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"Development of functional cosmetic ingredient using plants from Okinawa"

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1. Introduction

The functionalities of components extracted from the fresh or dried peel of the *Citrus* genus (Rutaceae family) are well known. For example, citrus byproducts (peels, leaves and their extracts) enhance lipid breakdown, with peel extract demonstrating significant lipolytic activity in adipose tissue. Representative citrus taxa include *Citrus tangerina* Tanaka (tangerine), *Citrus aurantium* L. var *daidai* Makino (Daidai), *Evodia rutaecarpa* (A.Juss.) Hook. f. & Thomson (Evodia) and *Citrus junos* Siebold ex Tanaka (Yuzu) [1]. Peels of these citrus fruits contain polymethoxyflavonoids (PMFs), a class of bioactive compounds.

Kabuchii (*Citrus keraji* var. *kabuchii* hort. ex Tanaka), a citrus fruit unique to Okinawa Prefecture, is characterized by a thick peel, strong and fresh aroma, and sweet taste [2]. The name "Kabuchii" means "thick peel" in the Okinawan dialect. Large amounts of peels are obtained from juice residue, yet there are no effective utilization methods available to use them.

Kabuchii peel contains various PMFs, such as nobiletin, tangeretin, sinensetin, isosinensetin,

heptamethoxyflavone, and natsudaidain, which are known for their antioxidant, anti-inflammatory, and anti-ageing effects [3–6]. These compounds act on oxidative stress and inflammatory pathways such as NF-κB and MAPK, suggesting the potential of Kabuchii peel extract as a functional cosmetic ingredient targeting skin health and the visible signs of ageing.

Therefore, the aim of this study was to develop a Kabuchii peel extract as an upcycled functional cosmetic ingredient using juice residues (**Figure 1**). The effects of extraction conditions on the PMFs and functional properties of the peel extract were evaluated. This upcycling reduces waste, adds value to local resources, and supports Okinawa's unique genetic resources in the circular cosmetics economy.

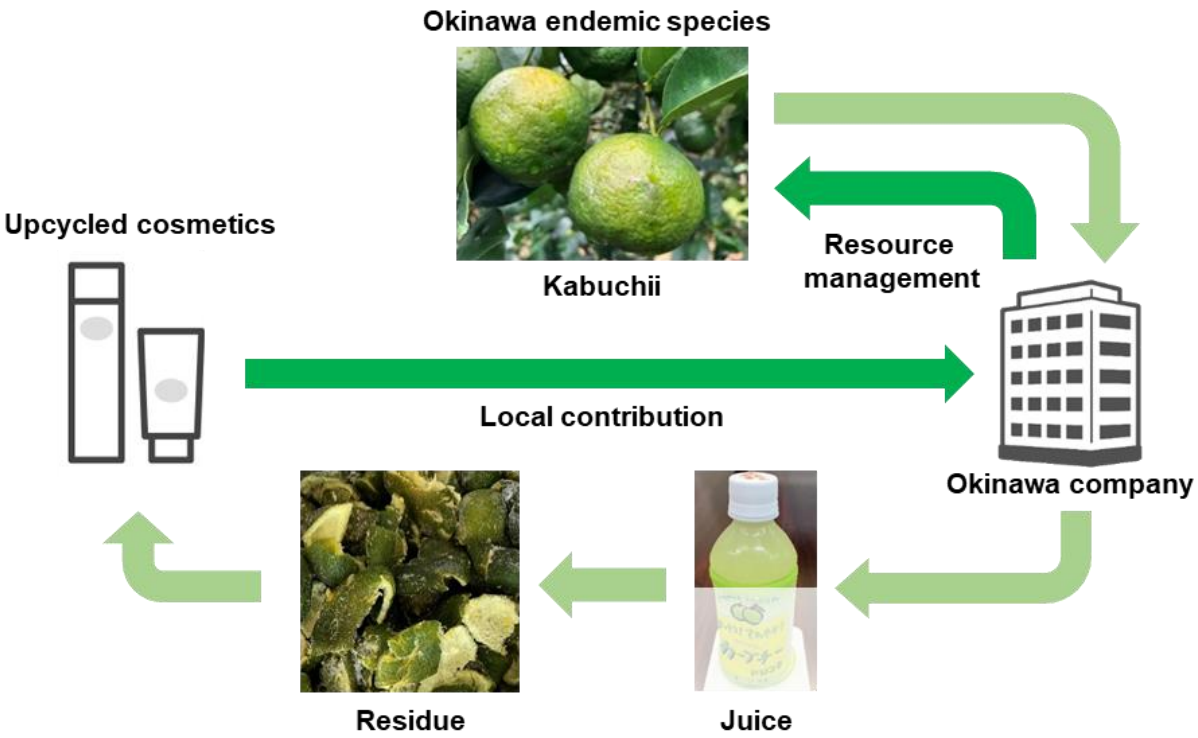

Figure 1. Upcycling Kabuchii waste from Okinawa for cosmetic applications.

2. Materials and Methods

2.1. Kabuchii peel

The peel of Kabuchii grown at Katsuyama Shikuwasa farm in Okinawa was used. The peel

was separated from the residue after juicing the Kabuchii fruits and was used as the raw material. The peel was washed with hypochlorous acid water and then dried with a vacuum dryer at 40 °C. The dried peels were ground to less than 1.0 mm using a crusher. To prevent the degradation of the raw materials, grinding was performed immediately before the extraction process.

2.2. Extraction methods

Ten grams of dried Kabuchii peel powder was extracted with 100 mL EtOH and BG at solvent concentrations from 30 to 100 vol%, as well as with water for 1, 2, 4, or 24 h with stirring at room temperature (RT), 50, and 80 °C. The extract was separated by vacuum filtration using 7 µm filter paper, then dried using a rotary evaporator at 40 °C overnight. The obtained solid components were redissolved in 30 wt% BG. The solution was placed at 4 °C overnight, precipitated solid components were removed using a 0.45 µm membrane filter and a 0.22 µm membrane filter (2 times). The final concentration was adjusted to 1.0 wt% using 30 wt% BG. The solution was sterilized by membrane filtration on a clean bench and placed in a sterilized container.

2.3. Evaluation methods

2.3.1. Quantitative analysis of PMFs

The PMFs in each extract were quantified using high-performance liquid chromatography (HPLC) with NexeraX2 system (Shimadzu). The quantified compounds included sinensetin, isosinensetin, nobiletin, heptamethoxyflavone, natsudaidain and tangeretin, using a YMC-Pack ODS-AM column (particle size 5 µm, inner diameter 2.1 mm, length 150 mm) at 30 °C and a flow rate of 1.0 L/min. Water (A) and methanol (B) were used as mobile phases. The injection volume was 10 µL. The detection wavelengths were 330 nm (except for tangeretin) and 367 nm (tangeretin). All analyses were performed in duplicate, and the results are presented as mean ± standard deviation. Statistical analyses were conducted using R software

(version 4.4.3; R Core Team) on Google Colaboratory. Differences between groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Results were considered statistically significant if $p < 0.05$.

2.3.2. Functional test

The antioxidant activity of Kabuchii peel extract and PMFs standards (sinensetin, isosinensetin, nobiletin, heptamethoxyflavone, natsudaidain, and tangeretin) was measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and superoxide dismutase (SOD) inhibitory activity. Elastase inhibitory activity was also measured as anti-aging activity [7]. For comparison, commercial *Citrus unshiu* (Unshu mandarin orange) and *Citrus depressa* (Shiikuwasha) peel extracts were used as reference materials. All assays were performed in triplicate, and the results are presented as mean ± standard deviation. Statistical analyses were performed using the same methods as those used for the quantitative analysis of PMFs.

ABTS radical scavenging activity

A 7 mM ABTS working solution (10 mL) was mixed with 140 mM potassium persulfate (176 µL). The mixture was then incubated in the dark at 25 °C for 12-16 h. Subsequently, the solution was diluted 30-fold with EtOH. The sample solution (20 µL) was added to the ABTS working solution (200 µL). The mixture was vortexed and incubated at 37 °C for 4 min. The absorbance was measured at 734 nm using a microplate reader (Infinite M200 PRO, TECAN). ABTS radical scavenging activity was calculated as follows:

$$\text{ABTS radical scavenging activity}\ (\%) = \frac{A_{734}(blank) - A_{734}(sample)}{A_{734}(blank)} \times 100 \qquad (1)$$

The IC_{50} values were determined from the concentration-response curve based on the percent inhibition. Ascorbic acid was used as positive control.

SOD inhibitory activity

Antioxidant activity was measured using SOD Assay Kit-WST (DOJINDO LABORATORIES). The samples and the reagents were mixed and incubated at 37 °C for 20 min. Absorbance was measured at 450 nm using a microplate reader (Infinite M200 PRO, TECAN). The SOD inhibitory activity was calculated using the following equation:

$$\text{SOD inhibitory activity } (\%) = \frac{A_{450}(blank) - A_{450}(sample)}{A_{450}(blank)} \times 100 \qquad (2)$$

The half-maximal inhibitory concentration (IC_{50}) was calculated as the percentage decrease in sample concentration. Chlorogenic acid was used as the positive control.

Elastase inhibitory activity

The elastase inhibitory activity of each extract and the PMFs standard was measured. A crude enzyme solution from human fibroblasts was reacted with the test sample (either the Kabuchii extract or the PMFs standard) and a pseudo-elastase substrate (glutaryl-Ala-Phe-4-methoxy-β-naphthylamide), and the fluorescence of the substrate degradation product (4-methoxy-β-naphthylamine) was measured using the spectrometer (SpectraMax i3x, Molecular Devices) at 340 nm (excitation wavelength), 425 nm (fluorescence wavelength) to calculate the elastase inhibitory activity.

2.3.3. CAMSAP3 Gene expression analysis using human keratinocytes

Normal Human Epidermal Keratinocytes (NHEK) were seeded at a density of 5×10^4 cells/dish. Kabuchii extract and reference samples were added at concentrations of 3×10^{-4} wt%, 1×10^{-3} wt% and 3×10^{-3} wt% and the cells were incubated for 24 h. Control cells were cultured without the samples. Total RNA was extracted using TRI Reagent (Merck KGaA, Darmstadt, Germany) and cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) with Oligo dT primers. Quantitative PCR was performed using Luna Universal qPCR Master Mix (New England Biolabs, MA, USA) and a LightCycler 96 system (Roche,

Basel, Switzerland), with the specific primer pairs designed using Primer3 for the target gene. Gene expression was analyzed using the ΔCt method, with GAPDH as the reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [8].

$$\Delta C_t = C_{qTarget} - C_{qGAPDH} \quad (3)$$

3. Results and Discussion

3.1. Effect of extraction conditions on PMFs

Figure 2 shows the effect of the solvent type and concentration on the extraction yield of PMFs from Kabuchii peel. EtOH showed a higher extraction yield than BG for all PMFs. Under these conditions, 50 and 75 vol% EtOH yielded the highest PMF concentrations. The temperature-dependence of the extracted PMFs was also investigated. The amount of PMFs was high at RT and 50 °C, but decreased at 80 °C (data not shown). In addition, the extracted PMFs concentration increased for up to 2 h, and no further increase was observed after 2 h. As a result, the optimal extraction conditions for PMFs from Kabuchii peel were 50-75 vol% EtOH, 50 °C, and 2 h.

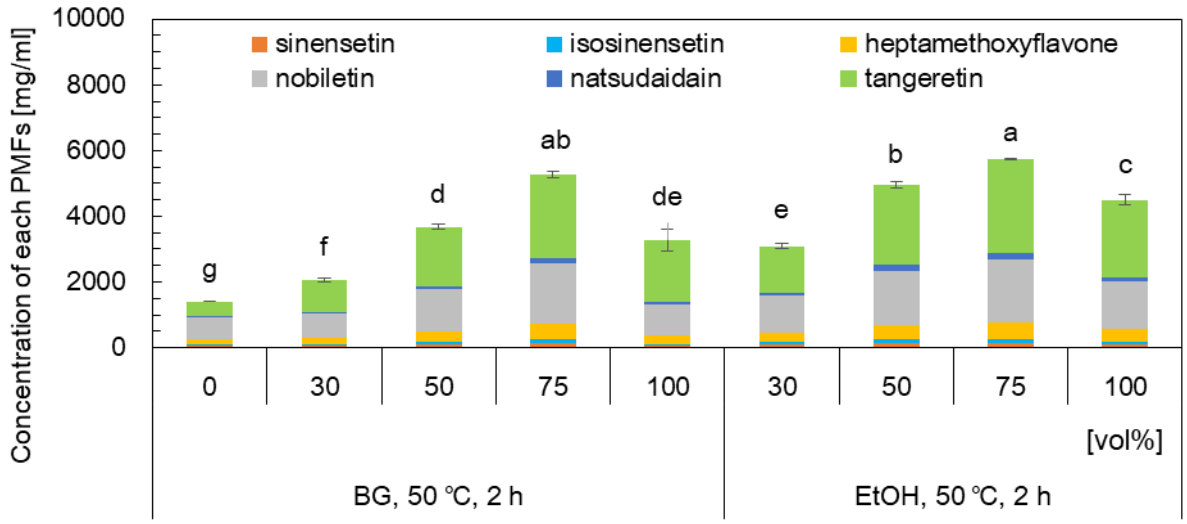

Figure 2. Relationship between solvent concentration and concentration of each PMF in Kabuchii peel extract. Error bars represent the standard error of the mean of total PMFs. Different letters indicate statistically significant differences between groups ($p < 0.05$). Highest mean values are described with the letter a.

3.2. Functionality test

3.2.1. SOD inhibitory activity, ABTS radical scavenging activity and elastase inhibitory activity

Figure 3 compares the SOD inhibitory and ABTS radical scavenging activities of Kabuchii peel extracts with those of commercially available Unshu mandarin and Shiikuwasha peel extracts. The extracts obtained using 50 and 75 vol% EtOH at 50 °C for 2 h showed superior performance compared to the commercially available products. In addition, extracts obtained at RT and 50 °C using 50 vol% EtOH for 2 h also showed higher activity than the existing products, as indicated by their lower IC_{50} values, which signify greater activity. As a result, the effective extraction conditions were determined using 50-75 vol% EtOH at 50 °C for 2 h. These results indicate that the PMF-rich Kabuchii peel extract is a promising functional cosmetic ingredient.

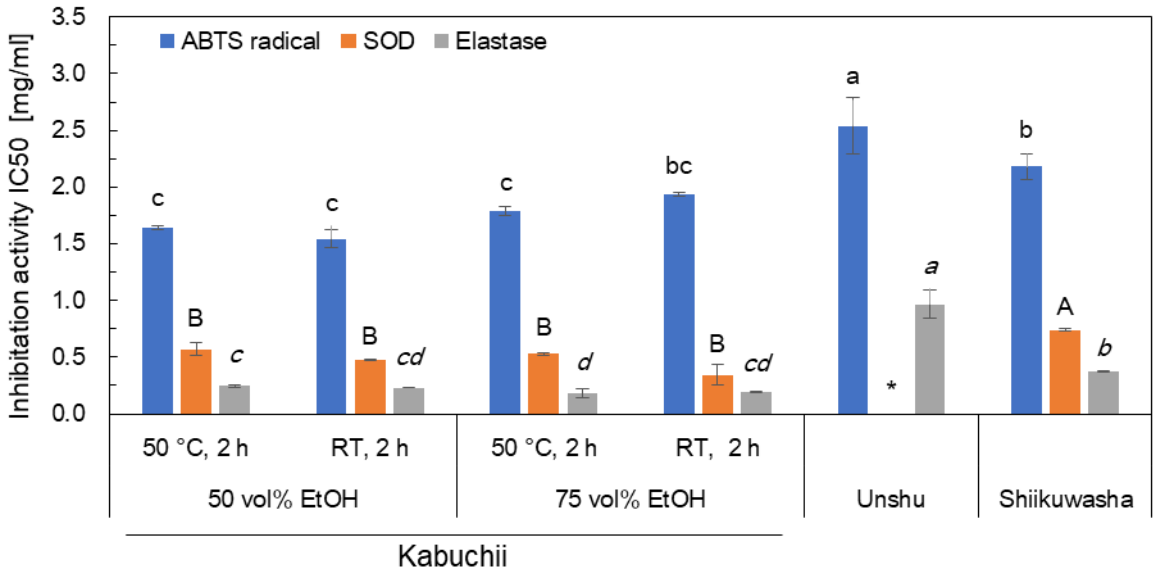

Figure 3. Comparison of IC_{50} values for ABTS radical scavenging, SOD and elastase Inhibitory activity in Kabuchii and commercially available Unshu mandarin and Shiikuwasha peel extracts. * indicates that the activity did not reach 50 % within the test concentration range. Error bars represent the standard error of the mean. Different letters indicate statistically significant differences between groups ($p < 0.05$) for ABTS (lowercase letters), SOD (capital letters), and Elastase (italic letters).

3.2.2. Functionality test results of standard products of PMFs

Functional tests were performed using the PMF standards. Table 1 summarizes the IC_{50} values obtained from the functional tests of the PMFs. Heptamethoxyflavone and nobiletin showed the highest SOD inhibitory activity. Therefore, it is suggested that heptamethoxyflavone and nobiletin contribute to the SOD inhibitory activity. Natsudaidain showed the highest ABTS radical scavenging activity, indicating that it is a significant contributor to the antioxidant function. In addition, sinensetin and nobiletin showed strong ABTS radical scavenging activities, further supporting their contribution to the ABTS radical scavenging activity of the extract. As a result, the Kabuchii peel extract exhibits SOD inhibitory activity, ABTS radical scavenging activity, and elastase inhibitory activity due to the presence of multiple PMFs components. Therefore, Kabuchii peel extract has potential antioxidant applications and can serve as a functional ingredient in anti-aging agents, active oxygen inhibitors, radical scavengers, and elastase inhibitors.

Table 1. IC_{50} values for ABTS radical scavenging, SOD and elastase Inhibitory activity of PMF standards.

PMF	SOD inhibition activity IC_{50} [mg/mL]	ABTS radical scavenging activity IC_{50} [mg/mL]	Elastase inhibition activity IC_{50} [mg/mL]
Natsudaidain	NT	0.06	NT
Heptamethoxyflavone	0.05	<1	NT
Isosinensetin	0.43	<1	0.12
Sinensetin	0.45	<1	0.06
Nobiletin	0.09	<1	0.15
Tangeretin	NT	<1	NT

< 1 indicates that activity did not reach 50% within the test concentration range.

NT; not tested (the reagent did not dissolve to test solvent)

3.2.3. Gene expression analysis in human keratinocytes

The mRNA expression levels of CAMSAP3 in human keratinocytes NHEK are shown in Table 2. CAMSAP3, a member of the CAMSAP family, contributes to non-centrosomal microtubule formation. In epithelial cells, the microtubules are located near the cell membrane and provide

polarity. In the skin, microtubules change from centrosome-dependent to non-centrosomal microtubules depending on the turnover of the epidermis, contributing to the tissue function [9]. The extract promoted the expression of CAMSAP3 1.4 to 9.5 times more effectively than the control. Therefore, CAMSAP3-mediated promotion of non-centrosomal microtubule formation may regulate epidermal turnover and contribute to the maintenance of skin homeostasis.

Table 2. mRNA expression levels of CAMSAP3 in human keratinocytes NHEK.

Kabuchii peel extract	**Concentration of extract [wt%]**		
	3×10^{-4}	1×10^{-3}	3×10^{-3}
Relative gene expression	1.4	2.4	9.5

3.3. Upcycling unused waste

To produce 100 kg of Kabuchii peel extract containing 1.0 wt% solids, 5.7 kg of dry powder is required. The yield of dried peels from frozen juice residue was 11.8%, indicating that 48 kg of residue was used to produce 5.7 kg of dry powder. Therefore, 48 kg of juice residue could be effectively converted into 100 kg of extract. These results show that juice residues can be effectively used as raw materials for cosmetic ingredients. The upcycling of Kabuchii, a citrus fruit unique to Okinawa, reduces waste and adds value to local resources. This approach provides a sustainable model of circular economy practices in cosmetics.

4. Conclusion

Kabuchii peel, a previously unused by-product of juice production, was successfully upcycled as a functional cosmetic ingredient. The most effective extraction conditions were determined using 50-75 vol% EtOH at 50 °C for 2 h. Kabuchii peel extract contains PMFs and demonstrated superior antioxidant and elastase inhibitory activities compared to commercial citrus peel extracts. In addition, it promoted CAMSAP3 expression in human keratinocytes, suggesting its potential role in supporting skin homeostasis. We have developed a manufacturing method for upcycled cosmetic ingredient with high antioxidant activity from Kabuchii peel, an unused resource. This upcycling reduces waste, adds value to local resources, and supports

Okinawa's unique genetic resources in the circular cosmetics economy.

5. References

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