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Anti-influenza A activity of C-geranyl flavonoids isolated from *Paulownia tomentosa* and *Maclura pomifera*

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C-Geranyl flavonoids have received considerable attention as a simple flavanone with antioxidant, anti-carcinogenic, and anti-inflammatory effects. Influenza virus infection causes thousands of deaths and millions of hospitalizations worldwide every year and the emergence of resistance to anti-influenza drugs has prompted scientists to seek for new natural antiviral materials. In this study, we screened six natural flavonoids from different sources to identify the most potent antiviral flavonoid A/PR/8/34 (H1N1) against human influenza. The two methoxy group flavonoids including 3′-O-methyl-5′-O-methyldiplacone showed potent anti-influenza activity. Therefore, 3′-O-methyl-5′-O-methyldiplacone may be of value as a virus-protector to healthy tissue surrounding influenza A during chemotherapy to obtain better antiviral control with a significant dose.

Key words: C-Geranyl flavonoids, anti-influenza, influenza A, virus-protector.

INTRODUCTION

Influenza viruses affiliated to the *Orthomyxoviridae* family have caused significant morbidity and mortality in humans through epidemic or pandemic diseases [1]. Influenza virus infection remains an important health problem, particularly for babies, young and elderly people, and imposes significant social-economic costs [2]. The main strategies for dealing with influenza involve vaccination and antiviral drugs. Even though annual vaccination is the core preventive strategy for influenza infections, natural antiviral drugs development is necessary to provide additional preventive and therapeutic benefits [3]. *Paulownia tomentosa* (Thunb.) Siebold&Zucc. ex Steud. belongs to the family *Scrophulariaceae* in deciduous trees distributed throughout Korea, China, and Japan. Previous publications have reported polyphenolic compounds, such as iridoids, phenolic glycosides, flavonoids, and phenylethanoids in the MeOH and EtOH extracts of *P. tomentosa*. Iridoids, lignans and flavonoids have been reported as bioactive compounds of *P. tomentosa* [4,5]. Among them, geranylated flavonoids are known as the main bioactive constituents. In fact, most of the substances that have been isolated so far are polar, usually glycosides [6]. Diplacone (6-C-geranyl-eriodictyol), is a prenylated flavonoid, a simple flavanone, among four geranyl falvonoids that have been known for inhibitory effects on inflammations and/or cancer. Furthermore, we reported that the hydroxylation patterns of the flavonoid B ring play a critical role in the cellular function of the C-geranyl flavonoids group [7]. *Maclura pomifera* commonly called osage orange in deciduous trees including *cudrania* and other genera of *Moraceae* and flavonoids has been reported as a bioactive compound of *Maclura pomifera*. Among them, prenylated isoflavones are known as the main bioactive constituents. Previous publications have reported polyphenolic compounds, such as osajin and pomiferin in the MeOH and EtOH extracts of *Maclura pomifera* [8]. In this study, we tested the efficacy of the flavonoid B ring of C-geranyl flavonoids against the influenza A virus.

EXPERIMENTS AND EQUIPMENT

Preparation of the C-geranyl flavonoids

Diplacone analogues (PT1~PT4) were kindly provided by Professor Karel Šmejkal of the University of Veterinary and Pharmaceutical Sciences Brno as standards for isolation from *Paulownia tomentosa* [6]. Osajin (PT5) and pomiferin (PT6) were recrystallized from the methanolic solution after preconcentration under vacuum from *Maclura pomifera* [9]. PT1~PT6 were diplacone, 3′-O-methyl-5′-hydroxydiplacone, 3′-O-methyl-5′-O-methyldiplacone, 3′-O-methyl diplacol, osajin and pomiferin. A 50 mM stock solution of PT1~PT6 was made in absolute ethanol, and working dilutions were prepared directly with

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dimethyl sulfoxide (DMSO; Sigma-Aldrich). Tamiflu (oseltamivir) was kindly provided by the Immunogenetics Laboratory, Department of Animal Biotechnology, Konkuk University. The control vehicle was culture media containing amounts of DMSO equivalent to those present in PT1~PT6.

Virus, cells, and reagents

Influenza virus A/Puerto Rico/8/34 (A/PR/8/34) was kindly provided by the Immunogenetics Laboratory, Department of Animal Biotechnology, Konkuk University. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC CCL-3) and maintained in minimum essential media (MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and 100 U/ml penicillin/streptomycin (Gibco). Before virus infection, MDCK cells were washed with PBS and cultured in virus growth medium (MEM without FBS) supplemented with 10% bovine serum albumin (Sigma-Aldrich, USA), 100 U/ml penicillin/ streptomycin, and 2 μg/ml trypsin TPCK (Gibco).

Cell viability and antiviral assays

MDCK cells were seeded in a 96-well plate at 2 ×104 cells/well for the determination of cell viability. When the cells reached confluency 24 h after seeding, they were washed twice with PBS and treated with the indicated concentrations of flavonoid. After incubation at 37°C for 48 h in a 5% CO_2 incubator, cell viability was determined using an MTT assay kit (Sigma-Aldrich) and an xMarkTM spectrophotometer (Bio-Rad, USA) to measure absorbance at 490 nm. For the antiviral assay, cells were infected with A/PR/8/34 virus at 100 TCID50 when they reached 80–90% confluence. Two hours post-infection, the virus-containing medium was replaced with virus-free growth medium containing flavonoids. Cell viability was determined 48 h post-infection by a MTT assay using a spectrophotometer to measure absorbance at 490 nm.

Statistical analysis

The results were expressed as the mean ± SE. Each value represents the mean of at least three independent experiments in each group. The statistical significance of the difference between two cell populations was determined using the two-tailed Student's t-test (Origin software; OriginLab). P values equal to or less than 0.05 and 0.01 were considered significant. A difference was considered to be significant at $P<0.05$.

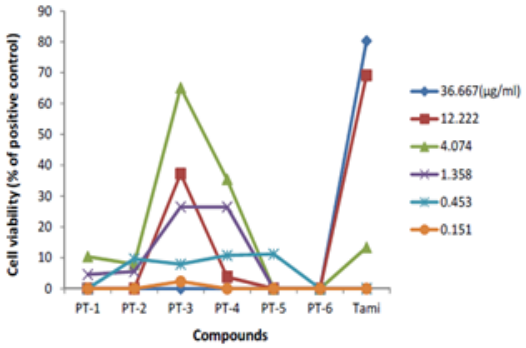

Fig. 1. Screening for antiviral flavonoids with antiviral assays. Structure of the two methoxy group flavonoids showing the positions of the free hydroxyl groups located on the B ring on the PT-3.MDCK cells infected with A/PR/8/34 virus at 100TCID50. Two hours post-infection, the medium was replaced with virus-free growth medium containing C-geranyl flavonoids. The MTT assay was performed 48 h after infection (*P<0.05).

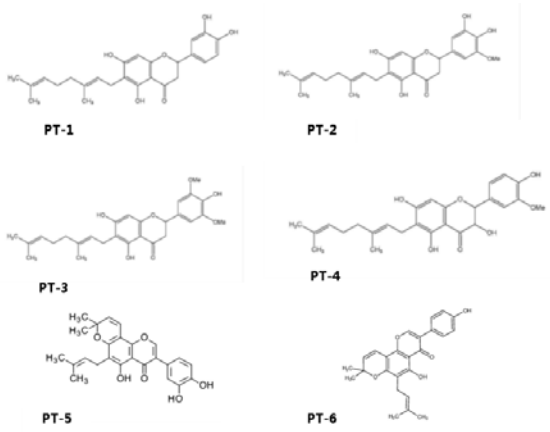

Fig.2. Structures of the PT 1~6

RESULTS AND DISCUSSION

Antiviral effect of the C-geranyl flavonoids. To screen for antiviral flavonoids, we first assessed cell viability after treating cells with the six flavonoids listed in Fig.1. The C-geranyl flavonoids (diplacone, 3′-O-methyl-5′-hydroxydiplacone,3′-O-methyl-5′-O-methyldiplacone, 3′-O-methyldiplacol, osajin and pomiferin) and Tamiflu increased the viability of MDCK cells (Fig. 1). We then tested the antiviral effect of the six flavonoids, which have free hydroxyl groups positioned at different locations on the B ring (Fig. 2). The 3′-O-methyl-5′-O-methyldiplacone (PT-3) exhibited a more potent antiviral effect than the other C-geranyl group flavonoids (Fig. 1). The antiviral effect of the two methoxy group flavonoids was quantified using the selectivity index (SI), which was calculated using the 50% cytotoxic concentration (CC50) (Fig

1). The results indicate that the two methoxy group 3′-O-methyl-5′-O-methyldiplacone, PT-3 may be an effective natural antiviral compound.

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АНТИ-ГРИПНА А-АКТИВНОСТ НА С-ГЕРАНИЛОВИ ФЛАВОНОИДИ, ИЗОЛИРАНИ ОТ *Paulownia tomentosa* И *Maclura pomifera*

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(Резюме)

С-геранил-флавоноидите са заслужили значително внимание като прости флаванони с антиоксидантна, анти-карциногенна и противовъзпалително действие. Грипните вирусни инфекции причиняват всяка година хиляди смъртни случаи и милиони хоспитализации по света. Необходимостта от противо-грипни лекарства е накарала учените на търсят нови природни противо-вирусни вещества. В тази работа ние подбрахме шест естествени флавоноиди от различни източници и идентифицирахме най-мощния антивирусен флавоноид A/PR/8/34 (H1N1) срещу грипа при хора. Два флавоноида с метокси-групи в молекулите си, включително 3′-О-метил-5′-О-метилдиплакон показва висока анти-грипна активност. Затова 3′-О-метил-5′-О-метилдиплаконътможе да бъде полезен като вирус-протектор на здравите тъкани срещу вирусите от грип А по време на хемотерапията за по-добър противо-вирусен контрол при значителна доза.