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Extraction Preparation and Anti-Wrinkle Biomarkers from *Ilex x wandoensis C. F. Miller* and M. Kim Hybrid nov., a New Plant

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Abstract: *Ilex x wandoensis* and M. Kim hybrid nov. (IW) is a new variety formed through the natural hybridization of *Ilex. cornuta* Thunb and *Iles. integra* Thunb. It was first discovered on Wando Island, Korea. IW has not been studied to date; we investigated the industrial application potential of IW leaf for the first time. We prepared hot water and solvent extracts and tested for biological activity. IW extract was confirmed to have antioxidant and anti-wrinkle effects. The hot water extract had a high antioxidant effect, and the hexane, ethyl acetate, and acetone extracts showed excellent elastase inhibitory activity. The HPLC and GC-MS analyses of β-amyrin and erythrodiol identified them as biomarkers for anti-wrinkle activity. Here, we report for the first time the active ingredients in IW leaf, representing a new plant variety, and suggest that IW extract could be developed as a cosmetic material.

Keywords: *Ilex x wandoensis* C. F. Miller & M. Kim hybrid nov leaf; Antioxidant; anti-wrikle; elastase

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1. Introduction

Ilex x wandoensis C. F. Miller and M. Kim hybrid nov. (IW) is a species of camphor tree native to Wando island in Korea [1]. Due to climate change, the growth area of IW in southern Korea has increased. Hence, it is necessary to study the possibilities of using IW for various purposes. Until now, the components of IW leaves have not been identified, and there have been no studies of its biological activity. To the best of our knowledge, this is the first report to demonstrate the IW leaf's composition and biological activity.

IW is a new plant formed by natural hybridization of *Ilex. cornuta* Thunb and *Ilex. integra* Thunb. The biological anti-inflammatory and anti-obesity activities of *Ilex. cornuta* Thunb have been reported. Liu et al. reported that *I. cornuta* hot water extract had anti-obesity effects that occur through the reduction of protein expression by peroxisome proliferator-activated receptors γ (PPARγ), and adipose differentiation-related protein (ADRP) [2]. Kim et al. reported that *I. cornuta* extract has anti-inflammatory effects, and that *I. cornuta* leaf extract reduced LPS-induced inflammatory factors in RAW 264.7 cells, confirming the presence of kaempferol (2.7%), vanillic acid (0.03%), isoquercetin and hyperin (2% isoquercitrin plus hyperin) for each gram of extract, by liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS). These were reported to be biomarkers of anti-inflammatory efficacy [3]. IW is a hybrid of *Ilex integra* Thunb, and the active substances of *I. integra* have been investigated, although there have been no other studies of triterpenes (rotundic acid, ursolic acid, and pedunclosides) with antibacterial and antifungal activity [4].

The extraction procedure used on plant sources a very important because, depending on the extraction solvent or extraction conditions, the amount of active substance or its biological activity can be affected. Extraction solvents such as ethanol, acetone, ethylacetate, hexane, and water may be selected. Organic solvents are advantageous for efficient extraction of non-polar, highly bio-active substances. Water extraction is relatively safe and is easier than using organic solvents. However, many non-polar substances have relatively low extraction points [5]. Solvent extraction, subcritical extraction, and supercritical extraction are representative extraction methods, and solvent extraction or hot water extraction are generally used [6]. Various bioactive substances are produced during supercritical and subcritical extraction, but considering the scale of the equipment required, organic solvent extraction or hot water extraction are held to be advantageous for the development of functional food sources. For cosmetic materials and pharmaceuticals, it is thought that supercritical or subcritical extraction have the advantage of obtaining various biologically active substances.

The most appropriate extraction process depends on the intended use of the extract. When the extract is to be used as a functional food, hot water extraction or ethanol extraction have advantages in extract preparation [7–9]. For cosmetic materials, organic solvent extraction is gnerally advantageous for obtaining active substances. Therefore, for easy processing of a product that can be used for two or more purposes, it is necessary to establish an optimal extraction solvent and suitable extraction conditions.

In a previous study we identified several biological substances, and among these the physiologically active substances overlapping between *I. cornuta* Thunb and *Ilex integra* Thunb were isoquercitrin and ursolic acid. In a preliminary study, we found that IW extract exhibited antioxidant and anti-wrinkle effects. Therefore, various solvent extracts and hot water extracts were prepared, and active ingredients and their biological properties were evaluated.

In the present study, water and organic solvent extracts of IW leaf were prepared, in order to identify the optimal product with respect to antioxidant and anti-wrinkle activity and phytochemical constituents. We used GC-MS and HPLC for the chemical profiling of different extracts. Subsequently, we examined the antioxidant and elastase inhibitory activities of the leaf extracts. Antioxidant activity was confirmed by measuring 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and total phenolic contents.

2. Results and Discussion

2.1. Identification of Active Constituents

In the present study, it was confirmed by analyzing several IW extracts that the active material exists as a known component of IW leaves and can be used as a biomarker of IW.

This is the first study to report on optimizing the extraction process of pharmaceutically active indicators from IW leaves and comparing antioxidant and elastase activities of different solvent extracts.

As shown in Table 1, the IW extractive yield was greatest when using hot water, at 40%. The ethyl acetate and ethanol extract yields were each about 9%, and the methanol, hexane, and acetone extract yields were each less than 5%.

Table 1. Extraction yields of *Ilex x Wandoensis* leaf.

Extract	Yield (%, *w*/*w*)
Hexane	2.59 ± 0.05
Ethylacetate	9.00 ± 0.7
Acetone	2.55 ± 0.35
Methanol	4.05 ± 1.41
Ethanol	9.30 ± 1.45
Hot water	39.3 ± 1.26

Table 2 shows the resultant biomarker content of the IW extract. CGA, rutin, and isoquercitrin were not detected in the hexane extract. Three biomarkers were detected in the acetone, methanol, ethanol, and hot water extracts. The hot water extract had the highest CGA and rutin content (2.35 ± 0.07, 0.73 ± 0.03%). The ethanol extract showed the highest isoquercitrin content (0.15 ± 0.01%).

Table 2. Contents of three biomarkers from IW leaf extracts.

Extract	Contents (%, *w*/*w*)		
	Chlorogenic Acid	Rutin	Isoquercitrin
Hexane ex	-	-	-
Ethylacetate ex	0.05 ± 0.005	-	-
Acetone ex	0.53 ± 0.017	0.40 ± 0.011	0.09 ± 0.005
Methanol	0.78 ± 0.005	0.31 ± 0.003	0.07 ± 0.001
Ethanol	1.61 ± 0.074	0.64 ± 0.011	0.15 ± 0.010
Hot Water	2.35 ± 0.066	0.73 ± 0.029	0.05 ± 0.002

We quantified the six extracts by selecting biomarkers likely to help with wrinkle improvement, using GCMS. The selected biomarkers were β-sitosterol, β-amyrin, and erythrodiol, and the results for each constituent are shown in Table 3. The amounts of β-sitosterol, β-amyrin, and erythrodiol, which are nonpolar active substances, were highest in the hexane extract (0.55, 0.59, and 1.39%). The nonpolar bioactive substances showed lower content as the hot water extraction temperature increased.

Table 3. Contents of β-sitosterol, β-amyrin, and erythrodiol in IW leaf extract.

Extract	Contents (%, *w*/*w*)			
	β-sitosterol	β-amyrin	Erythrodiol	Total
Hexane ex	0.55	0.59	1.39	2.53
Ethylacetate ex	0.35	0.43	0.47	1.25
Acetone ex	0.34	0.36	0.52	1.22
Methanol	0.20	0.25	0.34	0.78
Ethanol	0.11	0.10	0.19	0.40
Water	0.00	0.00	0.00	0.00

2.2. Antioxidant Activity and Total Phenolic Contents of IW Leaf Extracts

In general, antioxidants are known to have various effects on skin repair. Therefore, the IW extract's antioxidant efficacy was confirmed. DPPH radical scavenging activity was highest in the methanol, ethanol, and hot water extracts (Figure 1).

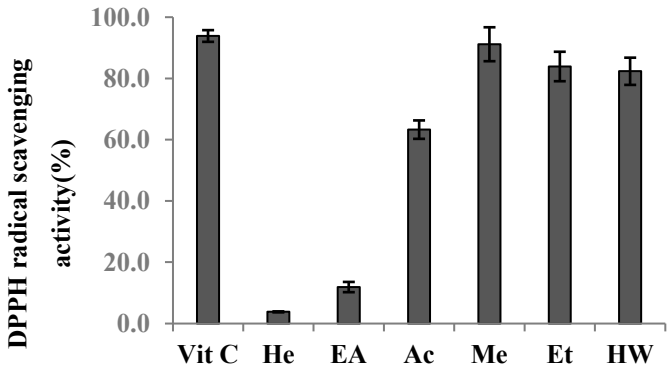

Figure 1. DPPH radical scavenging activity of various types of extracts from IW leaf (sample: 0.5 mg/mL, Asorbic acid: 25 μg/mL). He: hexane ex, EA: ethylacetate ex, Ac: acetone ex, Me: methanol ex, Et: ethanol ex, HW: hot water ex.

Hot water extract had the greatest activity, because of the IW extract's reducing power (67.55 ± 4.26%). The reducing power intensity fell with the increased extraction of the nonpolar solvent extract. Comparing measurements of total phenol, it was found that the hot water extract had the greatest phenol content (75.12 ± 2.58%). Extraction with a nonpolar solvent sharply reduced the total phenol content (Table 4).

Table 4. Reducing power and total phenolic content of IW extract.

Extract	Reducing Power (Ascorbic Acid eq. μg/100 μg Extract)	Total Phenolic Content (Gallic Acid eq. mg/g)
Hexane ex	1.31 ± 0.04	0.39 ± 0.007
Ethylacetate ex	2.76 ± 0.06	1.56 ± 0.03
Acetone ex	10.30 ± 0.35	11.56 ± 0.26
Methanol	12.18 ± 0.19	14.49 ± 0.13
Ethanol	20.05 ± 0.37	22.68 ± 0.22
Water	67.55 ± 4.26	75.12 ± 2.58

Table 5 shows that the hot water extract had the highest flavonoid content, and that using a nonpolar solvent decreased the flavonoid content (13.71 ± 0.33%).

Table 5. Total flavonoids of *Ilex x Wandoensis* C. F. Miller.

Extract	Total Flavonoids (mg/g eq.)
Hexane ex	-
Ethylacetate ex	0.36 ± 0.02
Acetone ex	0.72 ± 0.01
Methanol	1.44 ± 0.03
Ethanol	3.25 ± 0.05
Water	13.71 ± 0.33

2.3. Elastase Inhibitory Activity of Ilex x wandoensis C. F. Miller Leaf Extracts

Figure 2 shows the results of measuring the elastase inhibitory activity of IW extracts. Among the IW extracts, hexane, ethyl acetate, and acetone extracts showed superior elastase inhibitory activity, compared with methanol, ethanol, and hot water extracts when treated at the same concentration, the former group showed 50% or more activity.

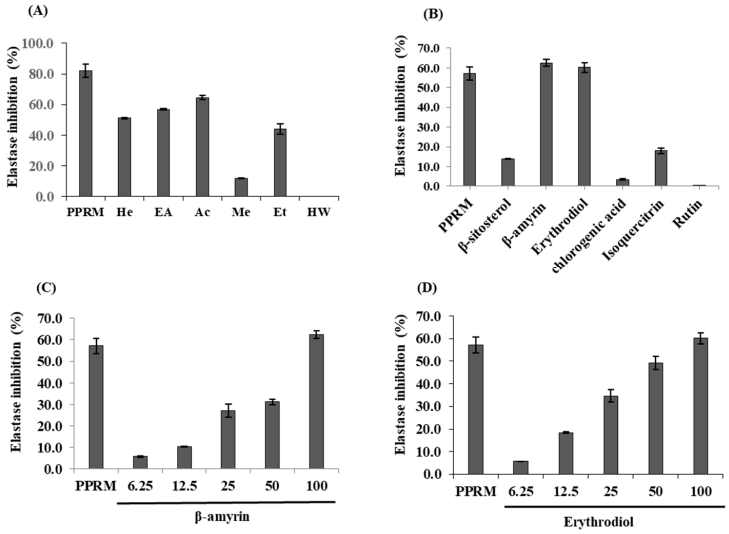

Figure 2. Elastase inhibition of IW extracts and biomarkers derived from IW extract: (**A**) elastase inhibitory activities in various solvent extracts of *Ilex x Wandoensis* (0.5 mg/mL); (**B**) elastase inhibitory activities of six biomarkers (0.1 mg/mL); (**C**,**D**) elastase inhibitory activity of β-amyrin and erythrodiol. The positive control was Phospharamdion (0.1 mg/mL).

When the common biomarkers analyzed in this study and the control material (PPRM) were tested at the same concentration (0.1 mg/mL), β-amyrin and erythrodiol showed the same inhibitory activity as the PPRM. These results confirmed that the anti-wrinkle biomarkers of the IW extract were β-amyrin and erythrodiol. The hexane extract contained 0.6% β-amyrin and 1.4% erythrodiol, accounting for about 2% of the total extract. This was considered a sufficient amount to use as a biomarker. The elastase inhibitory activities of β-sitosterol, chlorogenic acid, and isoquercitrin were each less than 10%, which were considered insufficient to have a significant effect on anti-wrinkle activity.

3. Experimental Section

3.1. Plant Material and Extract Preparation

Ilex x wandoensis C. F. Miller and M. Kim hybrid nov. leaves were supplied by Wando Arboretum (Wando, Korea). A voucher specimen (MNUCSS-CY-01) was deposited at the Mokpo National University (Muan, Korea). Air-dried IW leaves (20 g) underwent extraction twice with 100 mL of organic solvents (hexane, ethyl acetate, acetone, ethanol, and methanol) at 18 °C for 48 h, or with boiled water for 4 h. The resultant solution was dried for further experiments.

3.2. DPPH Free Radical Assay

The sample's antioxidant activity was determined following a DPPH radical scavenging assay. Briefly, the sample solution (1 mL) was added to the DPPH solution (1 mL, 0.4 mM) and mixed. The mixture was allowed to react at room temperature for 10 min. The mixture was measured at 517 nm using a microplate reader (Perkin Elmer, Waltham, MA, USA) [10].

3.3. Reducing Power

The reducing power of the sample was determined by the following assay method. The sample was mixed with sodium phosphate buffer and potassium at 50 °C for 20 min. Stop buffer was added to mixture (trichloroacetic acid). After centrifugation, the supernatant was mixed with distilled water and iron (III) chloride solution, then measured at 700 nm [10].

3.4. Determination of Total Phenolic Content

The total phenolic content was determined using a Folin–Ciocalteu assay [10]. The samples were mixed with sodium carbonate solution and Folin–Ciocalteu phenol reagent for 10 min. Absorbance was measured at 750 nm and compared with a gallic acid calibration curve. Results were expressed as milligrams of gallic acid equivalent per gram of sample [10].

3.5. Determination of Elastase Inhibitory Activity

The assay was performed according to the protocols of Chiocchio et al. [11]. Elastase (10 µL, 10 ug/mL conc) was mixed with Tris- HCl (90 µL, 0.2 M), STANA (100 uL, 2.5 mM, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide), and the sample (50 µL) at 37 °C for 30 min. This mixture was centrifuged at 15,000 rpm for 10 min. The absorbance was measured at 405 nm.

3.6. Identification and Quantitication of Biomarkers Using GC-MS

Analysis of the active constituents from IW leaf was performed using GC-MS according to a modified process [9]. Agilent 7890 gas chromatography (GC) and Agilent 5975 quadrupole mass spectrometry (MS) systems (Agilent Technologies, Palo Alto, CA, USA) were utilized to analyze molecular mass fragments (50–550 amu) of the IW leaf, with Agilent HP-5MS fused silica capillary column (30 mm l. × 0.25 mm i.d., 0.25 µm film thickness). The mass fragments were ionized under electron ionization (EI) conditions. A GC oven was isothermally programmed at 65 °C for 10 min at 10 to 300 min^{-1} with helium (He) as a carrier gas. All the data were compared with the system library (NIST 2017, Table 6).

Table 6. GC-MS analysis of hexane extract from IW leaf.

RT (min)	Hit Name	Quality	M.W.	Composition (%)
6.28	N,N′-Bis(trimethylsilyl)trifluoroacetamidine	97	256	0.10%
24.504	D-(−)-Fructofuranose	91	540	0.15%
24.951	Neophytadiene	97	278	0.23%
25.437	alpha.-D-Mannopyranose	95	540	0.18%
26.272	beta.-D-Allopyranose	93	540	0.15%
27.033	Hexadecanoic acid	99	328	4.91%
27.382	1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol	93	612	0.10%
27.932	Heptadecanoic acid	96	342	0.19%
28.109	9(E),11(E)-Conjugated linoleic acid	99	308	0.13%
28.201	Phytol	98	368	4.93%
28.544	Linoleic acid	94	352	1.08%
28.607	11-Octadecenoic acid	98	354	3.25%
28.819	Octadecanoic acid	99	356	0.61%
29.768	4,8,12,16-Tetramethylheptadecan-4-olide	99	324	0.07%
30.478	Eicosanoic acid	99	384	0.10%
31.227	1,2-Benzenedicarboxylic acid	90	390	0.09%
31.599	1-Monopalmitin	93	474	0.08%
32.017	Docosanoic acid	99	412	0.12%
32.75	Tricosanoic acid	99	426	0.05%
33.03	Glycerol monostearate	93	502	0.16%
33.327	Squalene	99	410	0.22%
33.9	delta.-Tocopherol,	99	474	0.08%
34.666	gamma.-Tocopherol	99	488	0.20%
35.977	alpha.-Tocopherol	99	502	0.12%
37.293	Campesterol	98	472	0.12%
37.608	Stigmasterol	99	484	0.73%
38.489	beta.-Sitosterol	97	486	6.20%
38.849	beta.-Amyrin	99	498	4.59%
39.742	Lupeol	99	498	42.04%
41.024	Erythrodiol	94	586	1.70%
41.876	Uvaol	94	586	11.41%
42.277	Oleanolic acid	95	600	1.68%
43.392	Ursolic acid	95	600	4.16%
43.793	Ursolic aldehyde	96	512	2.36%

3.7. Constituent Profiling by High-Performance Liquid Chromatography (HPLC) Analysis

Constituent profiling of IW extracts was performed with HPLC. All HPLC analyses were performed using the Alliance 2695 HPLC system (Waters; Milford, MA, USA) equipped with a photodiode array detector. The analysis method is described in Table 7 and chromatographic profiles are shown in Figure 3. Figure 4A shows the presence of three biomarkers in the sample. Sample concentrations were analyzed for content analysis of the three biomarkers by adjusting each biomarker to a concentration that did not interfere with surrounding peaks.

Table 7. Analytical conditions of the HPLC system for three markers.

Parameters	**Conditions**
Column	**Zorbax Extended-C18 (C18, 4.6 mm × 150 mm, 5 μm)**
Flow rate	0.8 mL/min
Injection volumn	10 μL
UV detection	330 nm
Run time	40 min

Table 7. *Cont.*

Parameters Column	Conditions Zorbax Extended-C18 (C18, 4.6 mm × 150 mm, 5 μm)		
	Time (min)	**A (%)** [1]	**B (%)** [2]
Gradient	0	10	90
	5	10	90
	23	20	80
	30	100	0
	35	10	90
	40	10	90

[1] Acetonitrile [2] 0.2% phosphoric acid.

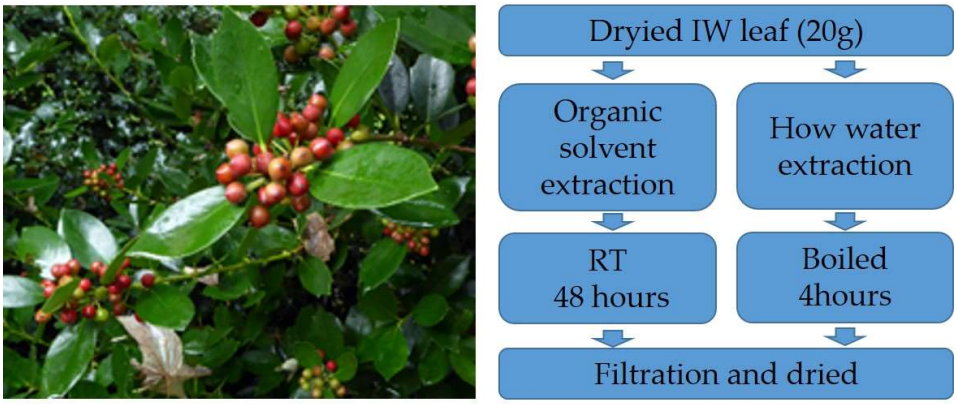

Figure 3. Picture of *Ilex x wandoensis C. F. Miller* and M. Kim Hybrid nov and scheme of IW extraction.

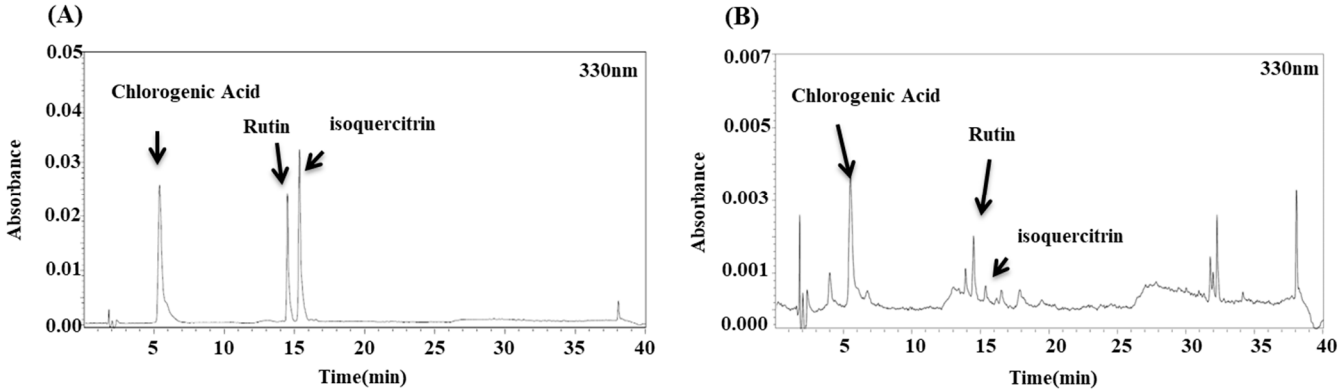

Figure 4. Chromatogram of standard and IW leaf extracts: (**A**) standard, (**B**) ethanol extract.

3.8. Statistical Analysis

The data were analyzed using Excel® software (ver.2017).

4. Conclusions

Ilex x wandoensis and M. Kim hybrid nov. (IW) is a new plant found in Korea. We have described the scientific identification of the active constituents and their biological efficacy. In the present study, we prepared various samples of IW leaf to assess the samples' basic properties, and for the first time we evaluated the antioxidant and elastase inhibitory activities of IW extracts. The antioxidant properties of IW extracts were tested; the hot water and methanol extracts showed the highest DPPH radical scavenging activities, total phenolic contents, and total flavonoids. Reducing power was greatest in the water extract. The acetone extract showed superior elastase inhibitory effect compared with PPRM. Our findings present basic information for the beneficial uses of IW leaf extracts for treating disease induced by oxidative stress. In this study, the different extracts that were analyzed exhibited different effects. Therefore, it is hypothesized that the applicability of IW leaf

depends on the extraction method. Hot water and acetone extracts have the potential to be used as antioxidants and anti-wrinkle materials. Several bioactive markers contained in IW leaf extracts were analyzed with GCMS and HPLC, and were found to possess antioxidant and elastase inhibitory activity. To the best of our knowledge, this is the first study to report the chemical profiling and biological effects of various IW leaf extracts, suggesting their beneficial uses in functional food sources and cosmetics.

Author Contributions: Conceptualization, S.-S.C., D.-H.P. and D.-S.O.; methodology and investigation, S.-Y.S., S.-H.L., M.-S.B. and S.-H.L.; writing and editing, S.-S.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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