

# Immunomodulatory Activities of Flavonoids, Monoterpenoids, Triterpenoids, Iridoid Glycosides and Phenolic Compounds of *Plantago* Species

Lien-Chai Chiang<sup>1</sup>Lean Teik Ng<sup>2</sup>Wen Chiang<sup>3</sup>Mei-Yin Chang<sup>1</sup>Chun-Ching Lin<sup>4</sup>

## Abstract

A number of *Plantago* spp. especially *P. major* has long been used in the treatment of diseases such as infection, inflammation and cancer. In this study, we evaluated the immunomodulatory activities of five chemical classes of pure compounds obtained from the *Plantago* genus on human peripheral blood mononuclear cells (PBMC). Studies were conducted on lymphocyte transformation by BrdU immunoassay and secretion of interferon-gamma (IFN- $\gamma$ ) using an ELISA assay. Results showed that the water-soluble compounds, namely aucubin, chlorogenic acid, ferulic acid, *p*-coumaric acid and vanillic acid, enhanced the activity of human lymphocyte proliferation and secretion of IFN- $\gamma$ . Among the water-insoluble compounds, with the exception of

luteolin, both baicalein and baicalin showed an enhancement of the human PBMC. Although oleanolic acid and ursolic acid of the triterpenoids did not significantly affect the proliferation of PBMC, they exhibited a strong stimulation of IFN- $\gamma$  secretion. Linalool, a monoterpene, showed a similar immunomodulatory activity as the triterpenoids. The present study concludes that the tested compounds, which possess immunostimulating activities, may contribute to the traditional claims of *Plantago*-based natural products used in treating cancers and infectious diseases.

## Key words

Immunomodulatory activity · *Plantago* species · Plantagiaceae · flavonoids · monoterpenoids · triterpenoids · iridoid glycosides · phenolics

## Introduction

*Plantago* spp. have long been used for treating diseases related to the skin, wound healing, inflammation, disorders of respiratory and digestive organs, reproductive system, blood circulation and cancer. Previous studies have shown that the *Plantago* genus contains five chemical classes of biologically active compounds (Fig. 1), namely flavonoids (i.e., baicalein, baicalin and luteolin), monoterpenoids (i.e., linalool), triterpenoids (i.e., oleanolic acid and ursolic acid), iridoid glycosides (i.e., aucubin) and phenolic compounds (i.e., caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid and vanillic acid) [1].

Chlorogenic acid potently enhanced human mononuclear cell proliferation and interferon-gamma (IFN- $\gamma$ ) production [2]. Ferulic acid reduced the murine interleukin-1-alpha or interleukin-8 production in response to influenza virus infections *in vitro* and *in vivo* [3], [4]. Oleanolic acid and ursolic acid stimulated the proliferation of splenocytes and enhanced the recovery of hematopoietic system in mice [5]. Baicalein inhibited humoral and cellular immunity in mice [6]. Luteolin suppressed migration and activation of leukocytes, degranulation of mast cells and interleukin-5 bioactivity [7], [8].

In traditional practice, *P. major* is used as a remedy for colds and viral hepatitis [9], [10]. The aqueous extract of this plant was reported to have a prophylactic effect on mammary cancer in mice

## Affiliation

<sup>1</sup> Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, R.O.C.

<sup>2</sup> Department of Food Science and Technology, Tajen Institute of Technology, Pingtung, Taiwan, R.O.C.

<sup>3</sup> Department of Clinical Pathology, Kaohsiung Medical University, Kaohsiung, Taiwan, R.O.C.

<sup>4</sup> Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan, R.O.C.

## Correspondence

Professor C. C. Lin · Graduate Institute of Natural Products · Kaohsiung Medical University · Kaohsiung 807 · Taiwan · R.O.C. · Phone: +886-7-3121101 ext 2122 · Fax: +886-7-3135215 · E-mail: aalin@ms24.hinet.net

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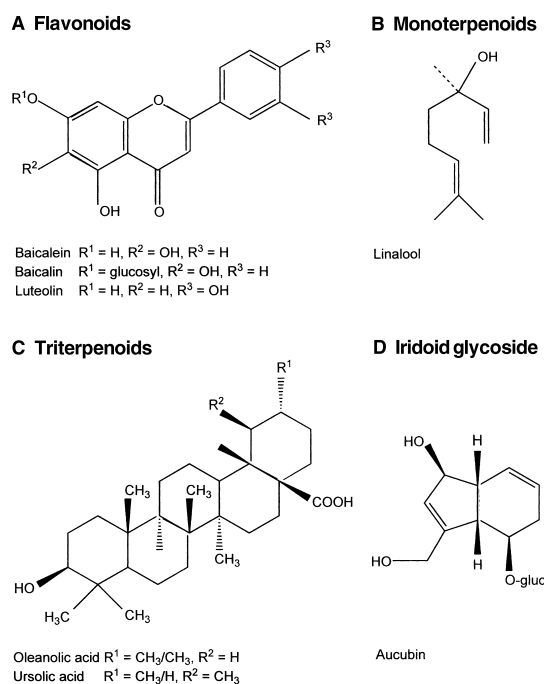


Fig. 1 Structure of bioactive compounds of the four chemical classes obtained from the *Plantago* genus.

[11]. It also exhibited chemotactic activity on neutrophils, but not the activity in inhibiting the intracellular neutrophils [12]. Its decoction is used for treating cancer [13].

In this study, our aim was to evaluate the immunomodulatory activities of the five chemical classes of twelve related pure compounds derived from *Plantago* genus on human peripheral blood mononuclear cells (PBMC).

## Materials and Methods

### Chemicals

Aucubin, baicalein, baicalin, chlorogenic acid and luteolin were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Caffeic acid, ferulic acid, linalool, oleanolic acid, *p*-coumaric acid, ursolic acid and vanillic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). The purity of these compounds was greater than 98%.

5-Bromo-2-deoxyuridine (BrdU) labeling and detection kit III was purchased from Roche Diagnostics GmbH (Germany). The human IFN- $\gamma$  immunoassay kits were obtained from R&D Systems Inc. (Minneapolis, USA). Deaminated heparin, Ficoll-Hypaque and phyto-mitogens of phytohemagglutinin (PHA) and concanavalin A (Con A) were obtained from Sigma-Aldrich Co. (USA).

### Human mononuclear cells

The healthy peripheral blood of volunteers was collected in a sterile syringe containing sufficient heparin to give a final concentration of 100 units/mL. Mononuclear cells were obtained by centrifuging (25 °C, 400 g, 30 min) the mixture of blood and normal saline (v/v:1/1) on Ficoll-Hypaque (2.4:1) gradients as described by the manufacturer's protocol (Sigma-Aldrich). Mono-

nuclear cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin G, 100  $\mu$ g/mL streptomycin and 0.25  $\mu$ g/mL amphotericin B (GIBCO BRL, New York, USA).

### Lymphocyte transformation test

The assay was conducted on a test tube containing 0.05 mL of unfractionated peripheral blood mononuclear cells, 0.15 mL fetal calf serum, 0.75 mL of RPMI 1640 and 0.05 mL of test sample. The concentration of the positive control was 5  $\mu$ g/mL and 10  $\mu$ g/mL for PHA and Con A, respectively. The medium with only dimethyl sulfoxide (DMSO) was used as a negative control. After a gentle mixing, 200  $\mu$ L per well of the mixture were added to a 96-well microculture plate in triplicate. The culture plate was then allowed to incubate for 3 days at 37 °C in a 5% CO<sub>2</sub> incubator. At the end of the incubation, 20  $\mu$ L per well of BrdU labeling solution were added and the culture plate was reincubated for additional 24 h at 37 °C. The culture plate was centrifuged at 300 g for 10 min, the labeling medium was then removed while the cells were dried at 60 °C for 1 h. Two hundred microliters per well of FixDenat solution were added to the cells, which were then incubated for 30 min at room temperature. After removing the FixDenat solution, 100  $\mu$ L of anti-BrdU-POD working solution was added to each well, followed by incubating further for 90 min at room temperature. The antibody conjugate was removed and the wells were rinsed three times with 200  $\mu$ L per well of washing solution. After washing, 100  $\mu$ L of substrate solution was added to each well, followed by incubating the culture plate for 30 min. At the end of the incubation, 25  $\mu$ L per well of H<sub>2</sub>SO<sub>4</sub> (1 M) was added, the culture plate was further incubated on the shaker set at 300 rpm for 1 min. Absorbance of samples was determined at 450 nm (test wavelength) and 690 nm (reference wavelength) using an ELISA reader (Multiskan EX, Labsystems) [14]. The stimulation index (SI) was determined by the ratio of optical density of test substance to the optical density of negative control.

### Enzyme-linked immunosorbant assay (ELISA)

The solid-phase sandwich ELISA procedure was performed according to the assay protocol provided by the supplier (R&D Systems Inc.). Briefly, the cultivation and treatment of human PBMC were done as previously described in the lymphocyte transformation test. After 3 days, particulates were removed from the supernatants by centrifugation, samples were then stored at -70 °C until use.

The assay was carried out by adding 100  $\mu$ L of sample diluent to each well (100  $\mu$ L/well), each of which then received either 100  $\mu$ L of IFN- $\gamma$  standard or supernatant sample. The well plate was covered with the adhesive strip and incubated at room temperature for 2 h. After incubation, each well was aspirated and washed, which was repeated for three times. Two hundred microliters of IFN- $\gamma$  conjugate (200  $\mu$ L/well) were further added to each well, followed by covering the culture plate with a new adhesive strip and then incubated at room temperature for 2 h. The same washing process was again performed on the wells. After adding 200  $\mu$ L per well of substrate solution, the samples were incubated at room temperature for 30 min, followed by adding 50  $\mu$ L of stop solution to each well. Absorbance of samples was measured at 450 nm (test wavelength) and 540 nm (reference wavelength) within 30 min using an ELISA reader.

### Statistical analysis

Results were expressed as means  $\pm$  standard errors. The unpaired Student's t-test was used to evaluate the difference between the control and test samples. A *p* value less than 0.05 was chosen as the level of significance.

### Results

The results showed that at concentrations lower than 20  $\mu\text{g/ml}$ , both baicalin and baicalein significantly ( $p < 0.05$ ) stimulated the proliferation of human PBMC and the secretion of IFN- $\gamma$  (Figs. 2 and 3). However, luteolin was found to possess inhibitory activity on the proliferation of human PBMC.

There was no clear difference in the immunoinhibitory activity between oleanolic acid and ursolic acid at concentrations between 1.25  $\mu\text{g/ml}$  and 20  $\mu\text{g/ml}$  (Figs. 2 and 3). However, ursolic acid showed a higher activity than oleanolic acid at concentration 40  $\mu\text{g/ml}$ . Oleanolic acid exhibited a weak activity in inhibiting the proliferation of human PBMC but with a strong activity in enhancing the secretion of IFN- $\gamma$  (258 pg/mL). Ursolic acid was active in inhibiting the proliferation of PBMC and enhancing the secretion of IFN- $\gamma$  at low concentration (1.25  $\mu\text{g/ml}$ ).

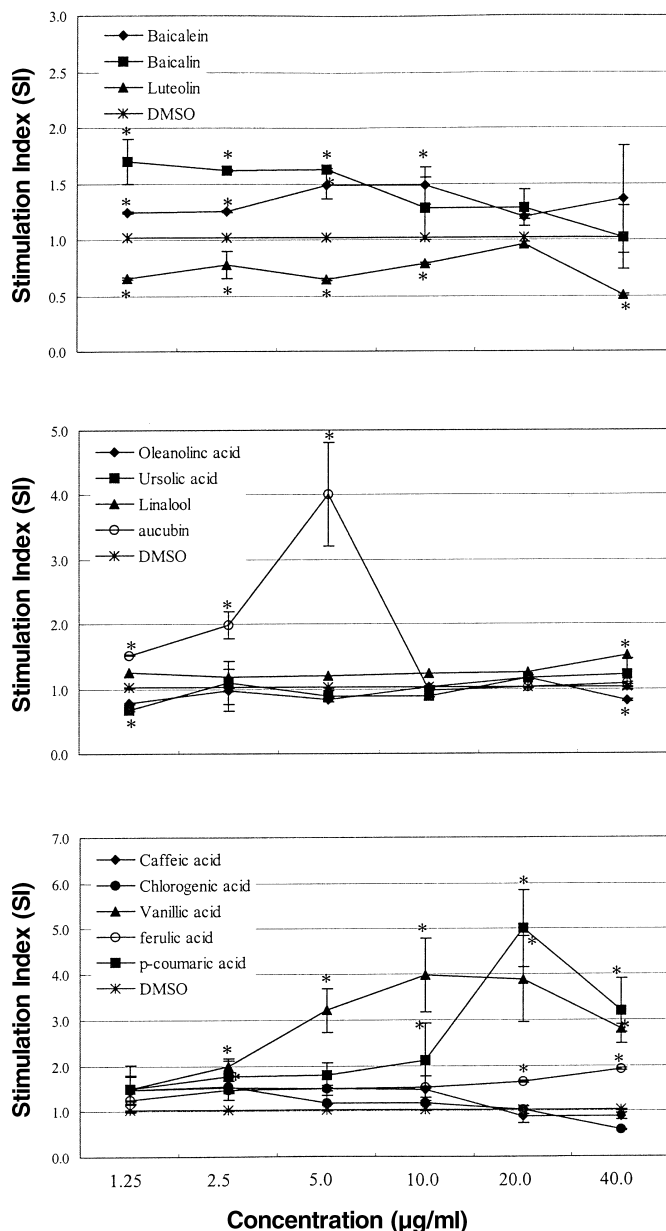
At concentrations lower than 5  $\mu\text{g/ml}$ , aucubin significantly stimulated the proliferation of human PBMC and enhanced secretion of IFN- $\gamma$ . Linalool exhibited a weak activity in stimulating the proliferation of human PBMC and a moderate stimulation of IFN- $\gamma$  secretion (Figs. 2 and 3).

Among the phenolic compounds tested, *p*-coumaric acid possessed the strongest immunostimulating activity, with a stimulation index (SI) equal to 4.59 for PBMC proliferation and secretion of IFN- $\gamma$  equal to 181 pg/mL. Vanillic acid exhibited a strong enhancement of PBMC proliferation and secretion of IFN- $\gamma$  at concentrations between 5  $\mu\text{g/ml}$  and 40  $\mu\text{g/ml}$ . Although the activity of ferulic acid was lesser than that of *p*-coumaric acid, it exhibited a trend of higher immunostimulating activity than chlorogenic acid and caffeic acid. However, chlorogenic acid exerted a higher secretion of IFN- $\gamma$  than ferulic acid and caffeic acid (Figs. 2 and 3, Table 1).

### Discussion

In traditional practice, *P. major* is used for treating diseases such as infection, inflammation and cancer [1], [13]. Its aqueous extract was reported to have chemotactic activity on neutrophils but not the activity in inhibiting the intracellular neutrophils [12].

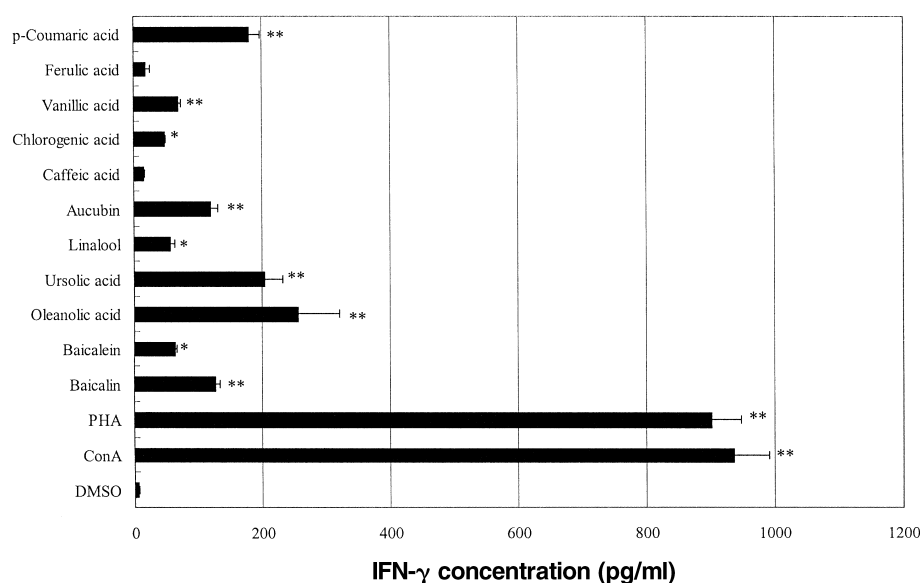
Previous studies showed that chlorogenic acid potently enhanced human mononuclear cells proliferation and IFN- $\gamma$  production [2]. In this study, a similar response was noted at concentrations below 10  $\mu\text{g/ml}$ . Ferulic acid reduced the murine interleukin-1-alpha or interleukin-8 production in response to influenza virus infections both *in vitro* and *in vivo* [3], [4]. However, our results indicated that ferulic acid stimulated the proliferation of human PBMC and enhanced the secretion of IFN- $\gamma$ .



**Fig. 2** The immunomodulatory activity of five classes of compounds from the *Plantago* genus, namely flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds. The various concentrations of test compounds were evaluated on their ability to directly stimulate PBMC without mitogen. Values represent the mean  $\pm$  SE of three independent experiments of triplicate measurements at each dose. Unpaired Student's t-test was used to evaluate the difference between test compounds and DMSO control at  $p < 0.05$ . The stimulation index of negative control (DMSO:  $1.02 \pm 0.01$ ) and positive controls (Con A:  $8.49 \pm 1.34$ , PHA:  $7.90 \pm 1.25$ ) was evaluated at the same time period.

The discrepancy in results between the two different studies could be due to the difference in the stimulating agent and host used in the study. Among the other phenolic compounds tested, *p*-coumaric acid showed a strong activity in stimulating the lymphocyte proliferation and secretion of IFN- $\gamma$  ( $p < 0.05$ ), whereas caffeic acid exhibited a weak activity (Figs. 2 and 3, Table 1).

Both oleanolic acid and ursolic acid stimulated the proliferation of splenocytes and enhanced the recovery of hematopoietic sys-



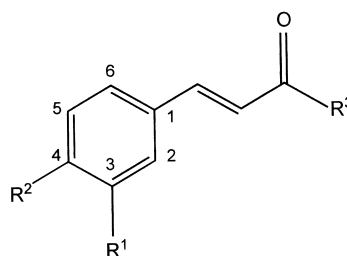
**Fig. 3** The IFN- $\gamma$  concentration present in the supernatant after treatment with five classes of compounds from the *Plantago* genus, namely flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds. The supernatants of the highest stimulation index (SI) of test compounds were used to determine the concentration of IFN- $\gamma$  by the solid-phase sandwich ELISA procedures. Values represent the mean  $\pm$  SE of three independent experiments of triplicate measurements at each dose. Unpaired Student's t-test was used to evaluate the difference between test compounds and DMSO control. \* $p < 0.05$  and \*\* $p < 0.01$  indicate significant differences between test compounds and DMSO. The IFN- $\gamma$  concentration in the supernatant after treatment with negative control (DMSO:  $5 \pm 2$ ) and positive controls (Con A:  $937 \pm 55$ , PHA:  $901 \pm 46$ ) was evaluated at the same time period.

tem in mice [5]. Surprisingly, in this study, inhibitory activity on the proliferation of human PBMC was noted at  $40 \mu\text{g/mL}$  for oleanolic acid and  $1.25 \mu\text{g/mL}$  for ursolic acid, but not at other concentrations tested. Triterpenoids such as oleanolic acid and ursolic acid strongly enhanced the secretion of IFN- $\gamma$  ( $p < 0.01$ ). A similar immunomodulatory activity was also noted on linalool, a monoterpene.

Baicalein inhibited humoral and cellular immunity in mice [6]. However, the present study showed that baicalein and its glycoside (baicalin) exhibited potently stimulatory activity on the proliferation of human PBMC and secretion of multipotent immunomodulatory cytokine IFN- $\gamma$ . The disagreement in two studies could be due to the difference in doses, species or method used in the study. Luteolin suppressed migration and activation of leukocytes, degranulation of mast cells and interleukin-5 bioactivity [7], [8]. These observations were further confirmed in the present study as demonstrated by the immunoinhibitory activity of luteolin on human PBMC proliferation at concentrations between  $1.25 \mu\text{g/mL}$  and  $40 \mu\text{g/mL}$ .

To date, several laboratories have demonstrated that IFN- $\gamma$  is able to activate non-specific cytotoxic activity in macrophages towards a variety of intracellular parasites and neoplastic cells [15], [16]. IFN- $\gamma$  was first identified in pHA-activated lymphocyte supernatants as an antiviral factor [17]. The direct antiviral action of IFN- $\gamma$  has been attributed mainly to the transcriptional induction of three genes, namely double-stranded RNA activated protein kinase, 2',5'-oligoadenylate synthetase, and dsRNA-specific adenosine deaminase [18]. IFN- $\gamma$  is a potent activator of mononuclear phagocytes which allow macrophages to kill tumor cells and phagocytose microbes. Mouse IFN- $\gamma$  (type II) preparations were 100-fold more active than type I IFN in inhibiting the outgrowth of tumors [19]. The present study has demonstrated that 75% of the twelve related pure compounds from *Plantago* genus possess immunostimulatory activity including enhancing the secretion of IFN- $\gamma$ . This finding suggests that immunomodulatory activities derived from the test compounds may participate in the therapeutic effects of *Plantago*-based natural pro-

**Table 1** Structure of phenolic compounds and their immunomodulatory activity



Compounds	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	Stimulation index (SI) <sup>a</sup>	Interferon- $\gamma$ (pg/mL) <sup>a</sup>
DMSO (control)				$1.0 \pm 0.01^d$	$5.0 \pm 2.0^e$
Con A				$8.5 \pm 1.34^a$	$937 \pm 55.0^a$
PHA				$7.9 \pm 1.25^a$	$901 \pm 46.0^a$
Caffeic acid	OH	OH	OH	$1.0 \pm 0.01^d$	$21.7 \pm 4.3^d$
Chlorogenic acid	OH	OH	$\delta$	$1.1 \pm 0.01^d$	$82.6 \pm 17.4^c$
Ferulic acid	OH	OCH <sub>3</sub>	OH	$1.8 \pm 0.08^c$	$60.9 \pm 19.5^c$
p-Coumaric acid	H	OH	OH	$5.0 \pm 0.20^b$	$182.6 \pm 17.4^b$

<sup>d</sup> 1,3,4,5-Tetrahydrocyclohexanecarboxylic acid.

<sup>a</sup> Values are mean  $\pm$  SE of three independent experiments of triplicate measurements at the concentration of  $20 \mu\text{g/mL}$ . Means in a column followed by the same letter are not significantly different at  $p > 0.05$  based on unpaired Student's t-tests.

ducts used in the folk medicine for treating cancer and infectious diseases [9], [10], [11], [12], [13].

The immunomodulatory activity of phenolic compounds on stimulation of PBMC was noted without a mitogen. It was interesting to find that the magnitude of activity varies with the differences in functional groups attaching to the common basic structure, which is the cinnamic acid. Compounds that contain the hydroxy group at the R<sup>2</sup> position (caffeic acid, chlorogenic acid) were found to possess lesser potent lymphocyte activating capability than compounds containing other groups at the same

position. The discrepancy in the stimulation of IFN- $\gamma$  secretion between caffeic acid and chlorogenic acid could be due to the difference at the R<sup>3</sup> position (Table 1).

In conclusion, five chemical classes of twelve pure compounds derived from the *Plantago* genus were found to possess immunomodulatory activity and enhance the secretion of IFN- $\gamma$ . The water-soluble compounds (aucubin, chlorogenic acid, ferulic acid, *p*-coumaric acid and vanillic acid) and water-insoluble flavonoids (baicalein, baicalin) except luteolin exhibited immunostimulatory activity. However, triterpenoids such as oleanolic acid, ursolic acid were not a potent modulator of lymphocyte proliferation but they exhibited strong activity in stimulating the secretion of IFN- $\gamma$ .

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