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Report

Gastroprotective Effects of Phellamurin Isolated from the Leaves of *Phellodendron Amurense* against Gastric Mucosal Injury in Rats

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Phellamurin, a flavonoid isolated from the leaves of *Phellodendron amurense* (Rutaceae), has not yet been investigated for its effects on the gastrointestinal tract. In this study, we evaluated its gastroprotective potential using an HCl/ethanol-induced gastric mucosal injury model in rats and an oxidative stress model in primary cultured rat gastric mucosal cells. Oral administration of phellamurin significantly reduced gastric lesion areas. Phellamurin also protected gastric mucosal cells against hydrogen peroxide (H₂O₂)-induced oxidative stress at concentrations that did not show detectable cytotoxicity under the present assay conditions. Quantitative PCR analysis showed no significant changes in the expression levels of antioxidant-related genes or cyclooxygenase (COX) genes. Phellamurin moderately inhibited *Helicobacter pylori* growth at 1 mM but did not inhibit urease activity. These findings suggest that phellamurin exerts gastroprotective effects, possibly through antioxidant-related cytoprotective mechanisms, rather than through direct antibacterial or urease-inhibitory activity.

Key words phellamurin, gastroprotection, oxidative stress, gastric epithelial cell, *Helicobacter pylori*

INTRODUCTION

Phellodendron bark (PHELLODENDRI CORTEX), derived from *Phellodendron amurense* Rupr. (Rutaceae) and related species, is widely used in traditional East Asian medicine and contains various bioactive constituents, including protoberberine alkaloids such as berberine.¹⁾ Berberine has been reported to exert diverse pharmacological effects, including gastrointestinal²⁾ and antimicrobial activities.³⁾ In addition to the bark, the leaves of *P. amurense* contain phellamurin, a flavonoid glycoside (Fig. 1). Flavonoids exert multiple beneficial effects on the gastrointestinal tract, including antioxidant, antibacterial, and cytoprotective activities.⁴⁾ Phellamurin has been reported to exhibit antitumor activity through apoptosis induction in osteosarcoma cells⁵⁾ and to inhibit intestinal P-glycoprotein activity.⁶⁾ However, the effects of phellamurin on the gastric mucosa remain unclear.

Peptic ulcer disease is a common gastrointestinal disorder caused by an imbalance between mucosal protective factors and aggressive factors, including gastric acid, non-steroidal anti-inflammatory drugs, and *H. pylori* infection.⁷⁻⁹⁾ Antioxidant defense and antibacterial activity are therefore considered important mechanisms for gastric protection.

In the present study, we investigated the effects of phellamurin on gastric mucosal injury. Specifically, we evaluated its gastroprotective effect in an HCl/ethanol-induced gastric injury model, its cytoprotective effect against oxidative stress

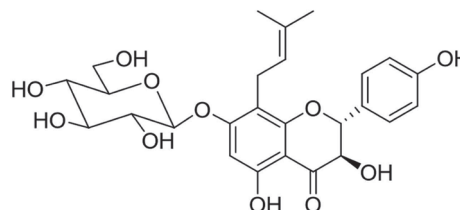


Fig. 1. Structure of Phellamurin

in primary cultured rat gastric mucosal cells, and its anti-*H. pylori* and anti-urease activities.

MATERIALS AND METHODS

Plant Materials The leaves of *P. amurense* were collected in Yamamoto Town, Watari District, Miyagi, Japan, in July 2019. The plant material was authenticated by Dr. Shirataki (Faculty of Pharmacy and Pharmaceutical Sciences, Josai University). A voucher specimen was deposited in the Laboratory of Natural Products and Phytochemistry, Faculty of

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Preparation of Extracts Fresh leaves of *P. amurense* (4.3 kg) were extracted with MeOH (30 L) at room temperature for 79 d. The solvent was removed under reduced pressure using a rotary evaporator to yield a crude extract (415 g). A portion of the crude extract (200 g) was suspended in water, adsorbed onto Diaion HP-20 synthetic adsorbent resin (8.5 × 13 cm), and successively eluted with H₂O, 25% MeOH, 50% MeOH, 75% MeOH, MeOH, and acetone to afford the corresponding fractions. The 25% MeOH-eluted fraction (12.4 g) was subjected to Diaion HP-20 column chromatography (8.5 × 13 cm), and the 75% MeOH-eluted fraction was purified by HPLC [column: Senshu Pak PEGASIL ODS SP100 (10 × 150 mm); mobile phase: H₂O/MeOH, 3:2; flow rate: 3 mL/min; UV detection: 254 nm] to yield compound **1**.

Identification Isolated compound **1** was obtained as a yellow amorphous solid. Its molecular weight was determined to be 518 based on FAB-MS data (m/z 517 [M – H][–]), and its molecular formula was estimated as C₂₆H₃₀O₁₁ from the ¹H- and ¹³C-NMR data. Compound **1** was identified as phellamurin by comparison of its spectroscopic data with literature values.¹⁰ The purity of isolated phellamurin was determined to be 91.7 ± 1.7% by quantitative ¹H-NMR using 3-(trimethylsilyl)-1-propane-1,1,2,2,3,3-*d*₆-sulfonic acid sodium salt (DSS-*d*₆) as an internal standard. The detailed qNMR conditions are provided in the Supplementary Materials.

Animals Male Wistar rats were purchased from Sankyo Labo Service Co., Inc. (Tokyo, Japan) and housed under controlled conditions (temperature, 23°C; light cycle, 07:00–19:00) with free access to standard rat chow and water. Rats weighing approximately 200–250 g were used in the *in vivo* experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University (Approval No. JU23101) and performed in accordance with the experimental animal guidelines of Josai University.

Protective Effect on Gastric Mucosal Injury The protective effect of phellamurin against HCl/ethanol-induced gastric mucosal injury was evaluated in male Wistar rats (200–250 g). The animals were randomly divided into three groups of six rats each and fasted for 24 h, with water withheld during the final 4 h. Phellamurin was prepared in 4% Tween 80 and orally administered at doses of 0, 2.5, or 5 mg/kg. Thirty minutes later, HCl/ethanol solution (5 mL/kg; 15 mM HCl in 90% ethanol) was orally administered to induce gastric mucosal injury. One hour after administration, the animals were euthanized, and the stomachs were excised and fixed in 2% formalin in phosphate-buffered saline. The stomachs were opened along the greater curvature and scanned. The total lesion area was quantified using ImageJ software and expressed as the gastric mucosal injury area (mm²).

Primary Cell Isolation and Culture Rat gastric mucosal cells were isolated as previously described.¹¹ Briefly, the stomach was excised from an anesthetized rat, everted, filled with Hank's balanced salt solution (HBSS), and incubated in 1,300 U/mL Dispase II (Godo Shusei Co., Ltd., Tokyo, Japan) at 37°C for 1 h. Surface mucous cells were detached by gentle agitation of the everted stomach in Ca²⁺- and Mg²⁺-free HBSS containing 0.1% BSA and 0.5 mM EGTA. The cell suspension was filtered through a 150-μm nylon mesh and cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Inc., CA, USA), 100 μg/mL

gentamicin sulfate, and 1 μg/mL Fungizone in collagen-coated 24-well plates at 37°C in a humidified atmosphere containing 5% CO₂. Cells were used for experiments after 2 d of culture.

Protective Effect of Phellamurin against Oxidative Stress in Primary Cultured Cells To suppress cell proliferation, the culture medium was replaced with DMEM/F-12 supplemented with 1% FBS before the assay. Phellamurin was prepared as a two-fold serial dilution in dimethyl sulfoxide (DMSO) and added to the culture medium (final concentration: 0.0625–1 mM; final DMSO concentration: <1%). Cells were pretreated with phellamurin for 4 h and then exposed to 300 μM H₂O₂ for an additional 4 h. Cell viability was evaluated using the MTT assay (Kanto Chemical Co., Inc., Tokyo, Japan). *N*-Acetyl-L-cysteine (NAC; 5 mM; Fujifilm Wako Pure Chemical Co., Osaka, Japan) was used as a positive control.

Gene Expression Analysis of Antioxidant-Related Genes and Cyclooxygenase Genes Cultured rat gastric mucosal cells were treated with DMSO (≤1%) as the vehicle control or with phellamurin (final concentration: 0.5 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 2 h. Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and purified according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Quantitative PCR (qPCR) for superoxide dismutase (SOD), glutathione S-transferase (GST), cyclooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2) was performed using a LightCycler® 96 System (Roche, Basel, Switzerland) with the KAPA SYBR Fast qPCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The thermal cycling conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Melting curve analysis was performed to confirm amplification specificity.

Primer sequences were as follows: SOD (forward, 5'-CCACTGCAGGACCTCATTTT-3'; reverse, 5'-CACCTTTGCCCAAGTCATCT-3'), GST (forward, 5'-ATGGGAGTTTGACCAGCAAG-3'; reverse, 5'-CCATGGCTCTTCAACACCTT-3'), COX-1 (forward, 5'-ACCCAAAGACTGTCCCACAC-3'; reverse, 5'-GATGCTCCTCCTTCAGCAAG-3'), COX-2 (forward, 5'-TACCCGGACTGGATTCTACG-3'; reverse, 5'-AAGTTGTGGGCTGTCAATC-3'), and GAPDH (forward, 5'-TGCCACTCAGAAGACTGTGG-3'; reverse, 5'-TCAGGAGACAACCTGGTCCTT-3'). Relative gene expression levels were calculated using the 2^{–ΔΔCt} method with GAPDH as the internal control.

Measurement of Anti-*H. Pylori* Activity Anti-*H. pylori* activity against *H. pylori* ATCC 43504 (Biofluid, Inc., Rockville, MD, USA) was evaluated as previously described.¹² Briefly, *H. pylori* was inoculated onto selective agar plates (Shimadzu Diagnostics Co., Ltd., Tokyo, Japan) and incubated at 37°C for 5 d. The resulting colonies were collected, suspended in medium, and adjusted to 1 × 10⁷ colony-forming units (CFU)/mL. Phellamurin was dissolved in DMSO and diluted with the medium. The bacterial suspension was added to the phellamurin solutions to yield a final bacterial density of 1 × 10⁶ CFU/100 μL per well. The mixtures were incubated at 37°C for 3 d. Clarithromycin was used as a positive control.

Urease Inhibition Assay Using the Phenol Red Method

Phellamurin (final concentration: 0.015–1 mM) was prepared as a two-fold serial dilution in DMSO. A 1- μ L aliquot of each dilution was added to 100 μ L of jack bean urease solution (4 U; Sigma-Aldrich, St. Louis, MO, USA) and preincubated for 3 h at room temperature. Acetohydroxamic acid was used as a positive control. After preincubation, 25 μ L of the urease solution (1 U) was dispensed into a 96-well microplate, followed by the addition of 200 μ L of substrate solution containing 0.5 M urea and 0.002% phenol red in 50 mM phosphate buffer (pH 6.8). The reaction mixture was incubated at room temperature for 10 min, and absorbance was measured at 570 nm.¹³⁾ Unless otherwise indicated, all *in vitro* experiments were performed independently at least three times.

Statistical Analysis Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using JMP Pro 17 software. Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Figure 2a shows the effects of phellamurin on HCl/ethanol-induced gastric mucosal injury, assessed as lesion area. Phellamurin reduced the lesion area compared with the control group, reaching statistical significance at 5 mg/kg. Representative macroscopic images (Fig. 2b) were consistent with these findings, showing attenuation of mucosal injury. The HCl/ethanol-induced gastric mucosal injury model is widely used to evaluate gastroprotective agents because it induces oxidative

stress and mucosal damage in the stomach.^{14,15)} Flavonoids are known to exert gastroprotective effects through several mechanisms, including antioxidant activity.⁴⁾ To examine whether antioxidant properties contributed to the effect of phellamurin, an oxidative stress assay using H_2O_2 was performed in cultured rat gastric mucosal cells (Fig. 3). Phellamurin significantly increased cell viability at concentrations of 0.25 mM or higher, indicating a cytoprotective effect. No cytotoxicity was observed up to 0.5 mM under the present assay conditions (data not shown). These findings suggest that antioxidant activity may contribute, at least in part, to the gastroprotective effect of phellamurin.

To further investigate the underlying mechanism, the expression of antioxidant-related genes was analyzed by qPCR in cultured gastric mucosal cells (Fig. 4). No significant changes were observed in the expression levels of SOD or GST. These results suggest that the protective effect of phellamurin is unlikely to be mediated by induction of antioxidant enzyme expression and may instead be associated with its intrinsic radical-scavenging activity. Because flavonoids have also been reported to modulate prostaglandin biosynthesis,¹⁶⁾ COX-1 and COX-2 mRNA expression was examined; however, no clear changes were detected. Collectively, these findings indicate that the gastroprotective effect of phellamurin is unlikely to involve transcriptional regulation of antioxidant enzymes or the COX pathway under the present conditions.

The anti-*H. pylori* activity of phellamurin is shown in Fig. 5. Phellamurin exhibited approximately 50% inhibition at 1 mM, whereas it showed no inhibitory effect in the urease assay (data not shown). These results indicate that phellamurin does not exert strong antibacterial activity against *H. pylori* under the present conditions and that urease inhibition is unlikely to contribute to its gastroprotective effect.

Although phellamurin demonstrated gastroprotective effects in the HCl/ethanol-induced gastric mucosal injury model, several limitations should be noted. The antioxidant effect was primarily evaluated *in vitro*, and further studies are required to

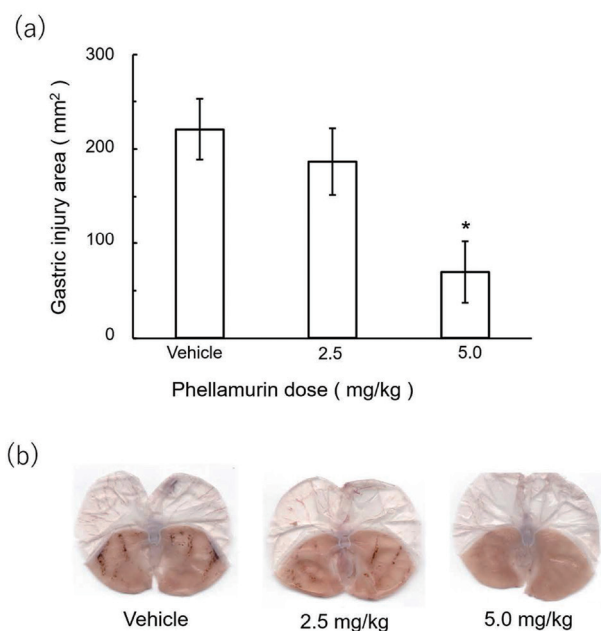


Fig. 2. Protective Effects of Phellamurin on HCl/Ethanol-Induced Gastric Mucosal Injury

(a) Gastric lesion area in rats treated with phellamurin. Phellamurin (2.5 or 5 mg/kg, p.o.) was administered 30 min prior to HCl/ethanol treatment. Data are expressed as mean $\pm$ SEM ($n = 6$). * $p < 0.05$ vs. vehicle-treated group (Dunnett's test). Vehicle: 4% Tween 80 in saline. (b) Representative macroscopic images of gastric mucosa from each group.

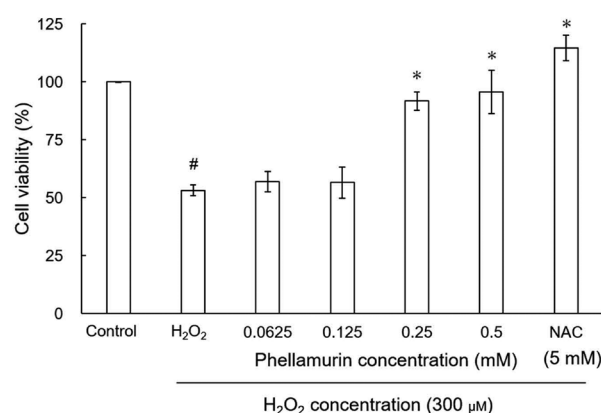


Fig. 3. Effect of Phellamurin on H_2O_2 -Induced Oxidative Stress in Cultured Rat Gastric Mucosal Cells

Cells were pretreated with phellamurin (0.0625–0.5 mM) for 4 h, followed by exposure to H_2O_2 (300 μ M) for an additional 4 h. Cell viability was assessed using the MTT assay. *N*-acetyl-L-cysteine (5 mM; NAC) was used as a positive control. Data are expressed as mean $\pm$ SEM ($n = 3$ independent experiments). # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. H_2O_2 -treated group (Dunnett's test).

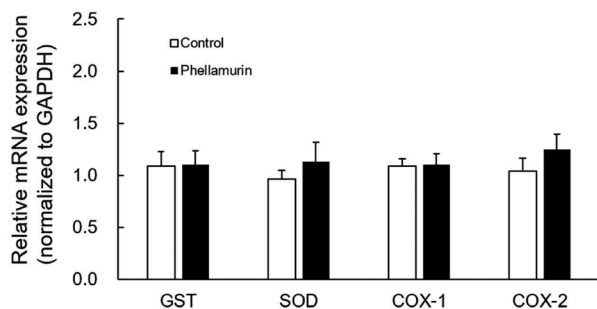


Fig. 4. Effect of Phellamurin on the Expression of Antioxidant-Related Genes and Cyclooxygenase Genes in Cultured Rat Gastric Mucosal Cells

Cells were treated with phellamurin (0.5 mM) for 2 h. mRNA expression levels of GST, SOD, COX-1, and COX-2 were analyzed by quantitative PCR. Gene expression was normalized to GAPDH and expressed relative to the control group. Data are presented as mean $\pm$ SEM ($n = 5$ independent experiments). No significant differences were observed between groups (Dunnett's test).

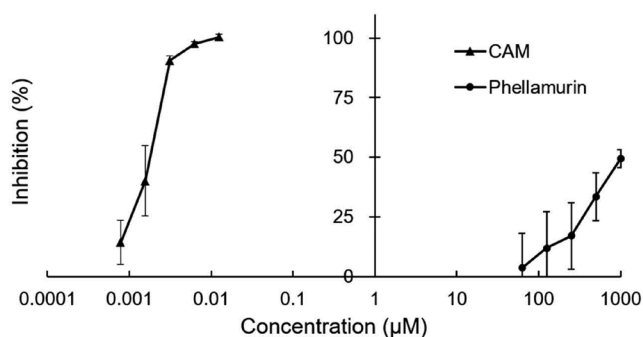


Fig. 5. Anti-*Helicobacter Pylori* Activity of Phellamurin

The inhibitory effect of phellamurin on *Helicobacter pylori* growth was evaluated after 3 days of incubation. Clarithromycin (CAM) was used as a positive control. Data are expressed as mean $\pm$ SEM ($n = 4$ independent experiments).

clarify the underlying mechanisms *in vivo*. In addition, the relatively high concentrations used in the cell-based assays may not fully reflect physiological conditions. Nevertheless, this study provides the first evidence that phellamurin exerts protective effects on the gastric mucosa and suggests its potential as a natural gastroprotective agent.

Conclusions The present study demonstrated that phellamurin exerts gastroprotective effects against HCl/ethanol-induced gastric mucosal injury in rats. *In vitro* experiments further showed that phellamurin protects cultured gastric mucosal cells against H_2O_2 -induced oxidative stress, suggesting the involvement of antioxidant activity. However, phellamurin did not significantly affect the expression of antioxidant-related genes or cyclooxygenase genes (COX-1 and COX-2), indicating that its effects are unlikely to be mediated through transcriptional regulation of these pathways. In addition, phellamurin exhibited only weak antibacterial activity against *H. pylori* and showed no urease inhibitory activity.

Collectively, these findings suggest that the gastroprotective effects of phellamurin are primarily attributable to its intrinsic

antioxidant/cytoprotective properties rather than to antibacterial mechanisms. Further studies are required to elucidate the underlying molecular mechanisms.

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Conflict of Interest Statement The authors declare no competing interests.

Data Availability Statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement Miku Goto: Investigation, Formal analysis, and Data curation. Ryuichiro Suzuki: Conceptualization, Methodology, Isolation and purification of phellamurin, Structural elucidation, Writing – original draft, Writing – review and editing, Supervision, and Project administration. Junki Tomita: qNMR measurement and Investigation. Aiko Sano: qNMR data analysis, Formal analysis, and Determination of the purity of phellamurin. Toru Kimura: Supervision and Project administration. Toru Tanaka: Conceptualization, Writing – original draft, Writing – review and editing, Supervision, and Project administration. All authors have read and approved the final version of the manuscript.

Ethics Approval All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University (Approval No. JU23101) and were conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

Informed Consent Not applicable.

Supplementary Materials This article contains Supplementary Materials. The detailed qNMR conditions used for the determination of the purity of isolated phellamurin are provided as Supplementary Materials.

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