Original Article

Inhibitory Potencies of Several Iridoids on Cyclooxygenase-1, Cyclooxygenase-2 Enzymes Activities, Tumor Necrosis factor-α and Nitric Oxide Production In Vitro

Kyoung Sik Park, Bong Hyun Kim and Il-Moo Chang

Natural Products Research Institute, College of Pharmacy, Seoul National University, 28-Yungun-dong, Jongro-ku, Seoul 110-460, Korea

To verify the anti-inflammatory potency of iridoids, seven iridoid glucosides (aucubin, catalpol, gentiopicroside, swertiamarin, geniposide, geniposidic acid and loganin) and an iridoid aglycone (genipin) were investigated with in vitro testing model systems based on inhibition of cyclooxygenase (COX)-1/-2 enzymes, the tumor necrosis factor-α (TNF-α) formation and nitric oxide (NO) production. The hydrolyzed-iridoid products (H-iridoid) with β-glucosidase treatment only showed inhibitory activities, and revealed different potencies, depending on their chemical structures. Without the β-glucosidase treatment, no single iridoid glycoside exhibited any activities. The aglycone form (genipin) also did not show inhibitory activities. To compare anti-inflammatory potency, the inhibitory concentrations (IC50) in each testing system were measured. The hydrolyzed-aucubin product (H-aucubin) with β-glucosidase treatment showed a moderate inhibition on COX-2 with IC50 of 8.83 μM, but much less inhibition (IC50, 68.9 μM) on COX-1 was noted. Of the other H-iridoid products, the H-loganin and the H-geniposide exhibited higher inhibitory effects on COX-1, revealing IC50 values of 3.55 and 5.37 μM, respectively. In the case of TNF-α assay, four H-iridoid products: H-aucubin, H-catalpol, H-geniposide and H-loganin suppressed the TNF-α formation with IC50 values of 11.2, 33.3, 58.2 and 154.6 μM, respectively. But other H-iridoid products manifested no significant activity. Additional experiments on NO production were conducted. We observed that only the H-aucubin exhibited a significant suppression with IC50 value of 14.1 μM. Genipin, an agycone form, showed no inhibitory effects on all testing models, implying the hydrolysis of the glycosidic bond of iridoid glycoside is a pre-requisite step to produce various biological activities.

Keywords: anti-inflammation – cyclooxygenase – iridoids – nitric oxide – tumor necrosis factor-α

Introduction

Iridoids represent a group of natural constituents with a monoterpene cyclic ring. They exist usually as glycosidic forms in nature, but are also found on rare occasions as an aglycone. Iridoids manifest dual facets of biological activities; one is to act as a defensive substance for certain plant species (1, 2), and the other is to produce a variety of pharmacological actions for animals. Many medicinal plants containing iridoids such as Plantago, Cornus, Rehmanniae, Scrophularia, Gentiana and Harpagophyllum have long been used to treat various ailments in the East and the West. The pharmacological activities are summarized: treatment of hepatic dysfunction (3), stimulation of bile acid excretion (4), antimicrobial activities (5), antitumor activities (6), antidotal activities for noxious Amanita mushroom poisoning (7), antiviral activities against Hepatitis B virus (8) and...
anti-inflammatory activities (9). A variety of iridoids including Scropolioside A, scrovalentinoside, verminoside and ipolamiide have been reported to possess significant anti-inflammatory activities in vitro and/or in vivo assay systems.

The present study is aimed to verify the anti-inflammatory potency of seven iridoid glucosides, and an aglycone form. Additional test samples derived from iridoid glycosides were also prepared by treating those seven iridoid glycosides with β-glucosidase: they are called the hydrolyzed-iridoid products (H-iridoid). Since our previous study (10) indicated that an iridoid glycoside, namely, aucubin exhibited various biological activities in vitro as it was treated with β-glucosidase to produce its hydrolyzed product (H-aucubin). As with the assay systems of anti-inflammatory activities, measurements of cyclooxygenases (COX-1 and COX-2) enzymes activities, tumor necrosis factor-α (TNF-α) formation and nitric oxide (NO) production were conducted in vitro. In addition, the inhibition concentration (IC₅₀) of each test sample was calculated to compare its potency.

**Methods**

**Materials**

Seven iridoid glucosides, namely, aucubin (99.5%), catalpol (>98%), loganin (>98%), gentiopicroside (>98%), swertiamarin (>98%), geniposide (99%), geniposidic acid (>98%) and one genin, genipin (>98%), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) (Fig. 1). Aspirin, piroxicam, meloxicam, rolipram, n-nitro-l-arginine methyl ester (l-NAME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), a lipopolysaccharide (LPS) (from Escherichia coli, 011: B4), arachidonic acid (AA), Griess reagent and β-glucosidase (EC 3.2.1.21) were obtained from Sigma Chemical Co. (St Louis, MO, USA). DMEM (Dulbecco’s Modified Eagle’s Medium) and RPMI1640 (Roswell Park Memorial Institute) medium and other reagents for cell cultures were obtained from Gibco BRL Life Biotechnologies (Gaithersburg, MD, USA). Enzyme-linked immunosorbent assays (ELISA) kits for the determination of PGE₂ (prostaglandin E₂) and TXB₂ (thromboxane B₂) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). An interferon (INF)-γ, antimate TNF-α monoclonal antibodies and a biotinylated secondary antibody were purchased from Pharmingen International (San Diego, CA, USA).

**MTT Cell Viability Assay**

The degree of mitochondrial respiration as an indicator of cell viability was assayed by measuring the mitochondria-dependant reduction of MTT to produce formozan crystals. Absorbance levels due to color changes were measured at 450 and 650 nm using the Automatic ELISA microplate reader (EL808, Bio-Tek Instrument Inc). Details of this experiment were reported previously (10).

**Cyclooxygenase-1(COX-1) Assay using Human Erythroleukemia (HEL) Cells**

The COX-1 assay with HEL cells (American Type Culture Collection, ATCC) was carried out using the method of Berg et al. (11). Briefly describing the culture condition: HEL 92.1.7 cells were grown in RPMI 1640 supplemented with a 10% heat-inactivated fetal bovine serum (HyClone) under 5% CO₂. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the culture medium. Then cells were harvested and re-suspended in a fresh medium at 1 × 10⁷ cells/ml. The stock solutions of test compounds dissolved in DMSO were diluted with the medium just before the tests. A portion of cell suspension (500 μl) was added to the testing sample solution (250 μl each), mixed and incubated for 30 min at 37°C. Then, AA was added to a final concentration of 30 μM, and incubated for an additional 15 min at 37°C. The reaction
was stopped by centrifugation (900 rpm) for 5 min at 4°C. To prepare the H-iridoid products, 1 mM of each iridoid glucoside was pre-incubated with the same volume of 0.5 mM of β-glucosidase (iridoid: enzyme = 1:0.5 v/v), and diluted by adding distilled water to the specified concentrations. Under this condition, almost all iridoid glycosides were easily converted by β-glucosidase into their hydrolyzed products (12). The concentration of TXB₂ in the supernatant was measured by using the ELISA method.

**COX-2 Assay using RAW 264.7 Cells**

A murine macrophage cell line, RAW 264.7 cells (ATCC), was maintained at 37°C in DMEM medium (Gibco BRL) containing 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured at a density of approximately 2 × 10⁵ cells/ml, then harvested and re-suspended in a fresh medium at a concentration of 1 × 10⁶ cells/ml. Immediately after adding hydrolyzed-iridoid samples (H-iridoids), the cells were incubated for 30 min at 37°C. Then the cells were stimulated with LPS (final concentration of 1 μg/ml) and incubated for an additional 6 h. Thereafter, similar assaying procedures were followed as done in COX-1 assay using HEL cells. However, instead of adding TXB₂, PGE₂ was added to the testing solutions, and the PGE₂ concentration in the supernatant was measured using the ELISA method.

**Assay of TNF-α Formation**

RAW 264.7 cells (2.5 × 10⁵ cells/well) were treated with LPS/IFN-γ in the presence or the absence of the testing iridoid samples for 4 h, and the assay was performed as described previously (10).

**Assay of NO Production**

Accumulated concentrations of NO₂⁻ in the medium were measured as an indicator of NO production. The assay procedure was the same as described previously (13). Briefly, RAW 264.7 cells were plated each at a concentration of 1.5 × 10⁶ cells/ml and stimulated with LPS in the presence or the absence of the testing iridoid samples for 24 h. Each separated supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethlenediamine dihydrochloride and 2% phosphoric acid) and incubated at room temperature in the dark for 10 min. The production of NaNO₂ was measured at 550 nm.

**Statistics**

Data expressed as the mean ± standard deviation were obtained from three or four separate experiments.

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**Table 1. Inhibition of TXB₂ (thromboxane B₂) formation in intact HEL cells by hydrolyzed-iridoids (H-iridoids)**

| Test sample       | 1 μM   | 10 μM  | 50 μM  | 100 μM |
|-------------------|--------|--------|--------|--------|
| H-aucubin         | 101.3± 3.0 | 97.6± 6.4 | 84.0±4.5 | 68.6±3.0* |
| H-catalpol        | 101.3± 3.0 | 91.3± 5.0 | 94.0±9.5 | 92.0±4.0  |
| H-loganin         | 91.0± 2.6 | 57.6± 5.5* | 55.0±5.2* | 46.2±6.4*  |
| H-gentiopicoside  | 101.3± 3.0 | 90.0± 3.4 | 92.0±8.5 | 88.6±3.0  |
| H-swertiaamin     | 98.0± 6.0 | 88.6± 5.1 | 89.0±7.0 | 91.6±8.1  |
| H-geniposide      | 91.6± 2.5 | 67.3± 3.0* | 54.6±9.5* | 51.6±3.2*  |
| H-geniposidic acid| 91.6± 6.5 | 92.0± 8.5 | 92.3± 5.6 | 89.0± 3.6 |
| genipin           | 88.3± 5.3 | 86.6± 4.2 | 84.0± 7.0 | 88.3± 6.1 |

Values are presented as mean±SD of the percentage of TXB₂ formation of cultures treated with vehicle (DMSO) only. *Statistically significant difference: P < 0.05.

Statistical analysis of the data was performed by one-way analysis of variances followed by Dunnett’s test. Values of P < 0.05 were considered statistically significant. Each IC₅₀ value was calculated by non-linear regression analysis (14).

**Results**

**Effects on Cell Viability**

Cell viability tests were performed throughout all experiments by using the MTT assay. In all iridoid test samples including H-iridoids at a concentration up to 100 μM, no significant cytotoxicity was observed (data not shown).

**Inhibitory Activities on COX-1 Enzyme**

To examine potential inhibitory activities of iridoid samples on COX-1, the amounts of TXB₂ reduction were measured in intact HEL cell culture (Table 1). The aspirin, a typical non-steroidal, anti-inflammatory drug, and the piroxicam, a specific COX-1 inhibitor, were employed as positive control drugs. Both control drugs showed inhibitory activities on COX-1 assay in dose-dependant manner with IC₅₀ of 7.23 and 0.31 μM, respectively (Table 2). None of iridoid glycosides without pre-treating β-glucosidase exhibited any significant inhibitory activities on COX-1 assay. Of those iridoid glycosides which were pre-treated with β-glucosidase, both H-geniposide and H-loganin produced high inhibitory activities on COX-1 assay, revealing IC₅₀ values of 5.37 and 3.55 μM, respectively. The H-aucubin showed an IC₅₀ value of 68.9 μM, indicating relatively less potency than H-geniposide and H-loganin. Other H-iridoid samples and genipin (aglycone) exhibited no significant inhibitory activities on COX-1 assay.
### Table 2. IC_{50} values of positive controls and hydrolyzed-iridoids (H-iridoids) for the inhibition of COX-1/-2 enzymes activities, the formation of TNF-α and the production of NO

|          | COX-1 (μM) | COX-2 (μM) | TNF-α (μM) | NO (μM) |
|----------|------------|------------|------------|---------|
| Aspirin  | 7.23       | 14.2       | –          | –       |
| Piroxicam| 0.31       | –          | –          | –       |
| Meloxicam| –          | 0.16       | –          | –       |
| Rolipram | –          | –          | 0.11       |         |
| L-NAME   | –          | –          | –          | 14.4    |
| H-Aucubin| 68.9       | 8.83       | 11.2       | 14.1    |
| H-Catalpol| ND         | ND         | 33.3       | ND      |
| H-Loganin| 3.55       | 131.0      | 154.6      | ND      |
| H-Geniposide| 5.37 | 32.4       | 58.2       | ND      |

ND, not determined.

### Table 3. Inhibition of PGE_2 (prostaglandin E_2) formation in LPS-stimulated RAW264.7 cells by hydrolyzed-iridoids (H-iridoids)

| Test sample   | 1 μM | 10 μM | 50 μM | 100 μM |
|---------------|------|-------|-------|--------|
| H-aucubin     | 92.3 ± 8.5 | 64.6 ± 6.4* | 41.3 ± 8.0* | 37.3 ± 4.0* |
| H-catalpol    | 88.0 ± 4.0 | 87.0 ± 7.2 | 76.0 ± 8.8 | 75.3 ± 4.9* |
| H-loganin     | 93.3 ± 6.1 | 88.0 ± 8.5 | 75.6 ± 9.2* | 55.3 ± 6.5* |
| H-gentiopicroside | 93.0 ± 6.5 | 95.6 ± 6.4 | 84.6 ± 4.7 | 86.0 ± 7.9 |
| H-swertianarin| 96.3 ± 9.2 | 89.0 ± 5.2* | 84.6 ± 14.5 | 86.0 ± 7.9 |
| H-geniposide  | 95.3 ± 5.0 | 89.3 ± 6.6 | 71.3 ± 9.0* | 64.0 ± 7.2* |
| H-geniposidic acid | 94.3 ± 6.6 | 93.6 ± 2.3 | 86.0 ± 7.9 | 78.0 ± 8.7 |
| genipin       | 92.3 ± 6.8 | 90.0 ± 6.2 | 84.6 ± 7.3 | 91.3 ± 6.7 |

Values are presented as mean±SD of the percentage of PGE_2 formation of cultures treated with vehicle (DMSO) only.
*Statistically significant difference: P < 0.05.

### Table 4. Inhibition of TNF-α (tumor necrosis factor) production in LPS-stimulated RAW264.7 cells by hydrolyzed-iridoids (H-iridoids)

| Test sample   | 1 μM | 10 μM | 50 μM | 100 μM |
|---------------|------|-------|-------|--------|
| H-aucubin     | 85.6 ± 11.0 | 63.6 ± 5.5* | 44.6 ± 10.5* | 24.0 ± 7.9* |
| H-catalpol    | 95.6 ± 4.0  | 92.3 ± 8.0 | 69.3 ± 6.5* | 67.3 ± 8.9* |
| H-loganin     | 96.6 ± 4.1  | 84.6 ± 5.1* | 71.0 ± 6.5* | 45.3 ± 3.5* |
| H-gentiopicroside | 89.6 ± 4.0 | 89.0 ± 6.0 | 82.6 ± 6.6 | 86.0 ± 6.5 |
| H-swertianarin| 89.6 ± 4.0  | 93.6 ± 2.0 | 86.3 ± 4.7 | 89.3 ± 4.0 |
| H-geniposide  | 93.3 ± 4.1  | 88.0 ± 8.5 | 71.0 ± 3.6* | 55.6 ± 4.0* |
| H-geniposidic acid | 100.0 ± 2.0 | 94.6 ± 12.5 | 84.0 ± 15.5 | 82.3 ± 7.3* |
| genipin       | 102.3 ± 8.8 | 97.6 ± 7.3 | 94.0 ± 9.3 | 96.6 ± 8.1 |

Values are presented as mean±SD of the percentage of TNF-α production of cultures treated with vehicle (DMSO) only.
*Statistically significant difference: P < 0.05.

### Table 5. Inhibition of NO (nitric oxide) production in LPS-stimulated RAW264.7 cells by hydrolyzed-iridoids (H-iridoids)

| Test sample   | 1 μM | 10 μM | 50 μM | 100 μM |
|---------------|------|-------|-------|--------|
| H-aucubin     | 96.3 ± 7.4  | 71.3 ± 9.0* | 49.0 ± 9.8* | 40.0 ± 3.6* |
| H-catalpol    | 96.0 ± 4.0  | 87.0 ± 7.2 | 86.0 ± 8.8 | 85.3 ± 5.9 |
| H-loganin     | 93.3 ± 6.1  | 98.0 ± 8.5 | 95.6 ± 9.2 | 105.3 ± 6.5 |
| H-gentiopicroside | 103.3 ± 6.5 | 95.6 ± 6.4 | 94.6 ± 4.7 | 96.0 ± 7.9 |
| H-swertianarin| 96.3 ± 9.2  | 89.0 ± 5.1 | 84.6 ± 14.5 | 84.2 ± 7.9 |
| H-geniposide  | 95.3 ± 5.0  | 89.3 ± 6.6 | 101.3 ± 9.0 | 104.6 ± 7.2 |
| H-geniposidic acid | 94.3 ± 6.6 | 93.6 ± 2.3 | 86.0 ± 7.9 | 98.3 ± 8.7 |
| genipin       | 104.6 ± 8.8 | 105.2 ± 10.0 | 111.3 ± 11.0 | 106.3 ± 13.4 |

Values are presented as mean±SD of the percentage of NO production of cultures treated with vehicle (DMSO) only.
*Statistically significant difference: P < 0.05.

### Inhibitory Activities on COX-2 Enzyme

Experiments using LPS-stimulated RAW 264.7 cells were conducted to examine inhibitory activities on COX-2 as described in Methods; the formation of the PGE_2 was measured and data were shown in Table 3. Both aspirin and meloxicam were used as positive control drugs for COX-2 inhibitors. Both control drugs exhibited inhibitory activities in dose-dependent manner with IC_{50} of 14.2 and 0.16 μM, respectively (Table 2). Contrary to the data obtained from COX-1 assay, we observed that H-aucubin exhibited a higher inhibition on COX-2 assay (IC_{50} value of 8.83 μM); whereas H-geniposide and H-loganin, produced much less inhibition with IC_{50} values of 32.4 and 131.0 μM, respectively.

### Suppression of TNF-α Formation

The assay was performed by using an ELISA method in RAW 264.7 cells that were stimulated with LPS/IFN-γ as described in Methods. The rolipram, a positive control drug, suppressed the TNF-α formation by 86% even at a concentration of 1 μg/ml. Unlike those results obtained from COX-1/-2 experiments, suppression of TNF-α formation was apparently more sensitive to a variety of testing iridoids as noted as four H-iridoid samples, H-aucubin, H-catalpol, H-geniposide and H-loganin, showed suppressive activities with IC_{50} values of 11.2, 33.3, 58.2 and 154.6 μM, respectively (Tables 2 and 4). Other H-iridoids and genipin did not have much influence on the suppression of TNF-α formation.

### Suppression of NO Production

To examine suppressive activities of H-iridoids on NO production, the LPS-stimulated RAW 264.7 cells were used (Table 5). The L-NAME, a well-known inhibitor, was used as a positive control. Treatments of L-NAME suppressed significantly the NO production in dose-dependent manners with an IC_{50} value of 14.4 μM (Table 2). Of H-iridoid samples tested so far, only H-aucubin exhibited a significant suppression with an
IC$_{50}$ value of 14.1 $\mu$M, indicating almost the same potency as that of the L-NAME treatment. In the view that the chronic suppression of NO may lead to heart disease, it is worth investigating the effect of H-aucubin on the cardiovascular system.

**Discussion**

Iridoids, naturally occurring substances, are found in many medicinal herbs including traditional Chinese herbs. They exist mostly as glycosides and some are found in genin forms without glycoside moiety in their structure. Those medicinal plants containing iridoids show a variety of biological activity as aforementioned. Of pharmaceutical products as well as dietary supplements containing such medicinal plant materials, agents (e.g. devil’s claw products made of Harpagophyllum) for treating inflammatory ailments including rheumatic inflammation have been marketed and used for long time. To verify the molecular mechanism of anti-inflammatory activities of iridoid analogs, we carried out the present study.

Based on the results obtained so far, we confirmed our previous findings in which iridoid glycoside itself is in inactive forms, and it should be converted to its active form by enzymatic hydrolysis of glycosidic bond, leading to its H-iridoid products (1, 10). Although no definite structure of H-iridoid products has been determined yet, it is presumed to produce a cleavage of monoterpenic rings, resulting in the active form of the drug (2, 12). To support such a postulation, we challenged the genipin (aglycone) without $\beta$-glucosidase treatment into all assay models in vitro; the animal cells (HEL and RAW 264.7) do not have $\beta$-glucosidase and aglycone form of genipin is more easily transported into the cell membrane than iridoid glycosides are. As anticipated, no inhibitory activities were noted in all three assay models. These results imply a similar bio-process occurs in whole animal systems for pharmacological actions of iridoid-containing herbs. In this connection, it is also mentioned that some iridoids such as geniposide and gardenoside are converted to nitrogen-containing metabolites (genipinine and gardenine) by intestinal bacterial flora (15). However, it is uncertain what kinds of biological action can occur with these genins.

Taking into consideration the structural differences versus potency of anti-inflammatory activities, only a minute structural difference renders a marked different potency to each iridoid. H-geniposide and H-loganin exhibited high inhibitory potencies on COX-1; whereas H-aucubin showed very low inhibition on COX-1. However, H-aucubin produced a moderate inhibition on COX-2. In addition, H-aucubin only exhibited suppressive activities on both NO production and TNF-$\alpha$ formation. But H-catalpol, which is a very similar structure with H-aucubin, revealed no significant inhibition on COX-1/2 and NO production. In this regard, further studies are needed with respect to the structural determination of H-iridoid products. A single medicinal herb usually contains a mixture of different analogues of iridoids and different contents of iridoids, depending on the plant’s parts. Such differences may render different pharmacological activities to each medicinal herb containing iridoids. Nonetheless, the present study provides important information for selecting more effective anti-inflammatory herbs among the many medicinal plants containing iridoids.

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