2. 3-Methoxynobiletin and nobiletin from JCCD-EXT degrade ERα proteins

In our previous study [10], the two major components of JCCD-EXT were identified to be nobiletin and 3-methoxynobiletin as shown in Fig. 2A. Though inhibition of tumor cells proliferation of nobiletin and 3-methoxynobiletin is incontrovertible, they exhibited different effects on the stability of ERα protein due to different degrees of methoxy substituents. 3-Methoxynobiletin decreased the proteins level of ERα in a dose-dependent manner. Moreover, the down-regulation effect was diminished with increasing time (Fig. 2B). Unexpectedly, the nobiletin showed slowly ERα degradation ability when treated with the high concentration and prolonged work time of up to 72 h (Fig. 2C). Although both nobiletin and 3-methoxynobiletin increased ERα ubiquitination which was then further augmented by proteasome inhibitor MG132, 3-methoxynobiletin may be easier to increase the ubiquitination of ERα proteins than nobiletin (Fig. 2D). These results indicated that the two major components (nobiletin and 3-methoxynobiletin) from JCCD-EXT induced ERα degradation in MCF7 cells *via* the ubiquitin-proteasome pathway and their difference in the degradation ability was caused by substituents at position 3. However, we also realized that some small amounts of other components in JCCD-EXT, such as

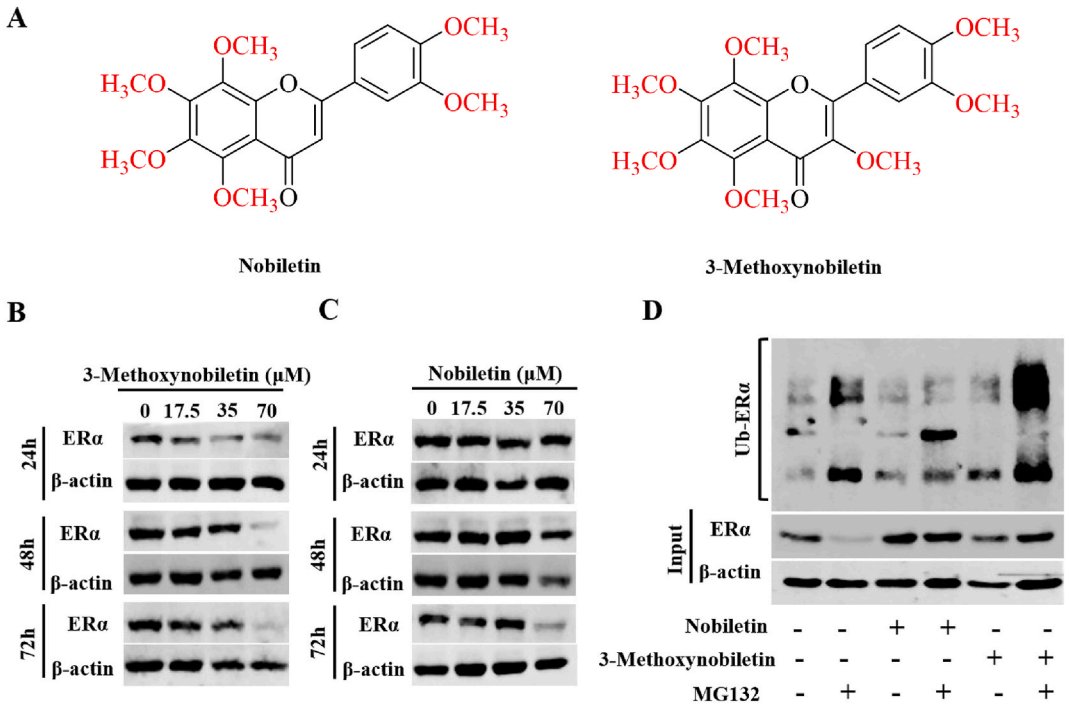

Fig. 2. 3-Methoxynobiletin and nobiletin from JCCD-EXT degrade ERα protein *via* ubiquitin-proteasome system. (A) The chemical structures of nobiletin and 3-methoxynobiletin from JCCD-EXT. (B, C) The protein levels of ERα in MCF7 cells treated with 3-Methoxynobiletin and nobiletin, respectively. (D) The ubiquitination status of ERα protein in MCF7 cells treated with nobiletin (70 μM) or/and 3-methoxynobiletin (70 μM) in the absence or presence of MG132 (5 μM) for 24h.

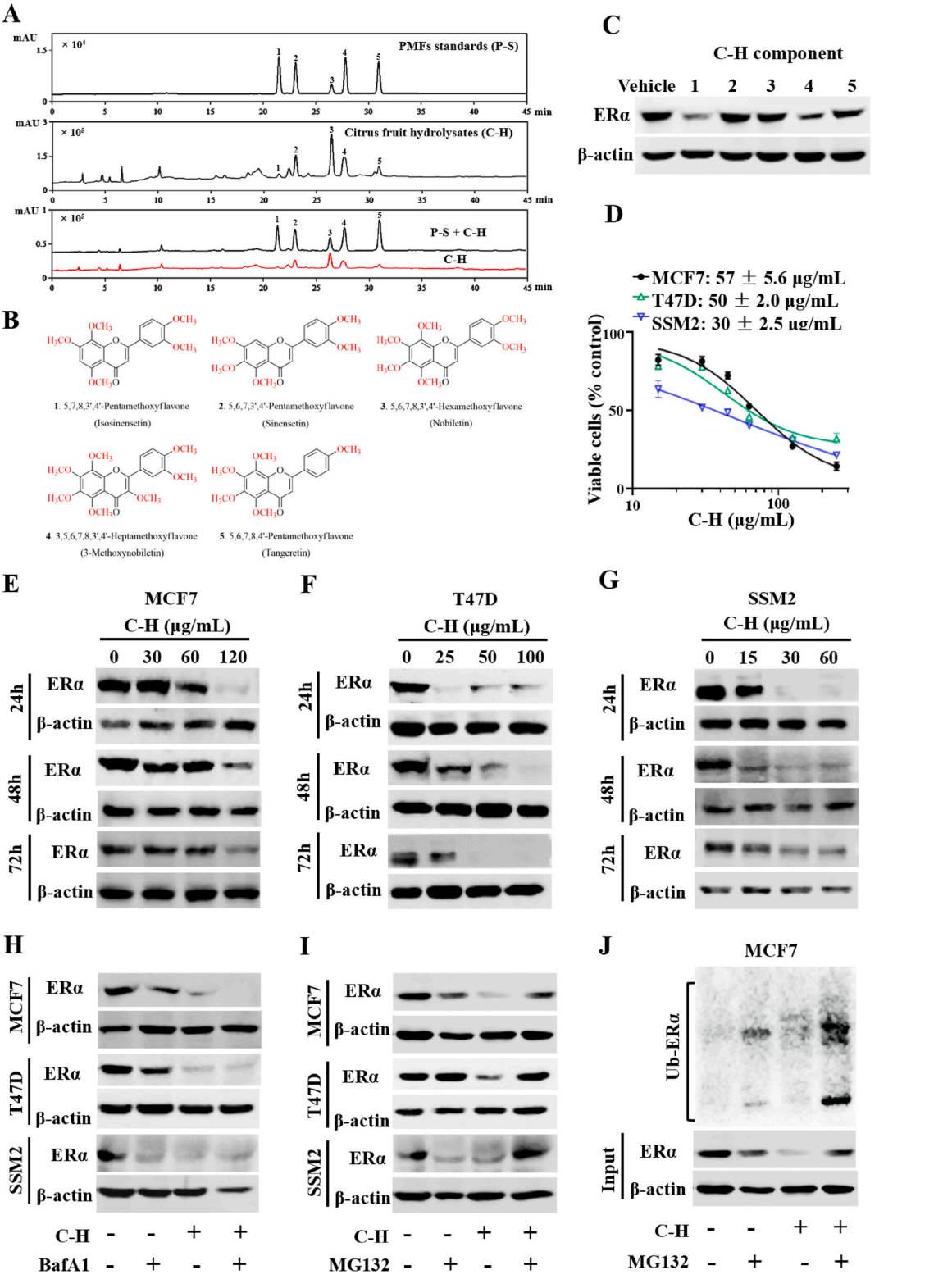

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Fig. 3. C–H degrades ERα proteins *via* ubiquitin-proteasome system. (A) HPLC analysis of C–H. (B) Chemical structures of five major components in C–H. (C) The protein levels of ERα in MCF7 cells treated with components **1**–**5** at their IC_{50} values (**1**: 0.4 mM, **2**: 0.28 mM, **3**: 0.07 mM, **4**: 0.07 mM, **5**: 0.14 mM) for 24h, respectively. (D) Cell viability assays of MCF7, T47D and SSM2 cells treated with C–H for 72 h. The single concentrations were related to the IC_{50} respectively. (E, F, G) The decrease of ERα levels in MCF7, T47D and SSM2 cells treated with C–H in a dose- and time-dependent manner. (H, I) The protein levels of ERα in MCF7, T47D and SSM2 cells treated with C–H (MCF7: 60 μg/mL, T47D: 50 μg/mL, SSM2: 30 μg/mL) for 24 h in the absence or presence of lysosomal inhibitor BafA1 (1 μM) or proteasomal inhibitor MG132 (5 μM). (J) The ubiquitination status of ERα protein in MCF7 cells treated with C–H (60 μg/mL) for 24 h in the absence or presence of MG132 (5 μM).

6-demethoxytangeretin, tangeretin, naringenin, tangerine and limocitrin, may have similar potential of ERα degradation. So, a wide range of citrus flavonoid screening for development of ERα degradation reagent and elucidation of structure-activity relationship is vital topic in the further study.

3.3. C–H degrades ERα proteins via ubiquitin-proteasome system

To overcome industrial resource constraints of JCCD-EXT and further in *vivo* pharmacological assays, we have developed an acid-driven method for extraction of PMFs from citrus and prepared PMFs-rich hydrolysate (C–H) in this study. HPLC analysis was performed and the five major components were identified, which used the PMFs standards (P–S) as reference substances, and the respective mixing ratio of components **1**–**5** in C–H is 1.4: 9.9: 71.6: 12.2: 4.9 (Fig. 3A).

The PMFs in the C–H accounted for the largest proportion, with isosinensetin (**1**), sinensetin (**2**), nobiletin (**3**), 3-methxoynobiletin (**4**) and tangeretin (**5**) making up the major components (Fig. 3B). Nobiletin (**3**), 3-methxoynobiletin (**4**) and tangeretin (**5**) in C–H are identical to those in JCCD-EXT. The five PMF-components in C–H were tested for their influence on the protein levels of ERα in MCF7 cells and the results showed that both isosinensetin (**1**) and 3-methxoynobiletin (**4**) could decrease ERα levels (Fig. 3C). Due to the

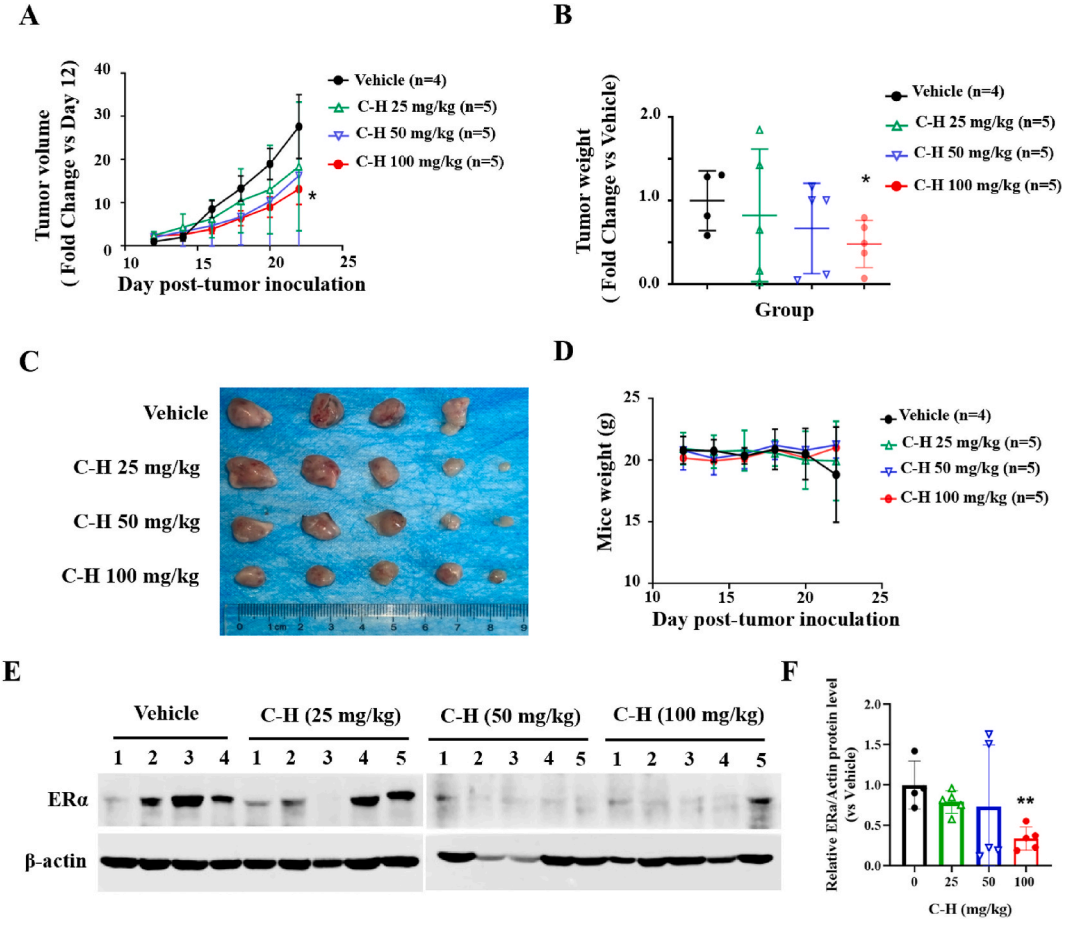

Fig. 4. C–H inhibits ER^+ breast cancer growth in *vivo*. (A) The tumor growth of SSM2 in 129 mice treated with indicated dosage of C–H. (B) The tumor weight of SSM2. (C) The tumors of SSM2 were harvested at the end of experiment and photographs were showed. (D) The body weights of mice in all groups were measured every two days. (E, F) The protein levels of ERα in tumor tissues were determined by Western blot and quantitative analysis was done. *$p < 0.05$ compared to vehicle group.

trace amount of isosinensetin (**1**) in C–H, the ERα degradation effect of C–H was postulated to be caused by the major components.

In addition to the anti-proliferation activities of C–H against ER^+ breast cancer cells MCF7, T47D and SSM2 (Fig. 3D), we also further validated the effect of C–H on ERα protein stability in these three cell lines according to the IC_{50} as shown in images. These results exhibited that C–H could degrade ERα proteins in a concentration- and time-dependent manner (Fig. 3E–G). Subsequently, the pathway of ERα protein degradation *via* ubiquitin-proteasome system also uncovered by using proteasome inhibitor MG132 and lysosomal inhibitor BafA1 in three ER^+ cell lines (Fig. 3H and I). Moreover, C–H (50 μg/mL) increased ERα protein ubiquitination in MCF7 cells following 24 h of incubation. Compared with the treatment with C–H alone, the ubiquitination levels of ERα protein in MCF7 cells were significantly enhanced with the combined treatment with C–H and MG132 (Fig. 3J). Taken together, C–H containing the same major components of JCCD-EXT reduced ERα stability through the ubiquitin-proteasome system.

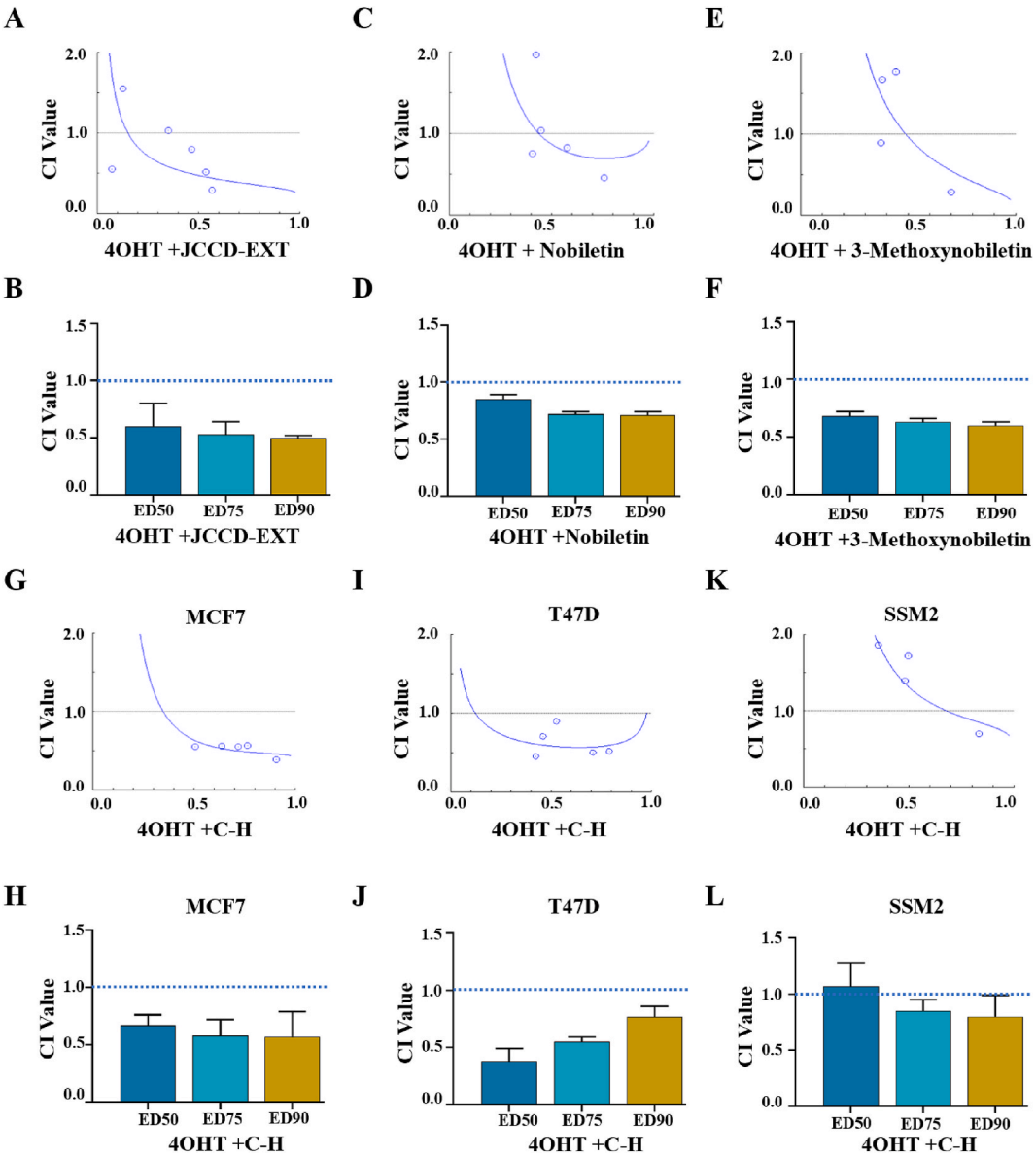

Fig. 5. C–H combined with 4OHT synergistically inhibit the cells proliferation of ER^+ breast cancer in *vitro*. (A, C, E) Fa-CI plots of combined treatment with 4OHT and JCCD-EXT/nobiletin/3-methoxynobiletin in MCF7 cells. (B, D, F) CI values for the synergistic effect of 4OHT and JCCD-EXT/nobiletin/3-methoxynobiletin in MCF-7 cells. (G, I, K) Fa-CI plots of combined treatments with C–H with 4OHT in MCF7, T47D and SSM2 cells. (H, J, L) CI values for the synergistic effect of C–H and 4OHT in MCF7, T47D and SSM2 cells. CI values were analyzed by CompuSyn software. CI value < 1 indicates synergy; CI value > 1 indicates antagonism.

3.4. C–H inhibits ER$^+$ breast cancer growth in vivo

To verify the anti-proliferation effect of C–H in *vivo*, the ER$^+$ breast cancer syngeneic model using SSM2 cell line was generated and treated with different dosages of C–H (25, 50, and 100 mg/kg). The highest dose group of C–H (100 mg/kg) displayed significant inhibitory effect against tumor growth and led to 50 % reduction in tumor volume and weight as compared to vehicle group (Fig. 4A–C). In addition, no significant mice weight loss or obvious abnormal cell morphologies were observed in the C–H treated groups (Fig. 4D and S1A). To further investigate the effect of C–H on the stability of ERα proteins in *vivo*, the tumor tissues were homogenized and processed to analyze the ERα expression. The result showed that the protein levels of ERα decreased in middle and high dose of C–H treated groups while less effect was observed in low dose of C–H treated group (Fig. 4E–F, Fig. S3). These results suggested that C–H could inhibit ER$^+$ breast cancer growth and down-regulate ERα protein levels in tumor tissue.

3.5. C–H combined with tamoxifen synergistically inhibit ER$^+$ breast cancer growth in vitro and in vivo

Since JCCD-EXT and C–H have the same major components and showed the potential of ERα degradation, the anti-cancer effect of those combinations with tamoxifen on ER$^+$ breast cancer was further evaluated (Fig. 5 and S2). The results showed that JCCD-EXT and 4OHT exhibited an explicit synergistic effect (CI < 1) in MCF7 cells (Fig. 5A and B). Though the combination of between nobiletin/3-methoxynobiletin and 4OHT achieved a good synergistic effect, the synergistic effect of nobiletin is obviously weaker than 3-methoxynobiletin/JCCD-EXT (Fig. 5C–F). As an alternative material, the synergistic cytotoxicity to ER$^+$ breast cancer cells simultaneously treated with C–H and 4OHT was evaluated in three cell lines including MCF7, T47D and SSM2. The co-treatment of C–H with 4OHT exhibited a strong synergistic effect (CI < 1) in hindering proliferation of ER$^+$ breast cancer cell lines MCF7 and T47D, whereas the synergistic effect is not obvious in SSM2 cells (Fig. 5G-L). In summary, the above results demonstrate that the simultaneous administration of JCCD-EXT/C–H/nobiletin/3-methoxynobiletin and 4OHT results in synergism, which is compatible with their effectiveness in inhibiting growth.

To further verify the synergistic effect in *vivo*, combined treatment of C–H and 4OHT was performed on 129 mice bearing orthotopic SSM2 tumors. As shown in Fig. 6A–C, treatment with C–H or tamoxifen by intraperitoneal injection every two days did not inhibit the tumor growth, while the combination therapy showed a significant inhibitory effect against SSM2 tumor, with a 50 % reduction both in tumor volume and weight compared to the vehicle group. The mean tumor weight also decreased significantly in the combined group compared to either C–H or TAM group. The weight of each group of mice did not change significantly, and there was no significant organ damage observed in all groups (Fig. 6D and S1B). These results suggest that combination C–H therapy with the endocrine therapy tamoxifen has more tumor suppressive effect in ER$^+$ breast cancer than either alone.

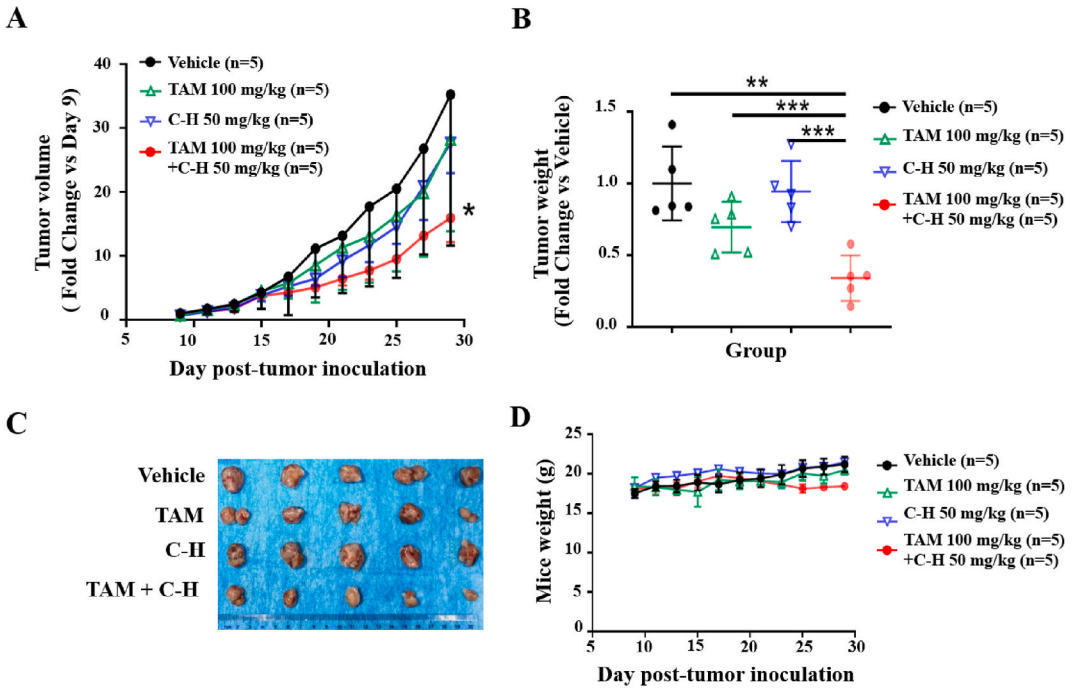

Fig. 6. C–H combined with tamoxifen inhibit of ER$^+$ breast cancer growth in *vivo*. (A) The tumor growth of SSM2 syngeneic model treated with C–H or/and TAM. (B) The tumor weight of xenograft tumors at the end of experiment. (C) Photographs of the tumors. (D) The body weights of mice in all groups were measured every two days. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.6. Discussions

Tamoxifen resistance in breast cancer results in late recurrence and death of some patients after 5 years of adjuvant endocrine therapy [16]. In order to increase tamoxifen sensitivity, drug synergy therapy has gradually become an important strategy for clinical treatment, such CDK4/6 inhibitors and chemotherapeutics as synergistic drugs [17,18]. Though the bioavailability of tamoxifen was improved in the presence of PMFs in *vivo* [19], their potential interaction and further pharmacologic properties were unrevealed. We reported that the PMFs extracted from citrus vinegar precipitation during ageing showed potent anti-proliferation of MCF-7 cells by inducing the G2/M phase arrest and breaking tubulin polymerization [10]. And some reports also showed PMFs had a wide range of biological activities [20]. In this study, we revealed that PMFs derived from citrus could decrease the ERa level and increase the sensitivity of tamoxifen in the treatment for ER^+ breast cancer.

Estrogen signaling pathway plays a pivotal role in cell proliferation of ER^+ breast cancer. And the therapeutic strategies targeting ERα have achieved greater clinical benefits [5]. Fulvestrant is an unshakable SERD in clinical treatment, and its poor bioavailability triggered the exploration of new SERDs [21], such as SAR439859, Elacestrant (RAD1901) and ERD-308 [22–25]. Up to now, these SERDs have been synthesized chemically, and there were few studies focusing on natural products inducing ER degradation.

Many studies have shown that the expression of ERα is down-regulated through a proteasome-dependent mechanism. However, few studies have focused on the regulation of flavonoids on the degradation of ERα *via* the ubiquitin-proteasome pathway. In our study, we have confirmed that the proliferative inhibition of JCCD-EXT, C–H, and their major components (nobiletin and 3-methoxynobiletin) against ER^+ breast cancer cell lines was achieved *via* the ubiquitin-proteasome-mediated ERα degradation, although they show different levels of ER ubiquitination and degradability. It would be interesting to further explore the correlation between the changes of methylation sites in flavonoid structure and ERa degradation.

Although SERD were effective as a treatment for tamoxifen-resistant breast cancer [26,27], loss of ER expression was observed in tamoxifen resistance cancer [28,29]. And the reason for ERa decrease during tamoxifen therapy remains elusive. Even sharing the same target, as fulvestrant and tamoxifen, the crosstalk between them in the treatment of breast cancer are hard to understand. It was reported that naringenin, which are abundant in citrus fruits, could down-regulated subtype of estrogen receptor ERa66 and combined with tamoxifen to play the anti-proliferative effects in ER^+ breast cancer [30]. In this work, the combined usage of C–H and tamoxifen in *vivo* inhibited the ER^+ breast cancer growth and boosted the effectiveness of tamoxifen therapy. In addition, C–H and JCCD-EXT exhibited superior and more stable ERα degradation ability to that of their two major components. Therefore, it would be necessary to isolate the unidentified components in C–H and JCCD-EXT and evaluate their potential for induced degradation of ERα.

4. Conclusions

In conclusion, this study provided a potentially candidate strategy using citrus PMFs for treatment of ER^+ breast cancer *via* the ubiquitin-proteasome pathway. Especially, it will help to hasten the development of anticancer drugs for overcoming the resistance of breast cancer to tamoxifen. Moreover, considering the attributions of health promoting of PMFs, C–H provides a potential source of functional ingredients for developing nutraceuticals.

Ethics statement

All animal experiments were carried out in accordance with the protocols approved by the Three Gorges University Laboratory Animal Center Committee on Use and Care of Animals, and Animal ethics approval number was 2020B010C. 129 mice (female, 6–8 weeks old, 18–20 g) were purchased from Beijing Vitalstar Biotechnology Co., Ltd. (Beijing, China) for ER^+ breast cancer model.

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Data availability statement

All relevant data are within the paper and its supporting information files. The original images of gel and blots supported figures [2 (B – D), 3(C, E – J)] were missed due to unexpected incidence. The authors warrant these results and relative conclusions of this study.

CRediT authorship contribution statement

Yiyu Wang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Meng Sun:** Writing – review & editing, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **Zhong He:** Methodology, Investigation. **Ying Han:** Resources, Methodology. **Yinhong Song:** Writing – review & editing. **Jianjia Liang:** Writing – review & editing. **Huimin Wang:** Writing – review & editing. **Ye Qin:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zhangshuang Deng:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Zhangshuang Deng reports financial support was provided by National Natural Science Foundation of China. Jianjia Liang reports financial support was provided by Yichang Applied Basic Research Project. Ye Qin reports financial support was provided by opening foundation of Tumor Microenvironment and Immunotherapy Key Laboratory of Hubei province in China. Zhangshuang deng has patent pending to China Three Gorges University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33104.

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