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GAS-EXPANDED LIQUID EXTRACTION OF ASCORBIC ACID AND POLYPHENOLS FROM UVC-TREATED MALPIGHIA EMARGINATA DC: ENHANCED BIOAVAILABILITY AND SKIN PIGMENTATION-REDUCING EFFECTS

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ABSTRACT

We investigated UVC treatment of acerola cherries (*Malpighia emarginata* DC) on the farm immediately after harvest to obtain extracts with the highest ascorbic acid content and intact polyphenols (hesperidin, rutin, tannins, and quercetin). Ascorbic acid and phenolic compounds were extracted from *Malpighia emarginata* DC using gas-expanded liquids (GXLs). 338.2 mg/g of ascorbic acid was found in the extract and 195.2 mg/g of ascorbic acid was found in the acerola juice powder. The antioxidant activity of the extracts increased as the concentration of ascorbic acid increased. Abundant polyphenolic compounds were detected in the phytochemical profile by LC-Q-TOF-MS/MS in the extract obtained using our extraction method. The bioavailability of the extract has been confirmed to be higher than that of synthetic ascorbic acid in humans. The extract was also found to be more effective in reducing skin pigmentation than other treatments.

1. INTRODUCTION

Vitamin C, also known as ascorbic acid, is an essential nutrient that plays important roles in the human body, such as collagen synthesis, wound healing, antioxidant activity and skin pigmentation. Acerola cherries (*Malpighia emarginata* DC) are known to be one of the richest sources of natural vitamin C, as well as other beneficial compounds, such as polyphenols.[1] However, the bioavailability of vitamin C in acerola cherries can be affected by various factors, such as processing methods and storage conditions.[2,3] Therefore, there is

a growing interest in developing effective methods to extract and preserve vitamin C and other beneficial compounds from acerola cherries.[4] In this study, we investigated the use of UVC treatment and gas-expanded liquids (GXLs) to extract ascorbic acid and polyphenols from acerola cherries immediately after harvest, aiming to obtain extracts with high ascorbic acid content and intact polyphenols. UVC treatment was utilized to extend the shelf-life of the fruit and preserve its quality due to acerola's high perishability and rapid loss of nutraceutical quality during postharvest storage. Meanwhile, GXLs were used to extract ascorbic acid and polyphenols under low-temperature conditions, considering that ascorbic acid is a highly unstable and thermolabile compound.[5,6] The obtained extract was evaluated for its antioxidant activity, phytochemical profile, and bioavailability, as well as its potential effect on reducing skin pigmentation.

2. MATERIAL AND METHODS

Raw material preparation and UVC Treatment

Acerola fruits (Malpighia emarginata DC) were obtained in 2021 from a local market in Brazil, where all fruits are sold one day after harvest at physiological maturity. Prior to UVC treatment, the fruits were sorted by color and size, washed with distilled water, dried, and divided into two groups - controls and treated fruits, respectively. The selected fruits had a moisture content of approximately 90%, approximately 10% soluble solids, and an average color value of Hue = 25°, L = 70, and Chroma = 25. The UVC treatment was conducted using a XeMaticA–2LXL System (SteriBeam Systems GmbH, Germany). The fruit was treated twice with UVC pulses of 0.3 J cm^{-2} (total dose of 0.6 J cm^{-2}). After treatment, UVC-treated and non-treated control fruits were immediately processed and sampled on day 0. The remaining half of the samples were stored in a refrigerator at 10 °C for seven days before processing. A synthetic l-ascorbic acid (Fisher Scientific Company, Pittsburgh, PA, USA) was prepared.

Quantification of ascorbic Acid and Phenolic content in raw material

The quantification of total ascorbic acid followed the method previously described by Gillespie and Ainsworth.[7] To summarize, a 50 mg sample powder was homogenized with 2 ml of 5% (w/v) trichloroacetic acid (TCA), filtered through four layers of cheese cloth, and then centrifuged at 16,000×g for 10 minutes at 4°C. The resulting supernatant was used for measuring the total ascorbic acid content. To determine the total phenolic content of the samples, the freeze-dried sample powder (1.5 g) was homogenized with 50 mL of 85%

methanol at 4 °C overnight to obtain a crude extract. The extract was fractionated into four groups of phenolics, namely phenolic acids, anthocyanins, flavonols, and procyanidins/polymeric anthocyanins, using solid-phase extraction with C18 cartridges. The individual phenolic compounds were identified using LC–MS/MS based on their retention time, UV spectra, and mass-to-charge ratio. The phenolic content was determined by injecting 10 µL of sample and quantifying the phenolic compounds using standard curves prepared with gallic acid, chlorogenic acid, cyanidin, kaempferol, quercetin, and myricetin. The phenolic content was expressed as mg of the corresponding phenolic equivalent per 100 g of dry weight, and the total phenolic content was determined as the sum of the concentration of individual phenolics.[8] The yields of the four phenolic fractions were F1—9.9 mg, F2—18.3 mg, F3—105.5 mg and F4—199.9 mg. The chromatograms were monitored at 520, 330, 280, and 210 nm, and complete spectral data were recorded in the range 200–600 nm.

Gas-Expanded Liquid (GXL) Extraction Procedure

The GXL process for extracting bioactive compounds from acerola was carried out in the following manner.[9,10] High-pressure extractions were performed using a PrepMaster supercritical fluid extractor under continuous flow mode. The PrepMaster unit (Suprex-Pittsburgh, PA, USA) maintained a CO_2 pressure of 7 MPa and a flow rate of 1 mL/min. Ethanol was pumped by a PU2080 HPLC pump (Jasco Corp., Tokyo, Japan) to achieve 50% CO_2 flow with a rate of 0.5 mL/min. The extraction cell and feeding tube (where the solvents are mixed) were placed in a hot air circulation oven set to 40 °C. The overall yield curve was constructed to determine the extraction time. Ethanol in the extracts was removed under nitrogen flow, and the extracted mass was used to calculate the extraction yield (g/100g). The extract was subsequently freeze-dried.

Quantification of ascorbic Acid and Phenolic content in the extract

The quantification of l-ascorbic acid was performed using UHPLC-q-TOF-MS with the following operating conditions.[12,13] To create a calibration curve, samples were prepared in the range of 5 to 100 µg/mL. Total phenolic content (TPC) was measured by the Folin-Ciocalteu method. A sample (10 mg/mL) of 10 µL was mixed with 600 µL of ultrapure water, and Folin-Ciocalteu reagent (50 µL) was added to the mixture. After 1 minute, 150 µL of 20% (w/v) sodium carbonate (Na_2CO_3) was added to the mixture, which was then adjusted to a final volume of 1 mL with water. The mixture was incubated in the dark at room

temperature for 2 hours with stirring. Then, 300 µL of the reaction mixture was transferred to a 96-well microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) and the absorbance was measured at 760 nm. The calibration curve (0-2 mg/mL) was prepared using standard gallic acid (Sigma-Aldrich, St. Louis, MO, USA) and the values were converted to mg GAE (gallic acid equivalent) per g of extract.

Antioxidant activities

Antioxidant activities were evaluated using two different assays[13,14] the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the Oxygen Radical Absorbance Capacity (ORAC) assay. The DPPH assay was conducted following the method described by Brand-Williams, Cuvelier, and Berset (1995), where 25 µL of the sample was mixed with 975 µL of a methanolic solution of DPPH (6 × 10−5 M) and incubated at room temperature for 1 hour in the dark. The absorbance was measured at 516 nm after transferring 300 µL to a 96-well microplate. The ORAC assay used fluorescein as a fluorescent probe and was conducted in a 96-well microplate containing 100 µL of extract at different concentrations (5–100 µg/mL) in ethanol/H_2O (1:9, v/v), 100 µL of peroxyl radical generator in 30 mM phosphate-buffered saline (PBS) at pH = 7.5, 25 µL of fluorescein (10 µM) in PBS buffer and 100 µL of PBS buffer. Fluorescence was measured every 5 minutes at 37°C for 60 minutes (λ excitation = 485 nm; λ emission = 530 nm). The results were expressed as EC_{50} in µg/mL, representing the concentration required to scavenge 50% of the radicals.

Study design I – Assessment of bioavailability

A total of twelve healthy female volunteers aged 22–27 years were enrolled in the bioavailability study. Their mean BMI was 20·3 kg/m2 and did not change significantly during the present study. These subjects took no dietary supplements or medications, did not smoke and were not habitual drinkers of alcoholic beverages. None of the subjects had a chronic illness or food allergy. All study participants were in good health on the basis of a medical history, a physical examination and normal results on clinical laboratory tests. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Clinical Research Ethics Committee, Tokyo, Japan. Written informed consent was obtained from all subjects. To assess the absorption and excretion of ascorbic acid alone, the subjects fasted overnight before taking a single dose of 500 mg along with distilled water as a reference in a crossover experimental design. The ascorbic acid solution of all doses contained 100 mL

distilled water. Each experiment was carried out with intervals of at least 14 days. Blood was collected from each subject at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 hours after the oral dose into tubes containing ethylenediaminetetraacetic acid (EDTA). The blood was immediately centrifuged at 1700 g for 15 minutes at 4 °C, and the resulting supernatant was used as plasma. Urine samples were collected and their volume recorded every 1 or 2 hours after the oral dose, with an overall collection time of 6 hours. The quantification of total ascorbic acid was carried out using high-performance liquid chromatography (HPLC)-electrochemical detection, which was previously described in reference.[15] To prepare the plasma samples, 4.5 volumes of 3% metaphosphoric acid (MPA) (Wako Pure Chemical, Osaka, Japan) were mixed with the samples and then centrifuged at 21000 g for 10 minutes at 4 °C. The resulting supernatants were stored at -80 °C until further use. Urine samples were also diluted with 10% MPA and similarly stored at -80 °C until use. The HPLC analysis was performed using Atlantis dC18 5 um columns (4.6 x 150 mm, Nihon Waters, Tokyo, Japan), and the mobile phase consisted of 50 mM phosphate buffer (pH 2.8), 0.2 g/l EDTA, and 2% methanol, with a flow rate of 1.3 ml/min. An electrochemical detector with a glassy carbon electrode at 0.6 V was used to record the electrical signals. To determine the creatinine levels in urine, a Creatinine Test Wako kit (Wako Pure Chemical, Osaka, Japan) was used following the manufacturer's instructions. Ascorbic acid levels in urine were then normalized by creatinine value.

Study design II – Improvement of hyperpigmentation

The improvement of hyperpigmentation study included 10 female participants aged 33 to 52 years with an average age of 43 years, who had skin hyperpigmentation on their face and phototype III or IV skin, according to the Fitzpatrick phototype scale (Fitzpatrick, 1975). However, pregnant or lactating women and women who may be pregnant, patients with a history of photoallergies or photosensitization, those who had been using steroid-containing skin external preparations for more than 1 month, those who had participated in a similar study within 6 months of the start of the current study, those with sensitive and irritable skin, those with skin abnormalities such as spots, acne, erythema, and expansion of capillaries, those who used the same or similar cosmetics or drugs on the same skin area within three months of the beginning of the study, and those who consumed medicine or food claimed to have skin-whitening effects were excluded from the study. To ensure a double-blind design, the test samples were prepared in liquid form, containing 20% ascorbic acid, while the control group samples contained 20% synthetic ascorbic acid. Both samples were provided in

the same container and were randomly assigned to the left and right sides of the face of each subject. The allocation was not disclosed to the subjects until the completion of the 8-week clinical study. The subjects applied each sample to the designated side of their face twice a day for the duration of the study. Prior to each evaluation, all subjects washed the tested skin area and rested for 30 minutes in a constant temperature and humidity chamber (20-24°C, 40-60% RH). Melanin index (M-index) was measured at the site of skin hyperpigmentation before and after using the sample, including immediately after sample application and at 5-day, 2-week, 4-week, and 8-week time points, to monitor treatment efficacy. The M-index was determined as the average value obtained by measuring the skin hyperpigmentation five times using a Mexameter® MX 18 (Courage+ Khazaka electronic, GmbH, Germany).

3. RESULTS AND DISCUSSION

The effect of UVC treatment

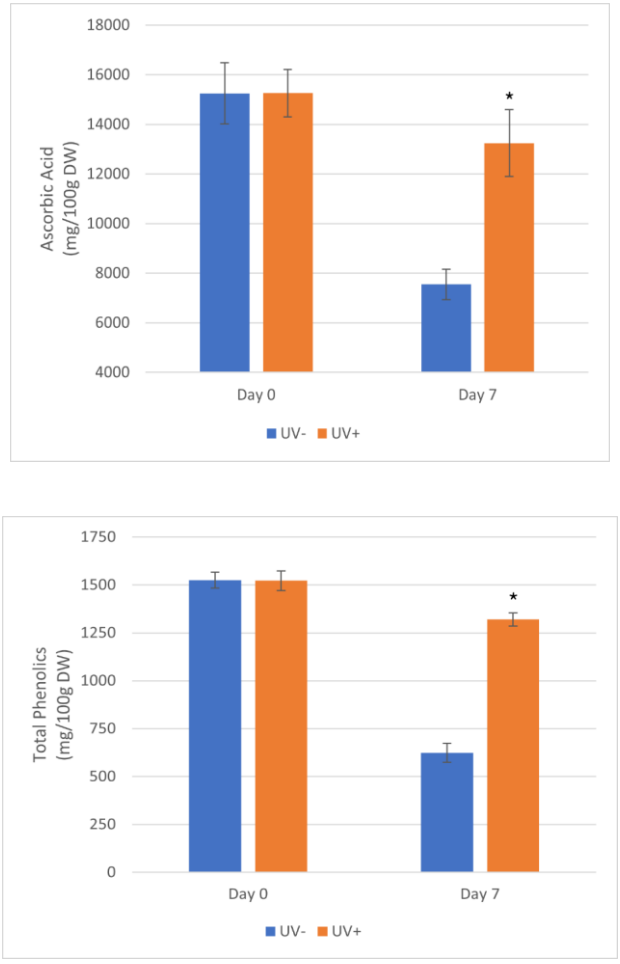


Figure 1: Effect of UVC on ascorbic acid and total phenolics metabolism. Two groups of Acerola fruits, one treated without UV (UV−) and the other with UVC treatment (UV+), were stored at 10°C for either 0 day (Day 0) or 7 days (Day 7). The total vitamin C

content was determined for each group (A). Additionally, the total phenolic compounds were analyzed by LC-MS and quantified for each group (B).

The UVC untreated group exhibited an initial mean ascorbic acid content of 15241mg/100g DW on Day 0, which significantly decreased to 7545mg/100g DW by Day 7. Meanwhile, the UVC-treated group exhibited an initial mean ascorbic acid content of 15256mg/100g DW on Day 0, which slightly decreased to 13243mg/100g DW by Day 7 (Fig. 1). Similarly, The UVC untreated group exhibited an initial mean total phenolic content of 1525mg/100g DW on Day 0, which significantly decreased to 624mg/100g DW by Day 7. Meanwhile, the UVC-treated group exhibited an initial mean total phenolic content of 1522mg/100g DW on Day 0, which slightly decreased to 1320mg/100g DW by Day 7 (Fig. 2). The study aimed to investigate the effect of UVC treatment on the metabolism of phenolic compounds and vitamin C in acerola during postharvest storage. Results showed that after 7 days of storage, UVC treated acerola samples had a significantly higher retention of phenolic compounds compared to the control group (Fig. 1). Previous studies have suggested that the measured amounts of phenolic compounds depend on the balance between their synthesis rate (k_s) and the rate of degradation or utilization (k_d).[16,17] The decrease in phenolic compounds observed in both UVC treated and untreated samples during postharvest storage (Fig. 1) may be attributed to a $k_d > k_s$. Additionally, the higher content of phenolic compounds in the UV-treated sample after 7 days of postharvest storage compared to the control (Fig. 1) may be associated with a k_s (UV+) > k_s (UV−). As a result, the UVC treatment may have altered the equilibrium between k_s and k_d, leading to the partial retention of total phenolic amounts and the alteration of their profiles.

Analysis of extract using the GXLs method

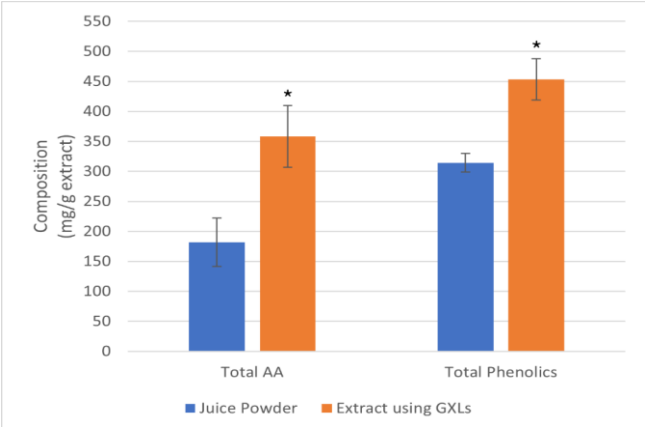


Figure 2: Composition of extract using GXLs Method and Acerola juice powder.

The extract obtained using GXLs method showed a measurement of 358.4 mg/g of ascorbic acid and 453.3 mg/g of phenolics, which are 197.2% and 144.1% higher than those found in each Acerola juice, respectively (Fig. 2) The extraction method using GXLs showed significantly higher levels of vitamin C and phenolic compounds compared to traditional methods. Considering that the antioxidant activity is proportional to the amount, these results suggest that acerola extract obtained using GXLs may be developed into a more potent drug or food with better pharmacological activity.

Antioxidant activity (EC50 µg/mL)

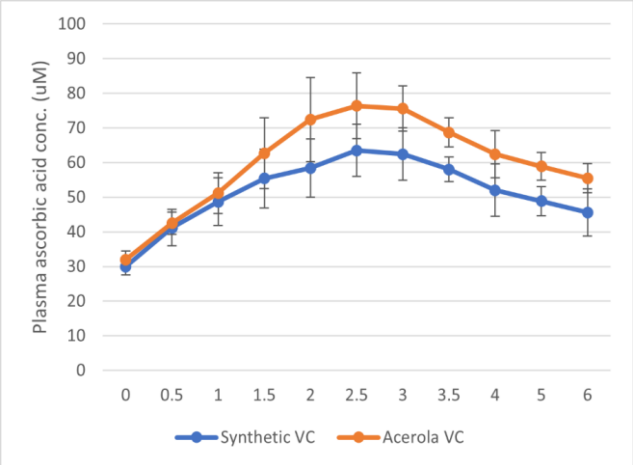
Table 1: Antioxidant activity of extract using GXLs method and acerola juice powder.

	Extract using GXLs	Acerola juice powder
DPPH	9.13 ± 0.87	4.14 ± 0.24
ORAC	3.29 ± 0.05	2.51 ± 0.27

The results indicate that TPC has an impact on antioxidant activity values, as evidenced by the greater antioxidant activity observed in the extract obtained using the GXLs method compared to the juice powder (Table 1). The antioxidant effect was found to be higher in proportion to the amount of the compounds present.

Assessment of bioavailability

3A



3B

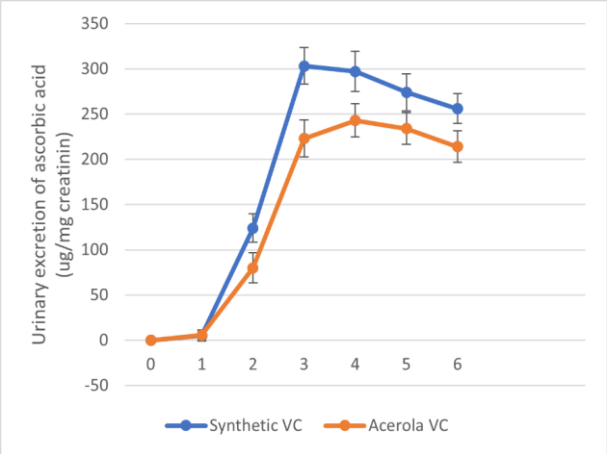


Figure 3A): Plasma concentration curve for fasting subjects ingesting 500 mg of ascorbic acid. 3B) Urinary excretion of ascorbic acid for fasting subjects ingesting 500 mg of ascorbic acid.

The plasma levels of orally ingested ascorbic acid were measured in all fasting participants, as illustrated in Figure 3A. The average concentration of ascorbic acid in the plasma of fasting individuals was found to be 32.91.1 mM. The quantity of ascorbic acid present in the three-day prescribed diets was measured, and the amounts were found to be 34.3, 14.3, and 11.3 mg, respectively. By controlling the participants' diets for three days and having them fast overnight, we ensured that accurate measurements of ascorbic acid levels in the plasma were obtained. The plasma concentration of ascorbic acid was significantly higher in the group that consumed acerola vitamin C, starting from 1.5 hours after ingestion compared to the group that received synthetic vitamin C. This trend became more pronounced at 2 hours and 2.5 hours after ingestion and was sustained until the final sampling time of 6 hours (Fig. 3A). Figure 3B shows the graph of the time it took for urinary ascorbic acid excretion after oral ingestion. The y-axis represents fractional excretion with creatinine collection. The peak times for ascorbic acid excretion were observed to be 3 to 4 hours after the oral ingestion of 500 mg. Starting from 2 hours after ingestion, the group that consumed acerola vitamin C showed lower levels of ascorbic acid excretion, which continued until the final urinary collection at 6 hours (Fig. 3B). GXLs extract showed higher absorption and lower excretion rates compared to synthetic vitamin C. This suggests that ascorbic acid contained in the GXLs extract was more utilized in the body, possibly indicating a greater distribution in tissues.

Effect on skin pigmentation

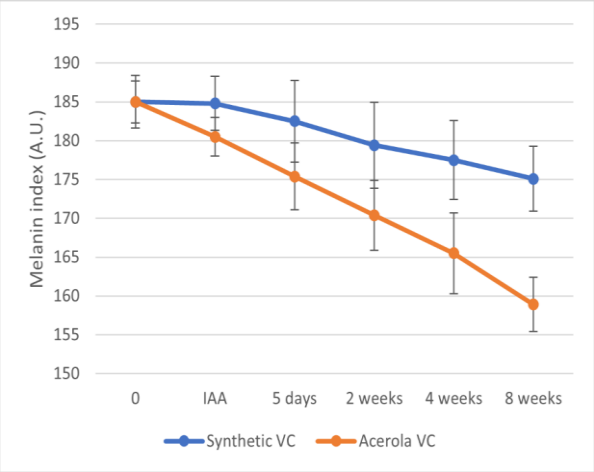


Figure 4: Changes in MI (Melanin index) following application for eight consecutive weeks. IAA: Immediately after application.

Statistically significant changes in the melanin index were observed immediately after application. The improvement rate of melanin index (MI) with synthetic vitamin C was 1.35% on the 5th day, 3.02% at 2 weeks, 4.05% at 4 weeks, and 5.35% at 8 weeks. In contrast, when using the GXLs method for the extract application, MI improvement rates were 5.18%, 7.89%, 10.54%, and 14.1%, respectively. Compared to synthetic vitamin C, the natural vitamin C applied using the GXLs method showed an immediate improvement in skin pigmentation as measured by the MI index, and had an overall efficacy that was 2.63 times higher.

4. CONCLUSIONS

In conclusion, the acerola extract obtained through GXLs extraction method after UVC treatment exhibited higher amounts of ascorbic acid and phenolic compounds compared to those extracted through conventional methods. As a result, it demonstrated improved antioxidant capacity, enhanced bioavailability, and superior efficacy in improving skin hyperpigmentation.

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