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Original Research

Bioaccessibility of Phenolic Compounds in Green Citrus Syrup During Simulated Gastrointestinal Digestion and Their Relation to Antioxidant Activity and Xanthine Oxidase Inhibition

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Abstract

Background: Green citrus is rich in phenolic compounds with potential antioxidant and xanthine oxidase (XO) inhibitory activities. However, the dynamic changes in its free and bound phenolic fractions during gastrointestinal digestion remain unclear. **Methods:** Green citrus syrup was subjected to *in vitro* simulated gastrointestinal digestion. Free and bound phenolic compounds were extracted at different digestion stages, and phenolic content, phenolic composition, bioaccessibility, antioxidant activity, and XO inhibitory activity were evaluated. Spearman correlation analysis and molecular docking were further performed to identify potential phenolic contributors to XO inhibition. **Results:** Simulated digestion markedly increased free phenolic content from 522.2 mg gallic acid equivalents (GAE)/L before digestion to 800.7 mg GAE/L after gastric digestion and 859.5 mg GAE/L after intestinal digestion. In contrast, whereas bound phenolic content decreased from 429.6 to 54.1 mg GAE/L. A total of 28 free and 21 bound phenolic compounds were identified. Phenolic bioaccessibility increased from 55.09% to 89.85% during digestion, accompanied by enhanced antioxidant activity and XO inhibitory activity. Correlation analysis identified 20 phenolic compounds positively associated with XO inhibition, and molecular docking suggested that hesperidin, naringin, hesperetin, hesperetin 7-O-glucoside, morin, and 6-gingerol could interact with XO mainly through hydrogen bonding. **Conclusions:** *In vitro* gastrointestinal digestion promoted the release and bioaccessibility of green citrus phenolics, thereby enhancing the associated antioxidant and XO inhibitory capacities. These findings provide a basis for the functional development and processing of green citrus syrup.

Keywords: green citrus; phenolic compounds; simulated digestion; antioxidant activity; xanthine oxidase

1. Introduction

Phenols are abundant in plant-based food and exert a broad spectrum of health-promoting effects [1]. They play a vital role in preventing oxidative damage to cells, which is linked to chronic diseases such as cancer and cardiovascular disorders, and help mitigate cellular senescence. Therefore, increasing intake of phenol-rich foods is widely recommended to support overall health [2]. Fruits abundant in phenolic acids, flavonoids, and other bioactive compounds are major dietary sources of phenols.

Phenolic compounds in plants typically exist in the free and bound phenols. Free phenols can be absorbed and utilized by humans after gastrointestinal digestion [3]. In fruits, vegetables, and cereals, 20–60% of phenols are generally present in bound forms, which are covalently linked to macromolecules in plant cell walls, such as structural proteins, cellulose, and pectin. Bound phenols often exhibit favorable biological activities, including antioxidant, prebiotic, anticancer, anti-inflammatory, antidiabetic effects, and neuroprotective effects [4]. The unreleased bound phenols during gastrointestinal digestion are ultimately metabolized by gut microorganisms [5]. However, the dynamic changes of bound phenols during gastrointestinal digestion have been insufficiently studied [4,6]. Green citrus, a member of the Citrus genus within the Rutaceae family, is rich in phenolic compounds, vitamin C, and carotenoids [7]. It exhibits various biological activities, including antioxidant, anticancer, anti-inflammatory, antihyperlipidemic, and antidiabetic effects [8]. Several citrus flavonoids, including hesperidin, naringin, tangeretin, and neohesperidin, have been reported to exhibit xanthine oxidase (XO)-inhibitory activity, suggesting their potential as antihyperuricemic agents [9]. Despite the well-recognized health benefits of citrus fruits, research on the phytochemical profiles, biological activities and bioaccessibility of green citrus during *in vitro* gastrointestinal digestion remains limited.

This study aims to investigate the changes of free and bound phenols in green citrus during *in vitro* simulated gastrointestinal digestion. The variations in the content, composition, antioxidant activity, and bioaccessibility of phenols in green citrus during *in vitro* digestion were analyzed. Then, the XO inhibitory ability of free phenols

during digestion was assessed. Finally, correlation analysis and molecular docking simulations were performed to explore the interactions between potential phenolic compounds and XO.

2. Materials and Methods

2.1 Materials and Reagents

Green citrus fruits identified as *Citrus reticulata* Blanco were harvested at the immature-green stage in September from Meijiang, Jiangmen City, Guangdong Province, China. The fruits were supplied by Nanchang Juena Food Co., Ltd. (Nanchang, China). XO (9002-17-9, 8.3 U/mg) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). α-amylase (A8755, 50 U/mg), pepsin (P8390, ≥250 U/mg), and pancreatin (P8150, 4000 U/mg) were obtained from Solarbio (Beijing, China). Other analytical-grade reagents, were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 In Vitro *Simulated Gastrointestinal Digestion*

Green citrus was squeezed to obtain its syrup, which was subjected to *in vitro* simulated digestion according to a previously reported method with minor modifications [10]. Briefly, 10 mL of green citrus syrup was mixed with 10 mL of simulated saliva, and incubated at 37 °C and 120 rpm for 2 min, then placed immediately in an ice bath to stop the enzymatic reaction. The simulated saliva (pH 7.0) consisted of $CaCl_2 \cdot 2H_2O$ (1.5 mM), KCl (15.1 mM), KH_2PO_4 (3.7 mM), $NaHCO_3$ (13.6 mM), $MgCl_2 \cdot 6H_2O$ (0.15 mM), $(NH_4)_2CO_3$ (0.06 mM), HCl (1.1 mM), and α-amylase (75 U/mL). Thereafter, the mixture was mixed with an equal volume of simulated gastric fluid and incubated at 37 °C and 120 rpm for 2 h. The simulated gastric fluid (pH 3.0) consisted of $CaCl_2 \cdot 2H_2O$ (0.15 mM), KCl (6.9 mM), KH_2PO_4 (0.9 mM), $NaHCO_3$ (25 mM), NaCl (47.2 mM), $MgCl_2 \cdot 6H_2O$ (0.12 mM), $(NH_4)_2CO_3$ (0.5 mM), HCl (15.6 mM), and pepsin (2000 U/mL). Subsequently, the resulting mixture was mixed with an equal volume of simulated intestinal fluid and incubated at 37 °C and 120 rpm for 3 h. The simulated intestinal fluid (pH 7.0) consisted of $CaCl_2 \cdot 2H_2O$ (0.6 mM), KCl (6.8 mM), KH_2PO_4 (0.8 mM), $NaHCO_3$ (85 mM), NaCl (38.4 mM), $MgCl_2 \cdot 6H_2O$ (0.33 mM), HCl (8.4 mM), bile salts (10 mM), and pancreatin (100 U/mL). During *in vitro* simulated digestion process, aliquots of the digest were withdrawn for subsequent extraction.

2.3 Extraction of Free and Bound Phenolic Compounds

Free phenolic compounds in green citrus syrup samples were extracted according to the method described by de Andrade et al. [11]. Briefly, 2 mL of the samples was mixed with 4 mL of 70% (v/v) ethanol, followed by sonication at 60 °C for 40 min. Afterward, the mixture was centrifuged at 10,000 × g for 10 min to collect the supernatant. The sediment was re-extracted using the same procedure. The supernatants from the two extractions were merged and concentrated using a rotary evaporator at 45 °C to obtain the free phenolic extracts.

Bound phenolic compounds in the residue from free phenolic extraction were extracted according to a previously reported method [12]. Briefly, 10 mL of 2 M NaOH was added to the residue, followed by hydrolysis at room temperature for 1 h. Subsequently, the mixture was neutralized with 6 M HCl (1.2 mL), and defatted with 10 mL of n-hexane and centrifuged (12,000 × g, 10 min) to collect the supernatant. The resulting supernatant was extracted five times using ethyl acetate to achieve the bound phenolic extracts.

2.4 Determination of Phenolic Content

The free and bound phenolic contents in green citrus syrup were determined using the Folin-Ciocalteu method [11,13]. Briefly, 0.5 mL of the phenolic extract was mixed with an equal volume of Folin-Ciocalteu reagent, followed by incubation in the dark for 5 min. Subsequently, 3.5 mL of distilled water and 3.25 mL of 7.5% Na_2CO_3 solution were added, followed by incubation at 37 °C for 2 h. The absorbance of the resulting reaction mixture was measured at 765 nm using a Varioskan LUX multifunctional microplate reader. The phenolic content was expressed as milligrams of gallic acid equivalents per liter (mg GAE/L). The total phenolic content was calculated as the sum of both free and bound phenolic contents.

2.5 Analysis of Phenolic Composition

The samples were analyzed using an Agilent 1290 Infinity II UPLC system coupled with an Agilent 6545 Q-TOF/MS (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 Rapid Resolution HD column (2.1 × 100 mm, 1.8 μm; Agilent Technologies, Inc., Santa Clara, CA, USA). The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). Separation was carried out at a column temperature of 40 °C with a constant flow rate of 0.3 mL/min, using a linear gradient elution program as follows: 0–1.5 min, 5% B; 1.5–10.5 min, 5%–26.5% B; 10.5–12.5 min, 26.5% B; 12.5–35 min, 26.5%–60% B; 35–45 min, 60%–100% B; 45–50 min, 100% B; 50–55 min, 100%–5% B. Mass spectrometric identification of eluted peaks was performed in negative ion mode with the following instrumental parameters: gas temperature, 325 °C; drying gas flow rate, 10 L/min; nebulizer pressure, 25 psi; octupole voltage, 750 V; skimmer voltage, 65 V.

2.6 Bioaccessibility of Phenols in Green Citrus

The free phenolic content in green citrus syrup before and after simulated digestion was determined. The bioaccessibility was calculated using the following equation:

Bioaccessibility (%) = (PC_{SF}/PC_{TM}) × 100

where PC_{SF} is the free phenolic content of the digested green citrus syrup; PC_{TM} is the total phenolic content of the undigested green citrus syrup.

2.7 Antioxidant Activity Assay

The antioxidant activity of the free phenolic extracts was determined using commercial assay kits for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, and ferric reducing antioxidant power (FRAP). DPPH radical scavenging activity was expressed as micrograms of Trolox equivalents per milliliter of green citrus syrup (μg Trolox/mL). ABTS radical scavenging activity was expressed as millimoles of Trolox equivalents per liter of green citrus syrup (mM Trolox). FRAP was expressed as millimoles of $FeSO_4$ equivalents per liter of green citrus syrup (mM $FeSO_4$).

2.8 XO Inhibition Activity Assay and Correlation Analysis

XO and xanthine were each separately dissolved in phosphate buffer (100 mmol/L, pH 7.5). A 200 μL aliquot of the free phenolic extract was mixed with 200 μL of XO solution (0.1 U/mL), followed by incubation at 37 °C for 5 min. Afterwards, 600 μL of xanthine (0.2 mM) was added to trigger the reaction. After 5 min of reaction, the absorbance of the reaction system was determined at 292 nm with a Ultraviolet–visible (UV-vis) spectrophotometer. The XO inhibitory activity was expressed as milligrams of allopurinol equivalent per milliliter of green citrus syrup (mg ALO/mL).

The correlation between XO inhibitory activity and the content of individual free phenolic compounds in the extracts was analyzed by Spearman's correlation analysis using SPSS 27.0 software (IBM Corp., Armonk, NY, USA).

2.9 Molecular Docking Analysis

The interaction between XO and individual phenolic compounds was analyzed using AutoDock Vina 1.5.6 (The Scripps Research Institute, La Jolla, CA, USA). The three-dimensional crystal structure of XO (PDB ID: 3ETR) was obtained from the RCSB Protein Data Bank, and the three-dimensional structure of the phenolic compounds was obtained from the PubChem database. A docking grid was created around the protein with center coordinates of x = –13.09, y = –1.745, z = 38.505, and dimensions of 150 × 86 × 94 Å. Docking calculations were performed using the Lamarckian genetic algorithm, and potential interactions between the compounds and XO were analyzed using PyMOL 3.0.3 (Schrödinger, LLC, NY, USA).

2.10 Statistical Analysis

The data were analyzed using one-way ANOVA followed by Tukey's post hoc test. All experiments were performed in at least in triplicate, and results are presented as means ± standard deviation (SD). Statistical significance were considered at $p < 0.05$.

3. Results and Discussion

3.1 Changes in Phenolic Content During In Vitro *Simulated Digestion*

Phenolic compounds associated with the food matrix may undergo release and degradation during gastrointestinal digestion. As shown in Table 1, the phenolic profile of green citrus syrup changed markedly throughout simulated digestion. Before digestion, the contents of free and bound phenolic compounds were 522.2 and 429.6 mg GAE/L, respectively. After completion of the sequential oral and gastric digestion phases, the free phenolic content increased markedly to 800.7 mg GAE/L, and further increased to 859.5 mg GAE/L after intestinal digestion. By contrast, the bound phenolic content decreased sharply, reaching 54.1 mg GAE/L after complete digestion. **Supplementary Table 1** further indicates that, although the proportions of free and bound phenolic fractions changed substantially during digestion, the total phenolic content remained relatively stable. These results suggest that simulated gastrointestinal digestion mainly promoted the conversion of bound phenolics into free phenolics rather than causing a substantial loss of total phenolics. In the present study, oral digestion had only a limited effect on phenolic release, whereas the gastric and intestinal phases were the major stages responsible for the liberation of bound phenolics. This result is consistent with Wang et al. [14], who reported minimal release of phenolic compounds from tomato pulp during *in vitro* oral digestion. The limited effect of the oral phase in our study may be attributed to the short digestion time and the relatively mild conditions, which were insufficient to extensively disrupt the citrus syrup matrix or hydrolyze matrix-bound phenolics. In contrast, the pronounced increase in free phenolic content after gastric digestion indicates that the acidic gastric environment played an important role in weakening the interactions between phenolics and matrix components, such as polysaccharides, proteins, or cell wall residues. This finding is broadly consistent with Bouayed et al. [15], who showed that approximately 65% of phenolic compounds in apples were released during gastric digestion, followed by a further 10% during the intestinal phase. However, compared with apples, green citrus syrup showed a continued increase in free phenolics during the intestinal phase, suggesting that some phenolics in this matrix may require both acidic hydrolysis and subsequent enzymatic or alkaline conditions for release. Similarly, Huang et al. [16] reported substantial release of bound phenolics from *Prinsepia roylei* seeds during gastrointestinal digestion, supporting the view that digestive conditions can promote the liberation of phenolics from plant matrices. Nevertheless, differences in the extent and stage-specific release of phenolics among green citrus syrup, tomato pulp, apples, and seeds may be related to

variations in food matrix structure, phenolic composition, and the binding strength between phenolics and macromolecules. Green citrus syrup is a processed citrus-based product, and heat treatment, sugar concentration, acidity, and pectin-rich components may influence the accessibility and stability of phenolic compounds during digestion. Mechanistically, the acidic gastric environment may promote the dissociation and hydrolysis of matrix-bound phenolic compounds by weakening ionic interactions between phenolics and the food matrix [17]. During intestinal digestion, deprotonation and digestive enzyme action may further facilitate the conversion of bound phenolics into free forms. Nevertheless, a proportion of bound phenolics remained unreleased after gastrointestinal digestion, suggesting that these compounds may reach the colon and undergo further hydrolysis and biotransformation by the gut microbiota [12,18].

Table 1. The content of free and bound phenols in *green citrus* during digestion.

Digestion phase	Phenolic content (mg GAE/L)		
	Free phenols	Bound phenols	Total
Undigested	522.2 ± 7.2^{b}	429.6 ± 4.5^{c}	951.8 ± 5.6^{a}
Gastric	800.7 ± 26.4^{a}	76.9 ± 8.0^{b}	877.6 ± 25.6^{a}
Intestinal	859.5 ± 44.9^{a}	54.1 ± 2.7^{a}	913.6 ± 131.5^{a}

GAE, gallic acid equivalents. Data are presented as mean ± standard deviation (SD) (n = 3). Within each sample group, different superscript letters (a, b, and c) indicate significant differences among treatments ($p < 0.05$), whereas values sharing the same superscript letter are not significantly different. Superscript letters were assigned independently within each sample group and should not be compared across different sample groups. Statistical differences were determined by one-way ANOVA followed by Tukey's post hoc test.

3.2 Changes in Phenolic Components During Simulated Digestion

Supplementary Tables 2,3 present the individual phenolic compounds identified in both free and bound phenolic extracts during simulated digestion. Simulated digestion changed both the qualitative profile and the quantitative distribution of the detected phenolic compounds. Qualitatively, a total of 28 free phenolic compounds and 21 bound phenolic compounds were identified, including four phenolic acids, namely 3,4-dihydroxybenzaldehyde, isoferulic acid, chlorogenic acid, and bergenin. The remaining identified compounds were flavonoids and their derivatives, such as hesperidin, poncirin, naringin, and hesperetin. Nakajima et al. [19] also reported that hesperidin, naringin, and eriocitrin were identified in the citrus fruits, with hesperidin being the dominant flavonoid and accounting for 53–75% of the total flavonoid content. Hesperidin has health-promoting effects, including anti-inflammatory, cholesterol-lowering, antihypertensive, and anticancer activities [8]. The dynamic changes of individual phenolic compounds during digestion are shown in Fig. 1. After gastrointestinal digestion, the number of free phenolic compounds was increased by 2, while that of bound phenolic compounds was decreased from 21 to 8. Quantitatively, the free phenolic content increased by 64.59% (from 522.2 to 859.5 mg GAE/L), while the bound phenolic content decreased by 87.41% (from 429.6 to 54.1 mg GAE/L); meanwhile, 94.76% of the initial total phenolic content was retained. During simulated gastric digestion, the content of 3,4-dihydroxybenzaldehyde, isoferulic acid, chlorogenic acid, and bergenin increased significantly. During simulated intestinal digestion, the concentration of bergenin and chlorogenic acid decreased, whereas the levels of hesperidin and naringin increased markedly. These changes indicate the release of phenolic compounds from the green citrus matrix. In addition, the alkaline intestinal environment has been reported to promote the conversion of certain citrus flavanones into chalcones [20]. Overall, these findings demonstrate that gastrointestinal digestion altered both the qualitative composition, as reflected by changes in the detected compound profile, and the quantitative composition, as reflected by changes in the relative abundance and partitioning of phenolics between the free and bound fractions.

3.3 Bioaccessibility of Phenolic Compounds

Fig. 2 presents the bioaccessibility of phenolic compounds in green citrus syrup throughout the *in vitro* simulated digestion. As digestion progressed, the bioaccessibility of phenolic compounds increased from 55.09% to 83.70% after oral and gastric digestion, and further rose to 89.85% after intestinal digestion. These results indicate that gastrointestinal digestion facilitated the hydrolysis and release of bound phenolic compounds into free phenolic forms, particularly during gastric digestion [21]. However, in the mildly alkaline environment of the intestine, phenolic compounds are more susceptible to deprotonation, degradation, and depolymerization, which may impair their structural stability and antioxidant activity. Additionally, the hydrolysis of flavonoid glycosides during digestion increases the content of free flavonoid aglycones [8]. Digestive enzymes can also cleave the flavonoid skeleton, resulting in the formation of lignans, phenolic acids, etc. [22]. Bound phenolic compounds that remain unhydrolyzed after gastrointestinal digestion will eventually reach the colon, where they are further degraded and metabolized by the gut microbiota [23]. Notably, the hydrolytic effects driven by acidic/alkaline environments and digestive enzymes during gastrointestinal digestion not only promote the release of phenolic compounds from the food matrix, but also trigger the structural transformation of phenolic compounds [24]. Both degradation and production of phenolic compounds occur during the process.

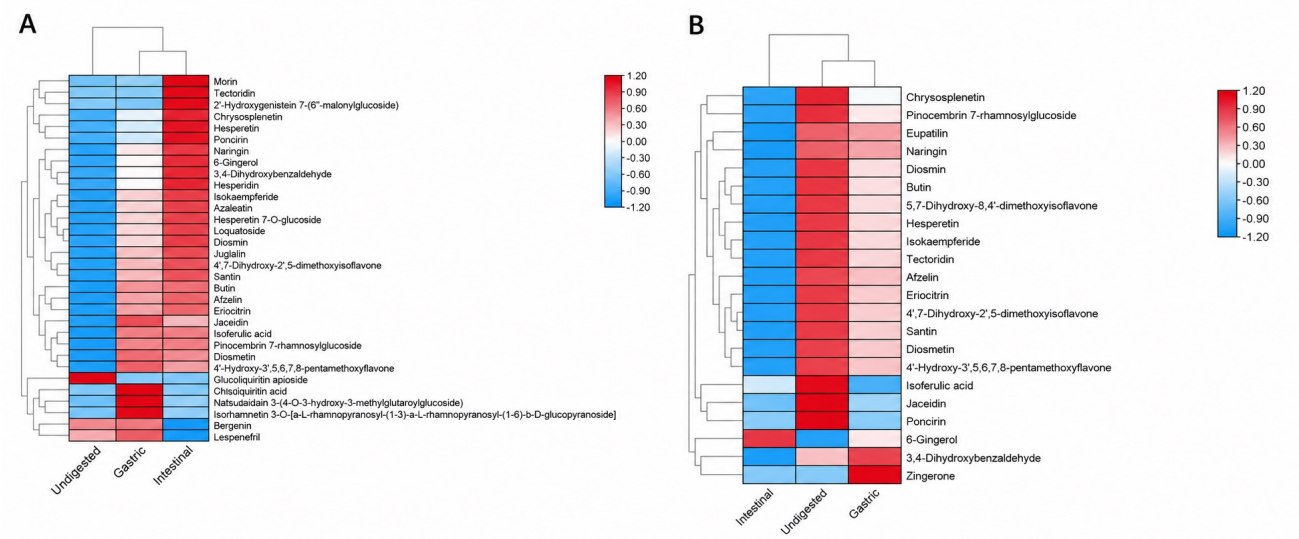

Fig. 1. Heat map and cluster analysis of free (A) and bound (B) phenolic compounds of *green citrus* during *in vitro* gastrointestinal digestion. n = 6.

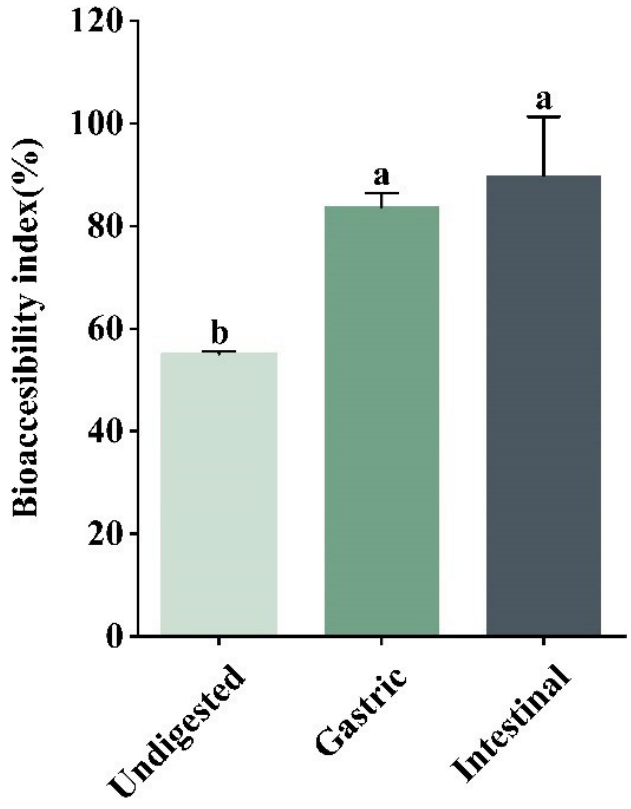

Fig. 2. The effect of *in vitro* gastrointestinal digestion on the bioaccessibility of *green citrus* phenols. Different letters indicate significant differences within the same group ($p < 0.05$). The error bars represent the SD. Data are presented as mean ± SD (n = 3).

3.4 Antioxidant Capacity of Free Phenols

The antioxidant activity of phenolic compounds is influenced by the number and position of hydroxyl groups [18]. Fig. 3 shows the antioxidant activity of free phenolic extracts from green citrus syrup at different digestion stages, including DPPH and ABTS radical scavenging activities, as well as FRAP. The DPPH radical scavenging activity increased from 639.59 to 1041.10 μg Trolox/mL after gastric digestion, and further increased to 1190.59 μg Trolox/mL after intestinal digestion. Similarly, the ABTS radical scavenging activity increased from 0.2690 to 0.2930 mM Trolox after gastric digestion and reached 0.3195 mM Trolox after intestinal digestion. FRAP increased to 3.79 mM $FeSO_4$ during gastric digestion, while it decreased to 3.09 mM $FeSO_4$ during intestinal digestion. This improvement in antioxidant activity is attributed to both the increased concentration of free phenolic compounds and the enhanced antioxidant activity of aglycones formed through the hydrolysis of phenolic derivatives [25]. In contrast, intestinal digestion may simultaneously promote the oxidation or degradation of phenolic structures that are particularly effective in Fe^{3+} reduction. The decrease in bergenin and chlorogenic acid and the increase in hesperidin and naringin also altered the relative contribution of individual compounds to the overall antioxidant response.

Additionally, the decline of FRAP should not be attributed solely to the change in pH. The transition from the acidic gastric environment to the near-neutral intestinal environment may induce the ionization, isomerization, oxidation, or biotransformation of pH-sensitive phenolic compounds. For example, chlorogenic acid can undergo substantial isomerization under simulated intestinal conditions [26]. In addition, pancreatin, bile salts, electrolytes, and interactions between phenolics and residual food-matrix components may affect the release, solubility, and effective availability of reducing compounds [27,28]. FRAP specifically evaluates electron-donating capacity through the re-

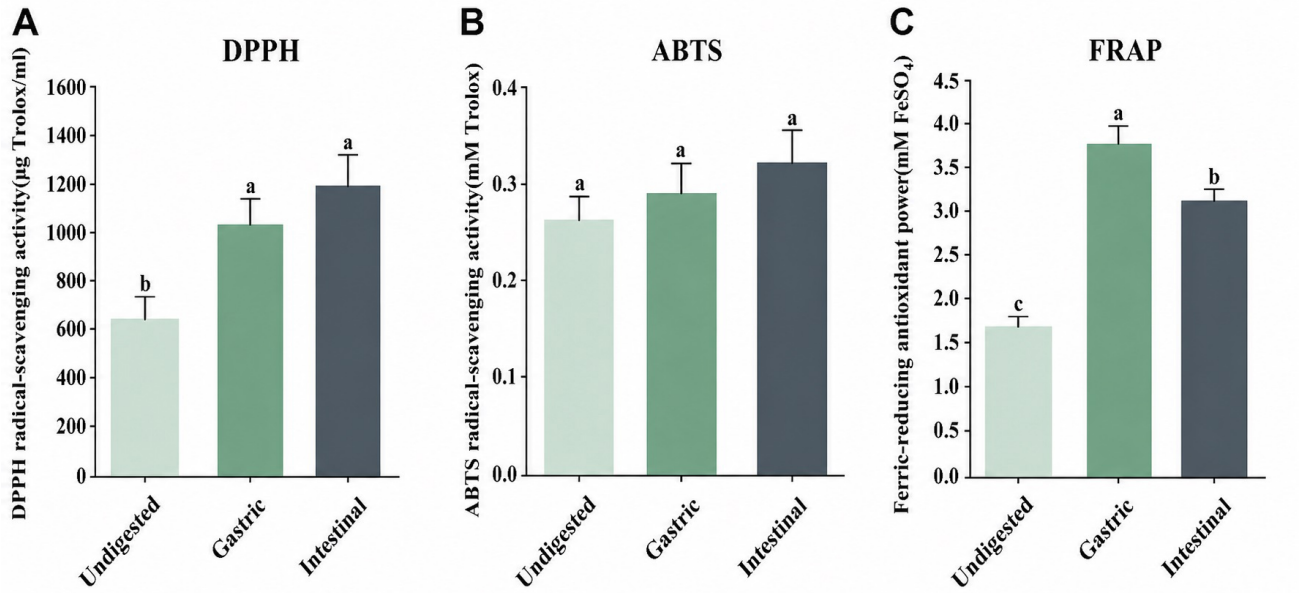

Fig. 3. Effect of gastrointestinal digestion on the antioxidant capacity of free phenols in *green citrus*. (A) 1,1-diphenyl-2-picrylhydrazyl (DPPH). (B) 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). (C) ferric reducing antioxidant power (FRAP). Different letters indicate significant differences within the same group ($p < 0.05$). The error bars represent the SD. Data are presented as mean ± SD (n = 3).

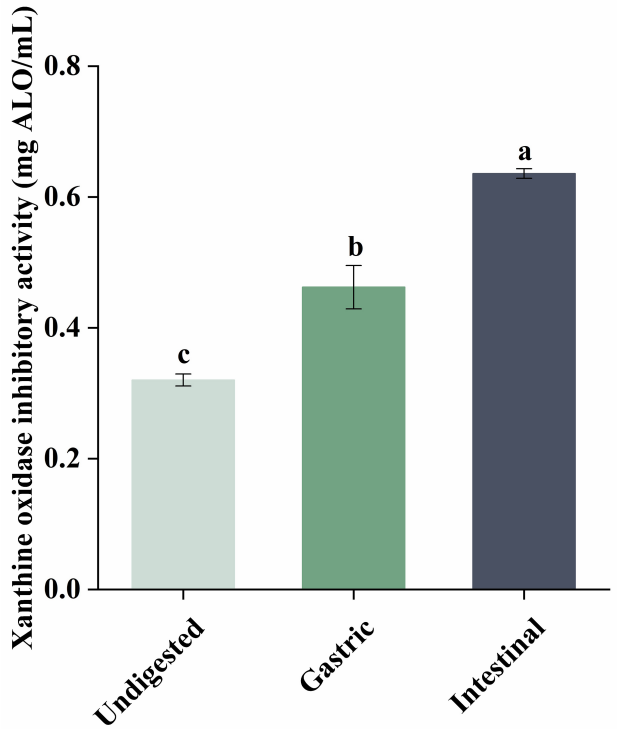

Fig. 4. Changes in the effect of gastrointestinal digestion on the xanthine oxidase (XO) inhibition of free phenols in *green citrus*. Different letters indicate significant differences within the same group ($p < 0.05$). The error bars represent the SD. Data are presented as mean ± SD (n = 3). ALO, allopurinol.

duction of Fe^{3+} to Fe^{2+} and is performed in an acidic reaction medium at approximately pH 3.6 [29]. Therefore, it does not necessarily follow the same trend as the DPPH and ABTS radical-scavenging assays, which respond differently to the structures and reaction kinetics of individual antioxidants [29,30]. Consequently, digestion-induced changes in the relative abundance and molecular structures of phenolic compounds may reduce ferric-reducing capacity even when free phenolic content and DPPH and ABTS activities increase, reflecting the assay-dependent nature of antioxidant capacity [31]. Notably, hesperidin was hydrolyzed to hesperetin during intestinal digestion, and the latter exhibits stronger inherent antioxidant activity [20]. Additionally, larger molecular bioactive substances may be degraded into smaller molecules with improved antioxidant activity during digestion [32].

3.5 XO Inhibition Activity of Free Phenols During Digestion

Phenolic compounds are well-known inhibitors of XO, which can help alleviate hyperuricemia [33]. Fig. 4 shows the XO inhibitory activity of free phenolic compounds from green citrus syrup at different stages of simulated digestion. The inhibitory activity increased progressively throughout digestion, from 0.32 mg ALO/mL before digestion to 0.46 mg ALO/mL after gastric digestion and 0.64 mg ALO/mL after intestinal digestion. This trend suggests that gastrointestinal digestion not only preserved, but also enhanced, the XO inhibitory capacity of the free phenolic fraction. Such an effect may be associated with the dynamic transformation of phenolic compounds during

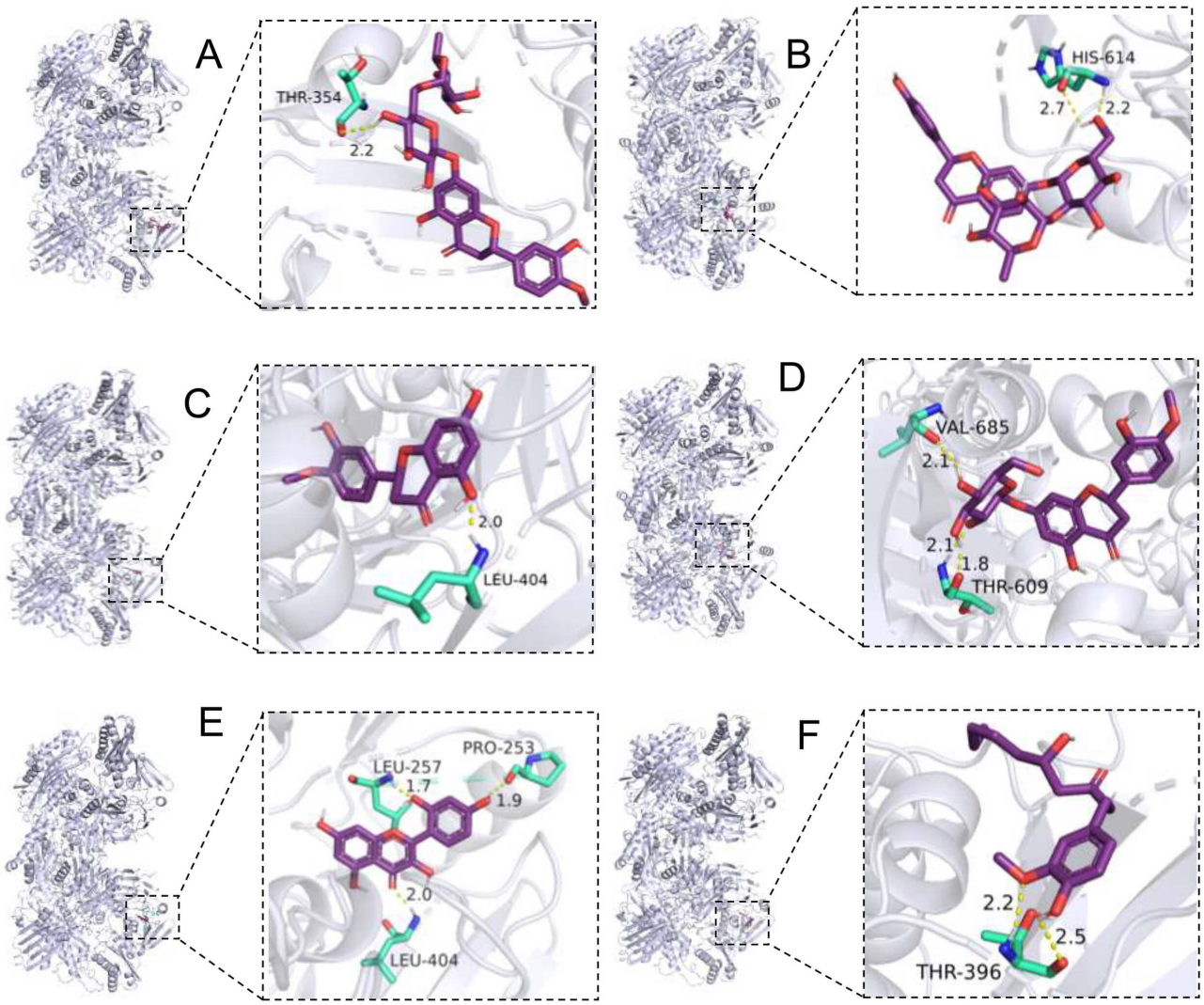

Fig. 5. Molecular docking of (A) Hesperidin, (B) Naringin, (C) Hesperetin, (D) Hesperetin 7-O-glucoside, (E) Morin and (F) 6-gingerol interacting with XO. The yellow dashed lines indicate hydrogen bond. The distance values of hydrogen bonds are expressed in angstroms (Å).

digestion, particularly the increase in the diversity of free phenolics, which could contribute to stronger XO inhibition. Moreover, flavonoids, the predominant phenolic constituents, have been reported to interact with XO via hydrophobic interactions and hydrogen bonding, thereby altering its conformation and suppressing its catalytic activity [34].

3.6 Correlation Analysis and Molecular Docking

To elucidate the contributors to the XO inhibitory activity of free phenolic compounds, correlation analysis was performed to explore the relationship between individual phenolic compounds and XO inhibitory effect (**Supplementary Table 4**). Among them, 20 phenolic compounds exhibited a strong positive correlation with XO inhibitory activity. Notably, naringin, hesperidin, hesperetin, morin, and 6-gingerol have been previously reported as effective XO inhibitors [9,35,36]. Based on their relative concentrations and correlation coefficients with XO inhibitory activity, six representative phenolic compounds, including hesperidin, naringin, hesperetin, hesperetin 7-O-glucoside, morin, and 6-gingerol, were selected for further molecular docking analysis. Typically, a binding free energy below 0 kcal/mol indicates favorable spontaneous binding between ligands and target macromolecules [37]. The molecular docking results showed that the binding free energies of the six selected compounds to XO were as follows: hesperidin (–4.84 kcal/mol), naringin (–1.43 kcal/mol), hesperetin (–5.98 kcal/mol), hesperetin 7-O-glucoside (–2.99 kcal/mol), morin (–5.75 kcal/mol), and 6-gingerol (–3.68 kcal/mol). For comparison, the binding free energy of quercetin, a well-recognized XO inhibitor, was reported to be –5.69 kcal/mol [38]. Fig. 5 shows the key amino acid residues and functional groups involved in the interac-

tions between the six phenolic compounds and XO. Specifically, hesperidin and hesperetin formed hydrogen bonds with Thr354 and Leu404 of XO, respectively. Naringin interacted with His614 through two hydrogen bonds, while 6-gingerol formed two hydrogen bonds with Thr396. Hesperetin 7-O-glucoside formed three hydrogen bonds with Val685 and Thr609, and morin formed hydrogen bonds with Leu404, Leu257, and Pro253. These results suggest that the phenolic compounds in green citrus syrup may reduce XO activity by interacting with the enzyme and altering its conformation via hydrogen bonding.

4. Limitations

The findings of this study should be interpreted in light of several limitations. First, although the *in vitro* digestion model provides a useful approximation of gastrointestinal conditions, it cannot fully recapitulate the complexity of human digestion, intestinal absorption, metabolism, and systemic distribution. Second, colonic fermentation and gut microbiota-mediated biotransformation of residual bound phenolics were not investigated, despite their potentially important contributions to the ultimate bioavailability and biological activity of citrus phenolics. Third, the mechanisms underlying the divergent changes observed in DPPH, ABTS, and FRAP activities remain speculative, as they were not directly validated through targeted identification of degradation products, assays using purified compounds, or matrix-matched controls. Fourth, the phenolic compounds identified by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS) were assessed primarily based on their relative abundance, except where absolute quantification using authentic standards was performed. Further confirmation using authentic standards and targeted quantitative liquid chromatography–tandem mass spectrometry (LC–MS/MS) is therefore warranted. Fifth, xanthine oxidase inhibition was evaluated only through *in vitro* assays and molecular docking; cellular and *in vivo* studies are needed to substantiate the potential anti-hyperuricemic effects. Finally, the green citrus syrup examined in this study was prepared from a single cultivar, geographical origin, harvest season, and production batch. Consequently, cultivar-, origin-, season-, and batch-dependent variability should be systematically evaluated before these findings can be generalized to other citrus-derived materials.

5. Conclusions

After gastrointestinal digestion, the free phenolic fraction of green citrus syrup showed increased antioxidant activity, greater bioaccessibility, and enhanced XO inhibitory activity. Correlation analysis revealed that 20 individual phenolic compounds were positively associated with XO inhibition. Among these, hesperidin, naringin, hesperetin, hesperetin 7-O-glucoside, morin, and 6-gingerol were identified as key candidate compounds responsible for XO inhibition, mainly through hydrogen-bonding interactions with the enzyme. These results improve the current understanding of the nutritional functionality of green citrus syrup and provide scientific support for its further development and processing.

Availability of Data and Materials

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

Author Contributions

YL, XO, AC, FP designed the research study. YL performed the research. XO, AC, FP provided help and advice throughout the study. YL, XO, AC, and FP analyzed the data. YL and AC wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JFSFQ53251.

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