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Bioavailability of Citrulline in Watermelon Flesh, Rind, and Skin Using a Human Intestinal Epithelial Caco-2 Cell Model

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Abstract: Watermelon produces many byproducts (watermelon rind and skin) even though those components contain various bioactive compounds, including citrulline. This study evaluated the citrulline concentration, total phenolic content, and antioxidant activity (DPPH and FRAP assays) of different parts of watermelon and investigated the bioavailability of citrulline from different parts of watermelon using an in vitro human intestinal epithelial Caco-2 cell monolayer model. Solid-phase extracted watermelon flesh, rind, and skin samples were treated on a Caco-2 cell monolayer for 1, 2, and 4 h. The collected basolateral solution at each time point was analyzed for the percentage of citrulline transport. Watermelon flesh had the highest citrulline content, but the watermelon skin had the highest total phenolic content and antioxidant activity compared to other watermelon parts. The citrulline bioavailability showed greater % transport in watermelon skin than in watermelon flesh, rind, and L-citrulline standard. It may be due to the different food matrices of watermelon parts. This suggests that the utilization of watermelon by-products such as skin would help develop value-added products with better bioavailability of citrulline. However, since this study was conducted with an in vitro cell model, more extensive research with in vivo studies will be needed.

Keywords: watermelon; citrulline; bioavailability; watermelon flesh; watermelon rind; watermelon skin; Caco-2 cells

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1. Introduction

Watermelon (*Citrullus lanatus*) belongs to the Cucurbitaceae family and is one of the most cultivated fruits, with approximately 102 million tons of world production in 2021 [1,2]. Watermelon is composed of flesh, rind, and skin. The flesh is the red pulp; the rind is the white or light green part between flesh and skin; and the skin is the green peel surface. The flesh part accounts for 40–50% of a watermelon, and the other 50–60% of the total mass of a watermelon is the rind and skin part [3,4]. The flesh is generally considered an edible part, and the rind and skin are discarded as by-products or used as animal feed [5]. The rind and skin produce an enormous amount of food waste from watermelon production to watermelon consumption, causing many environmental challenges [6].

Watermelon contains many nutrients and bioactive compounds, including vitamins, lycopene, citrulline, and phenolic compounds [7]. Due to these bioactive compounds, watermelon has shown many health benefits such as antioxidant, anti-diabetic, and anti-cancer effects [8–11]. Although watermelon rind and skin are normally discarded as by-products, watermelon rind and skin contain similar or higher total phenolic and citrulline content compared to watermelon flesh, showing strong antioxidant activities [12,13]. Thus, there has been increased attention towards the watermelon by-products and their

utilization [14–16]. Converting watermelon by-products with high bioactive value to value-added products would benefit functional food industries and the human diet and have a better environmental impact with less food waste [3].

Citrulline is a non-essential non-proteinogenic amino acid, and watermelon is one of the rich sources of citrulline [17]. Watermelon rind has especially shown to have greater citrulline content than watermelon flesh [12,18]. Citrulline is a product of the nitric oxide (NO) cycle and a precursor of arginine [19]. Since arginine is a conditionally essential amino acid related to the NO system, citrulline has a potential role in vasodilation and cardiovascular functions [20–22]. Citrulline also showed hydroxyl radical scavenging activities [23]. Thus, many studies have been on the therapeutic activities of citrulline in watermelon [24–26]. However, the bioavailability of citrulline needs to be considered since it depends on its intestinal absorption and different food matrix [24,27–29]. There have been some studies on the bioavailability of citrulline in watermelon [27,30,31], but little is known about the bioavailability of citrulline from different watermelon parts. Therefore, this study evaluated the chemical composition of different watermelon parts (flesh, rind, and skin) and investigated the intestinal uptake of citrulline from watermelon flesh, rind, and skin using the differentiated monolayer human intestinal Caco-2 cell model.

2. Materials and Methods

2.1. Materials

Pre-extracted, dried samples of watermelon flesh, rind, and skin were prepared as described previously [5]. All media components and reagents were obtained from Gibco® through Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Citrulline Concentration of Watermelon Flesh, Rind, and Skin

2.2.1. Solid Phase Extraction (SPE)

Solid phase extraction (SPE) was performed on pre-extracted watermelon flesh, skin, and rind samples before the analysis of citrulline. The pre-extracted watermelon samples (1 g) were reconstituted in 10 mL of deionized water. Sep-Pak® Vac 20 cc (5 g) cartridges (Waters Corp., Milford, MA, USA) were activated by washing with 20 mL of 100% methanol, followed by a 20 mL deionized water wash. The reconstituted aqueous extracts were bound to the columns, followed by a 20 mL deionized water wash. The remaining was eluted with 10 mL of 100% methanol. The methanol fraction was dried under nitrogen and reconstituted with 10 mL of deionized water.

2.2.2. Citrulline

Citrulline in watermelon samples was determined using the citrulline assay (Abcam, Cambridge, UK). Briefly, 50 µL of the sample was mixed with 5 µL of SDS solution and proteinase K solution in a tube. After 2 h incubation at 37 °C, assay reagents were added and incubated for 30 min at 95 °C. After cooling down at 4 °C for 5 min, the tube was centrifuged at 13,000× *g* for 10 min. The supernatant (200 µL) was transferred to a 96-well plate, and the absorbance was read on a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA) at 540 nm.

2.3. Total Phenolic Content and Antioxidant Activity

The total phenolic content in watermelon flesh, skin, and rind samples after SPE was determined using the modified Folin-Ciocalteu assay in Slinkard and Singleton [32], using gallic acid as standard. Briefly, samples were diluted 50 times in distilled water. After putting gallic acid standards and diluted samples (20 µL) in a 96-well plate, 100 µL of 0.2 N Folin–Ciocalteu reagent and 80 µL of 0.7 M sodium carbonate. The plate was incubated for 2 h at room temperature. The absorbance was read with a microplate reader (Synergy HT multimode microplate reader, BioTek, Winooski, VT, USA) at 760 nm. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

The free radical-scavenging activity of watermelon flesh, skin, and rind samples after SPE was evaluated using an adapted method of Akkari et al. [33] with 2,2-diphenyl-1-picrylhydrazyl (DPPH). Trolox was used as a reference standard. Samples were diluted 50 times in methanol. Trolox standards and diluted samples (10 µL) were added with 140 µL of 0.1 mM DPPH in methanol and incubated for 30 min in the dark at room temperature. The results were measured at 517 nm. The free radical-scavenging activity was calculated with the following equation: scavenging effect = [(A0 − A1)/A0] × 100, where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample. Data were expressed as micromole of Trolox equivalent (TE) per gram of sample, using the scavenging effect of the Trolox standard curve.

Ferric reduction antioxidant potential (FRAP) of watermelon flesh, skin, and rind samples after SPE was determined using QuantiChrom™ FRAP Assay Kit (BioAssay Systems, Hayward, CA, USA). Samples diluted 100 times were mixed with working reagent and incubated at room temperature for 40 min. The absorbance was measured at 590 nm. The results were expressed as micromole of ferrous (Fe^{2+}) equivalent per gram of sample.

2.4. Cell Culture and Measurement of Transepithelial Electrical Resistance (TEER)

Caco-2 (ATCC® HTB-37™) human intestinal epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% non-essential amino acids (NEAA), 10% fetal bovine serum (FBS), and 100 U/mL penicillin and 100 µg/mL streptomycin, named working media (WMEM) in this experiment and incubated at 37 °C in a humidified, 5% CO_2 incubator (VWR® Water Jacketed CO_2 incubator, VWR International, Radnor, PA, USA). Cells between passages 24 and 30 were used in the experiments. Caco-2 cells were seeded onto transwell inserts (polycarbonate membrane, 12 mm i.d., 0.4 µm pore size, Corning Inc., Kennebunk, ME, USA) in 12-well plates at a density of 1.0×10^5 cells/well. The media was changed every 2 days until the cells were used for the experiment 21 days after seeding. The integrity of the cell monolayer was measured by TEER using a Millicell-ERS Volt-Ohm Meter (MD Millipore Corporation, Billerica, MA, USA) at 21 days post-seeding. When TEER value reached 400 or greater ohms, the cells were treated with watermelon flesh, rind, and skin samples as well as L-citrulline standard to measure the rate of transport across the cell monolayer to determine the bioavailability of citrulline in watermelon from the three parts.

2.5. Cell Viability Assay

The concentration of watermelon flesh, rind, and skin samples for cell treatment was determined by cell viability assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega Co., Madison, WI, USA) according to the manufacturer's instruction. Caco-2 cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates and incubated in a 37 °C, 5% CO_2 incubator for 24 h. The cells were treated with 3 different citrulline concentrations (100, 200, and 300 µM citrulline) of watermelon flesh, rind, and skin and L-citrulline standard for 4 h. After 4 h, cell supernatant was replaced with fresh cell culture medium, and 20 µL of MTS reagent was added into each well. After 1 h incubation, the absorbance was read at 490 nm with a microplate reader. Cell viability was calculated as the percentage of living cells over control cells.

2.6. In Vitro Bioavailability Assay

Prior to each assay, WMEM in both apical and basolateral chambers of the transwell were aspirated. To measure the uptake of citrulline from the apical side of the Caco-2 cells, the cells were first deprived of amino acids (AA) in incubation for 15 min at 37 °C in phosphate-buffered saline (PBS). PBS was aspirated, and the cells were rinsed three times with ice-cold PBS. The treatment (500 µL) was added to the apical chamber, and 1.5 mL of WMEM was added to the basolateral chamber. The solution from the basolateral chamber was collected at 1, 2, and 4 h. The collected samples were mixed with 50 µL stop solution

(25 µL of 100% trifluoroacetic acid and 25 µL of 70% ethanol) and frozen immediately. The concentration of citrulline in the watermelon flesh, rind, and skin treatments and collected basolateral solution at 1, 2, and 4 h was measured using a citrulline assay kit (Abcam, Cambridge, UK), as described in Section 2.2.2.

2.7. Statistical Analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with a one-way analysis of variance test (ANOVA) and Tukey's test. Data were expressed as means ± standard deviation (SD) for citrulline, TPC, and DPPH assay and as means ± standard error of the mean (SEM) for cell culture experiments. A difference of $p < 0.05$ was considered significant.

3. Results

3.1. Citrulline Concentration of Watermelon Flesh, Rind, and Skin

Citrulline concentration of watermelon flesh, rind, and skin was measured using citrulline assay (Figure 1). The watermelon flesh contained the highest citrulline concentration (1505 ± 66.9 µM), followed by skin (1113.5 ± 92.8 µM) and rind (780.5 ± 232 µM). Citrulline concentration in watermelon flesh was significantly higher than in the skin ($p < 0.05$) and in the rind ($p < 0.01$).

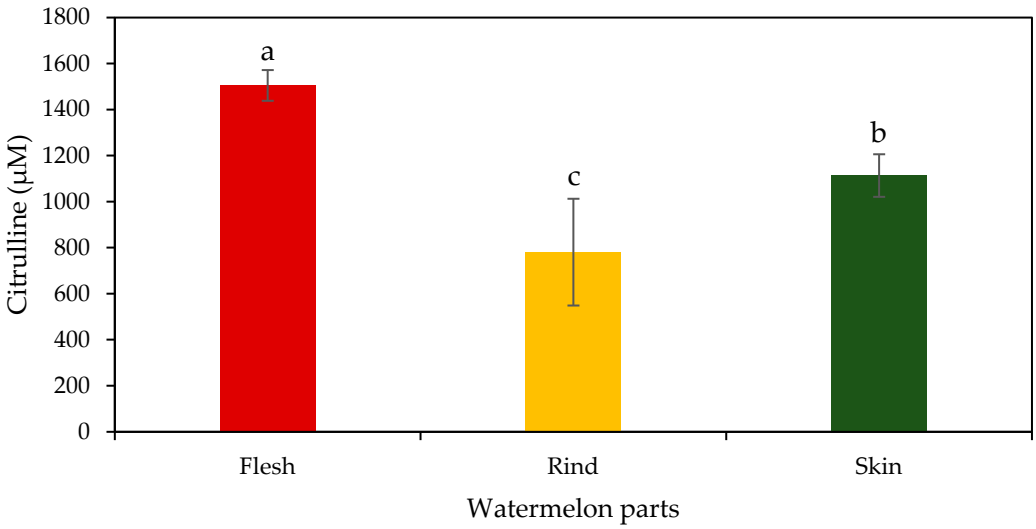

Figure 1. Citrulline concentration of watermelon flesh, rind, and skin. Data are expressed as the mean ± SD (n = 5). Means followed by a different letter are significantly different ($p < 0.05$).

3.2. Total Phenolic Content and Antioxidant Activity

The total phenolic content of watermelon flesh, rind, and skin was 12.7 ± 0.5, 2.4 ± 0.4, and 15.2 ± 0.6 mg GAE/g, respectively (Table 1). Watermelon skin contained the highest total phenolic content, followed by the flesh and rind. The total phenolic content of the rind was significantly lower than other parts ($p < 0.05$). Antioxidant activity of watermelon flesh, rind, and skin tested with DPPH and FRAP assays showed a similar pattern as the total phenolic content. As the result of DPPH assay, watermelon skin (31.1 ± 6.2 µmol TE/g) showed the highest antioxidant activity, followed by watermelon flesh (28.7 ± 3.5 µmol TE/g) and rind (7.0 ± 2.7 µmol TE/g). The free radical-scavenging activities of watermelon skin and flesh were significantly higher than the rind ($p < 0.05$). In FRAP assay, ferric reduction antioxidant potential of watermelon flesh, rind, and skin was 181.9 ± 7.2, 40.1 ± 9.0, and 240.0 ± 8.4 µmol Fe^{2+}/g, respectively. Watermelon skin showed significantly higher FRAP compared to the flesh ($p < 0.05$) and skin ($p < 0.01$).

Table 1. Total phenolic content and antioxidant activity of watermelon parts.

Sample	Total Phenolic Content (mg GAE/g)	DPPH (μmol TE/g)	FRAP (μmol Fe^{2+}/g)
Flesh	12.7 ± 0.5 ^{b}	28.7 ± 3.5 ^{a}	181.9 ± 7.2 ^{b}
Rind	2.4 ± 0.4 ^{c}	7.0 ± 2.7 ^{b}	40.1 ± 9.0 ^{c}
Skin	15.2 ± 0.6 ^{a}	31.1 ± 6.2 ^{a}	240.0 ± 8.4 ^{a}

Values represent mean ± SD (n = 20 for total phenolic content and DPPH; n = 15 for FRAP). Means within columns with different letters are significantly different ($p < 0.05$). DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = ferric reduction antioxidant potential; GAE = gallic acid equivalent; TE = trolox equivalent.

3.3. Cell Viability of Watermelon Flesh, Rind, and Skin

The cell cytotoxicity of watermelon flesh, rind, and skin on Caco-2 cells was determined by using MTS assay (Figure 2). Watermelon parts (flesh, rind, and skin) containing 100–300 μM citrulline and L-citrulline were treated onto Caco-2 cells for 4 h. As a result, all treatments containing 100 μM citrulline did not affect the cell cytotoxicity of Caco-2 cells. However, the rind with 200 and 300 μM citrulline and skin with 300 μM citrulline showed significant cytotoxicity compared to the control ($p < 0.05$). Therefore, watermelon parts with 100 μM citrulline were used as the treatment concentration for in vitro bioavailability assay.

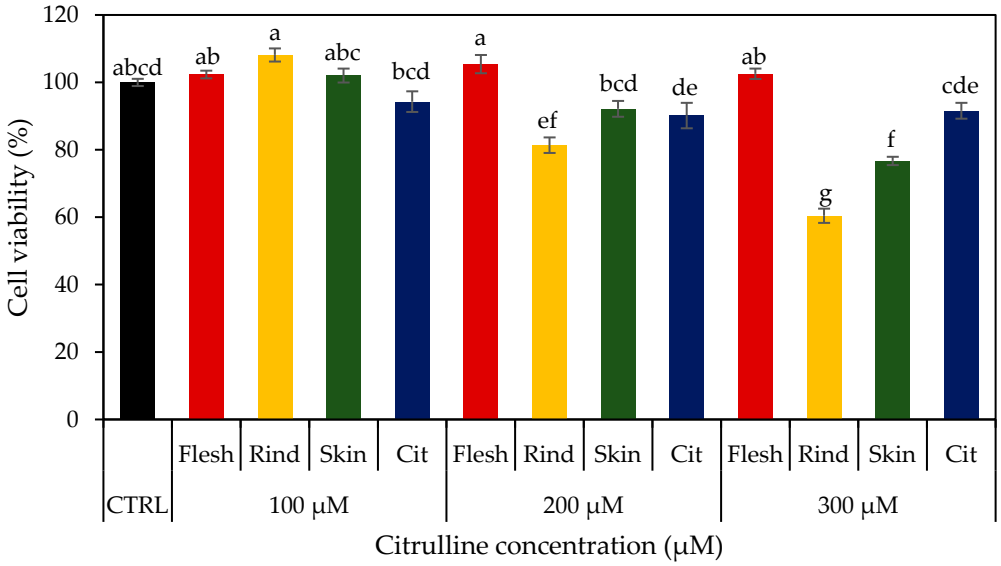

Figure 2. Cell viability of Caco-2 cells treated with different parts of watermelon samples and L-citrulline (100–300 μM citrulline). Data are expressed as the mean ± SEM (n = 12). Means followed by a different letter are significantly different at the 5% level. CTRL = control; Cit = L-citrulline.

3.4. In Vitro Bioavailability of Citrulline in Watermelon Flesh, Rind, and Skin

The concentration of citrulline in watermelon flesh, rind, and skin treatments and in basolateral solution collected at 1, 2, and 4 h was measured using citrulline assay (Figure 3). There were no significant differences in the % transport of each treatment among the different treatment times. This showed that the absorption of citrulline in different watermelon parts and L-citrulline were mostly processed in 1 h. The watermelon skin showed higher % transport at all time points than L-citrulline. Especially at 1 h treatment, % transport was significantly higher in the watermelon skin treatment compared to L-citrulline treatment. The highest % transport in each treatment occurred at 1 h for watermelon skin, at 2 h for watermelon flesh and L-citrulline, and at 4 h for watermelon rind. Overall, it showed transport of citrulline in all watermelon parts and the standard ranged from 30.3 to 36.6%.

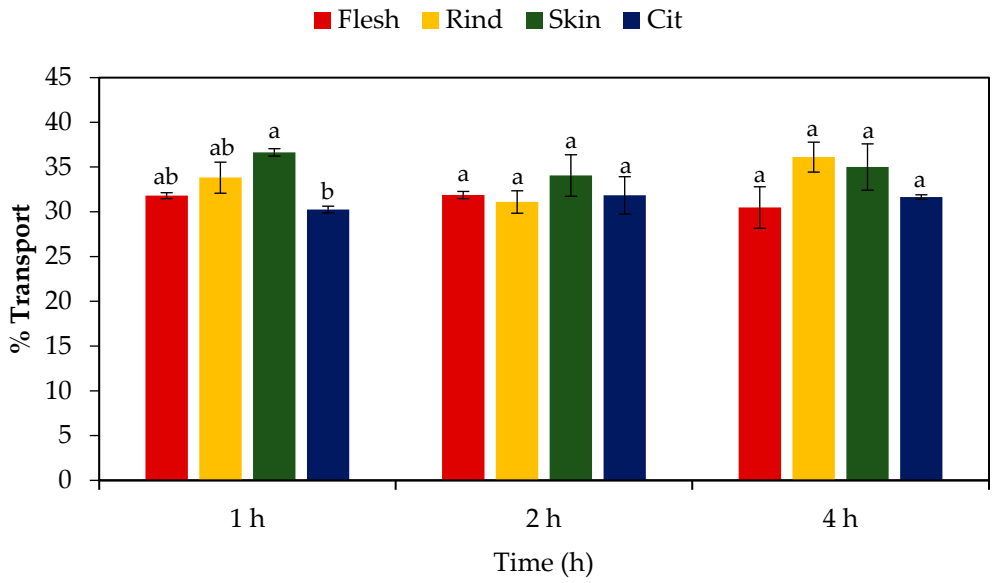

Figure 3. Transport of citrulline in watermelon samples using Caco-2 cell assays after 1, 2, and 4 h incubation. Data are presented as mean ± SEM (n = 2). % Transport was calculated as the percentage transported across the cell monolayer from the apical to the basolateral chamber. Different letters in the same treatment time indicate significant differences between results at the 5% level.

4. Discussion

Watermelon (*Citrullus lanatus*) has many nutritional and beneficial properties. A total of 71 phenolic and other polar compounds have been characterized in watermelon [34]. One of those compounds, citrulline, is a non-essential amino acid. Citrulline was first identified in the juice of watermelon [35,36]. It has been reported that watermelon juice contains ~2.33 g of citrulline per L of unpasteurized watermelon juice [37]. Citrulline has potent antioxidant activity and plays an important role in the nitric oxide (NO) system because it is a precursor of arginine. Citrulline may offer therapeutic strategies for controlling NO metabolism disorders and improving cardiovascular ability [4,24]. Citrulline has also been shown to reduce the recovery heart rate and muscle soreness after 24 h [31].

As the attention to food waste and byproducts for being potential biosources increased, many studies have identified and quantified citrulline content in watermelon rind and skin as well as watermelon flesh. Jayaprakasha et al. [18] measured the concentration of L-citrulline in three different varieties of watermelon juice and rinds. In all varieties, rinds contained higher L-citrulline (13.95–28.46 mg/g dry weight (dw)) compared to watermelon juice (11.25–16.73 mg/g dw). Ridwan et al. [38] investigated L-citrulline content in watermelons (flesh and rind) grown and consumed in Malaysia and showed that L-citrulline content was higher in the rind (45.02 mg/g) than in the flesh (43.81 mg/g) of red watermelon juice extract and showed similar trends in yellow crimson watermelon juice extract, with 16.61 mg/g in rind and 15.77 mg/g in flesh detected. Casacchia et al. [39] determined bioactive compounds extracted from watermelon pulp and rind with nine different watermelon cultivars from different origins. The concentration of L-citrulline in fresh rind was significantly higher than in fresh pulp except in watermelons from Latina, Italia, and Santana, Romania, showing up to 2.6 mg/g L-citrulline in fresh watermelon rind. Pp et al. [40] quantified citrulline from the 'Sugar Baby' variety of watermelon rinds, and the concentration of citrulline was 13.36 mg/g dw in the rind and 9.78 mg/g dw in the skin. Tarazona-Díaz et al. [12] determined the citrulline content in the rind and flesh of five different watermelon cultivars. Citrulline level in the rind (2.0–7.2 g/kg fresh weight (fw)) was higher than that in flesh (1.1–4.7 g/kg fw) in all five watermelon cultivars. Akashi et al. [41] investigated the accumulation pattern of citrulline in mature watermelons. Citrulline showed a bipolar accumulation pattern in mature watermelon, as the concentration of citrulline was highest in the outer peels (4.4 ± 0.8 g/kg fw), decreased in inner rinds (2.1 ± 0.94 g/kg fw) and

peripheral flesh fractions (0.83 ± 0.36 g/kg fw), but increased toward the center of flesh. Rimando and Perkins-Veazie [4] measured the citrulline level in the flesh and rind of watermelons with different flesh colors (red, yellow, and orange). A higher level of citrulline was detected in the rind (15.6–29.4 mg/g dw) than in the flesh (7.9–28.5 mg/g dw), but it was the opposite on a fresh weight basis, showing higher citrulline content in fresh flesh (1.0–3.5 mg/g fw) than in fresh rind (0.8–1.5 mg/g fw). This can be explained by the higher moisture level of rind (95%) than flesh (90%). Fan et al. [30] compared the bioactive components of watermelon flesh, rind, and seeds and showed that the highest citrulline content was detected in watermelon rind (19.3 ± 1.1 mg/100 kcal) followed by watermelon flesh (10.0 ± 1.0 mg/100 kcal) and watermelon seeds (1.4 ± 0.2 mg/100 kcal). These previous studies reported that watermelon rind has higher L-citrulline than watermelon flesh. The present study appears to have the opposite results showing the highest citrulline content in watermelon flesh, followed by the skin and rind (Figure 1). However, the opposite results can be explained by the word 'rind' used in some previous studies [12,18,30,38,39], because 'rind' in these previous studies indicated the white rind and green skin, which are all byproduct parts other than flesh.

In addition to the citrulline concentration, total phenolic content and antioxidant activity of watermelon parts were investigated in the present study. Watermelon skin showed the highest total phenolic content, free-radical scavenging activity, and ferric reduction antioxidant potential followed by watermelon flesh and skin. These results were consistent with previously reported studies [42,43]. Green rind (skin) showed the highest total phenolic content (0.7 mg GAE/g), followed by the flesh (0.3 mg GAE/g) and the white rind (0.2 mg GAE/g), with a similar pattern in DPPH radical scavenging activity [42]. The green rind (skin) extract treated at 250 °C showed the maximum ferric reducing power among the watermelon flesh, white rind, and green rind (skin) parts [42]. Yusoff et al. [43] extracted watermelon rind using ultrasound-assisted extraction, and it contained 15.1 ± 0.6 mg GAE/g, showing similar total phenolic content as the present study. Total phenolic content of red- and yellow-fleshed watermelon rind powders extracted using different solvents (water, methanol, ethanol, and acetone) reported values 1.1–2.2 mg GAE/g, which was lower than our result (rind 2.4 mg GAE/g) [44]. However, there were several studies not consistent with the present study [45,46]. The white part of watermelon peels (rind) contained 63.3 ± 1.5 mg TAE (tannic acid equivalent)/g, showing higher total phenolic content than the red flesh (47.3 ± 0.9 mg TAE/g) [45]. In Neglo et al. [46], watermelon skin showed the highest total phenolic content (0.087 ± 0.002 mg GAE/g) and DPPH (55.8 ± 2.4 %), but watermelon rind was higher than the flesh in both total phenolic content and DPPH.

It is important to investigate intestinal absorption of citrulline because the bioavailability of citrulline depends on intestinal absorption and different food matrices [24,27]. There have been several studies on citrulline uptake [27,30]. Bahri et al. [27] investigated the mechanisms and kinetics of citrulline uptake using a Caco-2 human intestinal epithelial cell model. They found that citrulline uptake was pH-independent and that the uptake rate went down without Na^+. Fan et al. [30] investigated the concentration of L-citrulline in plasma over 24 h after watermelon flesh, rind, and seed intake. The maximum concentration showed between 1 and 2 h after watermelon flesh, rind, and seed intake, especially watermelon flesh and rind showing significant increase compared to the control ($p < 0.05$). However, the transport of citrulline from different watermelon parts in the intestine is poorly understood. In the present study, the bioavailability of citrulline was slightly greater in the watermelon skin than in the watermelon flesh and rind (Figure 3). In addition, the bioavailability of L-citrulline standard was similar or lower than the bioavailability of citrulline in different watermelon parts. This difference may be because the citrulline standard was not contained within the matrix of the watermelon. Tarazona-Díaz et al. [31] performed an in vitro intestinal absorption of L-citrulline by using Caco-2 cells, with unpasteurized watermelon juice, pasteurized watermelon juice, and L-citrulline standard. L-citrulline in unpasteurized watermelon juice showed higher absorption than in the L-citrulline standard,

suggesting that the transport and bioavailability of L-citrulline are greater when it was contained in a matrix of watermelon juice than as a pure compound.

5. Conclusions

In this study, we evaluated the composition of different parts of watermelon and investigated the bioavailability of citrulline from different parts of watermelon using an in vitro human intestinal Caco-2 cell monolayer model. Watermelon flesh had the highest citrulline content, but the watermelon skin had the highest total phenolic content with higher radical scavenging activity and ferric reduction antioxidant potential than watermelon flesh and skin. The citrulline bioavailability showed higher % transport in watermelon skin than in watermelon flesh, rind, and even L-citrulline standard. It may be due to the different food matrices of watermelon parts. This suggests that the utilization of watermelon byproducts such as skin would help develop value-added products with higher bioactive compounds and better bioavailability of citrulline. However, since this study was conducted with an in vitro cell model, more extensive research with in vivo studies will be needed.

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