International Journal of Advanced Culture Technology Vol.7 No.3 120-125 (2019)
DOI 10.17703/IJACT.2019.7.3.120

IJACT 18-9-17

Biological Activities on Phenolic Compounds of Japanese anise (*Illicium anisatum* L) Extracts

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Abstract

In this paper, we have isolated six phenolic compounds, such as (+)-catechin ***(1)****, taxifolin* ***(2)****, taxifolin-3-O-β-D-(+)-xylose* ***(3)****, quercetin* ***(4)****, quercetin-3-O-α-L(+)-rhamnose (quercitrin)* ***(5)****, apigenin-8-C-rhamnosyl-(1‴→ 2")-glucoside (2"-O- rhamnosylvitexin)* ***(6)*** *from the EtOAc(Ethyl Acetate) and H_2O soluble fractions of Japanese anise(Illicium anisatum L) leaves and twigs. Also, we have evaluated antioxidative and antiviral activity for each isolated compound. The antioxidative test was DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity. According to the experimental results, all of the isolated compounds indicated the increased radical scavenging activities as the concentration increases and most of the isolated compounds indicated generally good antioxidative values compare to the controls, ascorbic acid and α-tocopherol. In the antiviral activities, all of the isolated compounds had no potentials in rhinovirus 1B* (HRV 1B)*. But in enterovirus 71* (EV 71) *and Influenza virus A/PR/8 (Influenza PR8), only quercetin* ***(4)*** *indicated the good antiviral activity compare to the control.*

Based on the above results, we found that the phenolic compounds of Japanese anise may be applied for one of the natural biomass sources that can be used as an antioxidant and an antiviral substance.

Keywords: *Japanese anise (Illicium anisatum L.), Phenolic compound, Biological activity, Antioxidative activity, Antiviral activity*

1. Introduction

Free radicals and reactive oxygen species are known to be harmful substances that damage lipids, proteins, nucleic acids and cause oxidative stress and cause various adult diseases and aging (Aviram [1], Novo and Parola [2]). Generally, antioxidants that regulate these reactive oxygen species are classified into natural and synthetic sulfuric acid. Typical synthetic sulfation agents are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and Troxol-C, which are often used in pharmaceuticals and food applications. However, due to concerns about the safety of synthetic antioxidants, studies on new natural antioxidants that can replace synthetic antioxidants in plants and trees have been carried out steadily (Kim *et al*. [3], Lim *et al*. [4], Williams *et al*. [5]). Recently, the emergence of new viruses such as SARS and AI have seriously threatened the lives of humans and livestock, and the social interest in the prevention of various viral diseases and new therapeutic agents is increasing. Most of the antiviral agents, such as IDU (iododeoxyuridine), ACV (acyclovir), and IFN (interferon), have been reported to exhibit various side effects such as cytotoxicity and neuropsychiatric

Manuscript received: June 22, 2019 / revised: July 10, 2019 / Accepted: July 24, 2019
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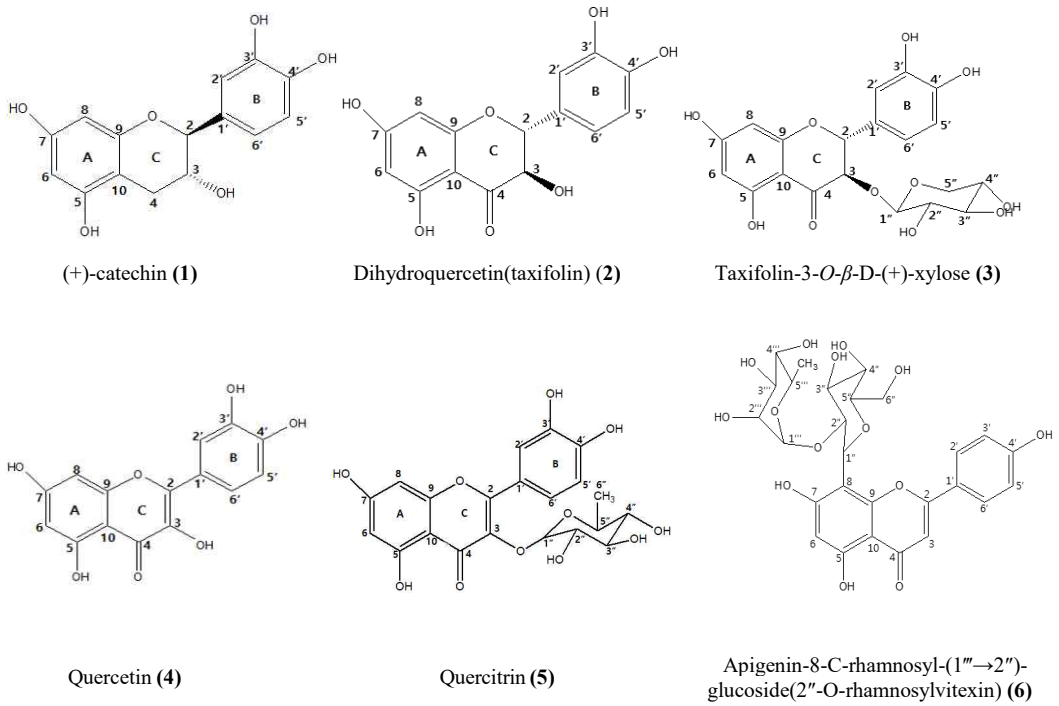
symptoms (Ong and Hayden [6], Tisdale [7]). So, there is a continuing need for the development of new antiviral agents with fewer side effects and safety.

From this point of view, research on herbicides using natural products with relatively low side effects and relatively high safety has been extensively carried out in various fields. In particular, researches on natural drug products using extractives of wood have been actively carried out. Japanese anise (*Illicium anisatum* L) is an evergreen species, which belongs to the genus such as the Chinese octagons (*Illicium verum Hook*.f.), which was used as a raw material for the Tamiflu, a new type of influenza (H1N1) treatment. It is distributed in Korea, China and Japan. In Korea, it is a species that lives in southern part and Jeju Island. And it is known that the leaves are narrow, long oval, shiny, 1.5 to 3.5 cm wide, and 1 to 1.5 cm long. It is also reported that the bark of the Japanese anise is used as a blood coagulant, and leaves and twigs are used as medicines and fragrances. But it is reported that fruit is toxic and should be cautious (Yamada [8]). However, unlike the Japanese anise, which is known to be toxic to nervous system, Chinese octagons (Star anise) are not toxic and fruit is used for spices and food. And it is known to have antifungal, antibacterial and antioxidant activity (Kim and Oh [9], Kim and Kim [10], Kim and Kang [11])**.** Therefore, it is considered that the Japanese anise that belong to the same genus may have various physiological activities useful for human body including antioxidant activity. However, there have been very few studies on the extracts of the Japanese anise.

Recently, some papers have reported several phenolic compounds such as taxifolin, quercetin, and quercitrin isolated from the Japanese anise leaves and twigs (Min and Bae [12], Shinn *et al*. [13]). In this study, antioxidation and antiviral tests were conducted on the phenolic compounds isolated from the previous studies, and the activities of the compounds were evaluated to obtain basic data for utilization as a natural biomass resource.

2. Materials and methods

2.1 Preparation of test materials



(+)-catechin **(1)** Dihydroquercetin(taxifolin) **(2)** Taxifolin-3-*O*-*β*-D-(+)-xylose **(3)**

Quercetin **(4)** Quercitrin **(5)** Apigenin-8-C-rhamnosyl-(1‴→2″)-glucoside(2″-O-rhamnosylvitexin) **(6)**

Figure 1. Structures of the isolated compounds

Table 1. IC_{50} values of antioxidative activities of the compounds

	Fraction	IC_{50}($\mu g/m\ell$)
compound	(+)-catechin	4
	Taxifolin	8
	Taxifolin-3-O-β-D-(+)-xylose	11
	Quercetin	4
	Quercitrin	16
	2″-O-rhamnosylvitexin	> 100
Control	Ascorbic acid	2.3
	α-tocopherol	12

Japanese anise (*Illicium anisatum* L.) leaves and twigs were collected and dried at room temperature for two weeks. Then it was extracted three times with 50 % aqueous acetone after grinding. The extracts were concentrated and sequentially fractionated with *n*-hexane, chloroform, EtOAc(ethyl-acetate) and H_2O(water) to get freeze dried powder. A portion of EtOAc and H_2O soluble fraction from the leaves and twigs were chromatographed on a Sephadex LH-20 column with various eluting solvents such as aqueous MeOH(methanol), 100 % MeOH and H_2O mixture. As a result, all six compounds were isolated (Fig.1). From the EtOAc soluble fraction of the leaves, two compounds were isolated: (+)-catechin **(1)**, taxifolin **(2)**, and then three compounds from H_2O soluble fraction: quercetin **(4)**, quercitrin **(5)**, 2″-O- rhamnosylvitexin **(6)**. From the EtOAc soluble fraction of the twigs, five compounds were isolated : (+)-catechin **(1)**, taxifolin **(2)**, taxifolin-3-*O*-*β*-D-(+)-xylose **(3)**, quercetin **(4)**, quercitrin **(5)** (Min and Bae [12], Shinn *et al*. [13]).

2.2 Antioxidation activity test (DPPH radical scavenging ability)

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method was used for the antioxidation activity test. The DPPH radical scavenging method was carried out by modifying the method of Blois [14] by using DPPH as a method for assaying the free radical scavenging activity. For the test, 100 $\mu\ell$ of diluted sample was added into 96 well microplate and then, 100 $\mu\ell$ of 0.2 mM DPPH solution was added. After reacting at room temperature for 30 minutes, the absorbance was measured with a microplate reader at 517nm. The control group was prepared by adding ethanol instead of the sample, and measured by the same method. In order to calibrate the color of the sample, ethanol was added instead of 0.2 mM DPPH solution and the inhibition rate was calculated by the equation (1).

$$\text{the inhibition rate}(\%) = (1- \frac{\text{Absorbance of the sample}}{\text{Absorbance of control group without addition}} \times 100 \quad (1)$$

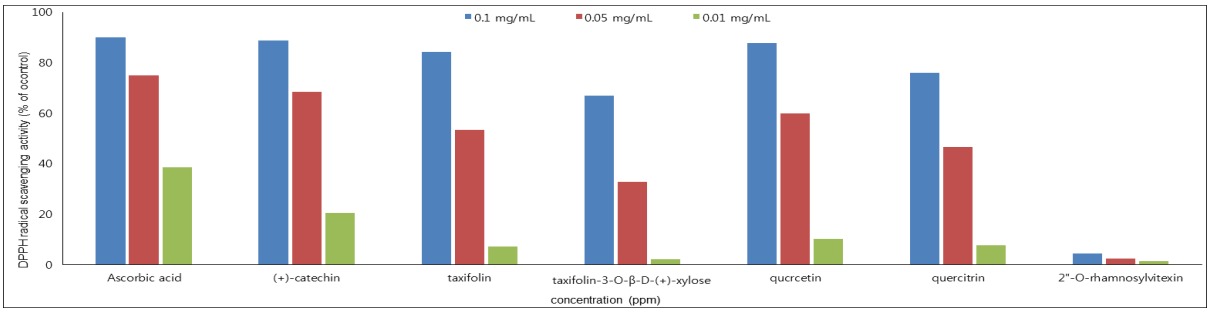


Figure 2. DPPH radical scavenging activities on the isolated compounds of *Illicium anisatum* Extracts

Also, the antioxidant effect of each sample was compared with the value of IC_{50}, which means the concentration of the sample required to reduce the absorbance of the control group without the sample to 50%.

2.3 Antiviral activity test

Antiviral activity and cytotoxicity were measured by SRB (sulforhodamine) assay using cytopathic effect (CPE) induced by viral infection. SRB assay is a method for measuring cell viability using sulforhodamine, a protein staining reagent, and is currently used for screening anticancer drugs. For the antiviral activity test, three viruses were purchased from ATCC(American Type Culture Collection): Influenza PR8 (Influenza virus A/PR/8), HRV 1B (Rhinovirus 1B) and EV 71 (Enterovirus 71).

First, 2 X 10^4 cells were prepared in each well of a 96-well culture plate and used for experiments when they grew to 90%. Twenty-four hours later, 90 ㎕ of each virus diluted to a concentration of 1% FBS and TCID 50 (tissue culture infection dose 50) was added, and 10 ㎕ of the antiviral agent at an appropriate concentration was added. Each antiviral agent was determined in four concentration ranges of 0.4, 2, 10, and 50 ㎍/㎖. Three wells were treated with the virus alone and not with the antiviral agent. And the other three wells were used as the cell control without the virus and antiviral agent. After incubation for 2 days at 37 ℃ and 5% CO_2 in a CO_2 incubator, the absorbance of the cells was measured. Absorbance was measured at 562

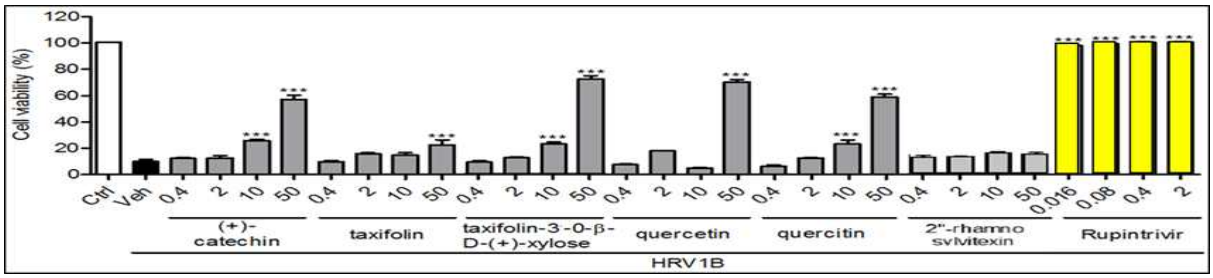


Figure 3. The effect of the compounds on Rhinovirus 1B

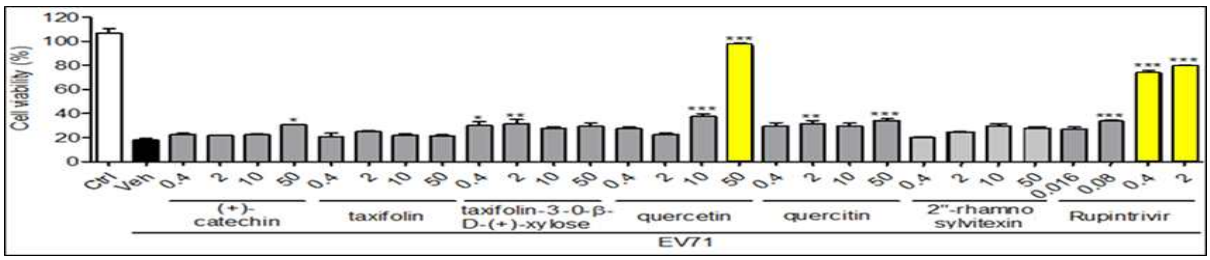


Figure 4. The effect of the compounds on Enterovirus 71

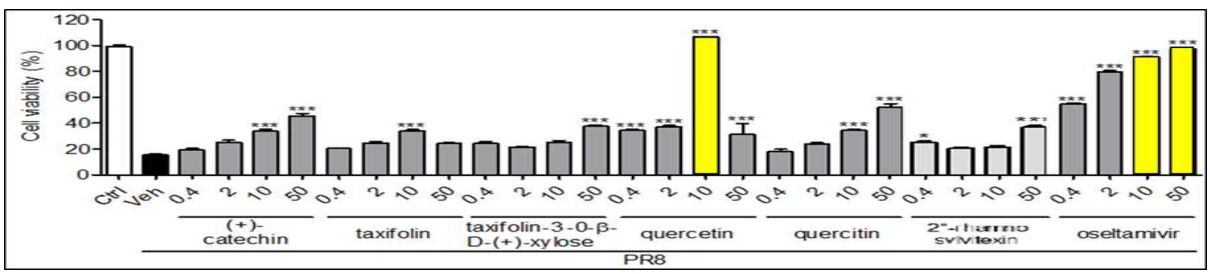


Figure 5. The effect of the compounds on Influenza A virus

nm using a Spectra Max i3 microplate reader (Molecular Devices, Palo Alto, Calif., USA) with a reference absorbance at 650 nm and cell viability was calculated for comparison based on the measured optical density.

3. Results and Discussion

3.1 Antioxidation activity test

The antioxidant activity of each of the compounds isolated from Japanese anise (*Illicium anisatum* L) was determined using the DPPH radical scavenging method. As shown in Table 1, the IC_{50} values of the compounds **1** and **4** were 4 ㎍/㎖, which is the lowest IC_{50} value among the six compounds, indicating the highest antioxidant activity. Compounds **2** and **3** showed lower antioxidation activity than ascorbic acid, but higher activity than α-tocopherol. On the other hand, in the case of compound **5**, the IC_{50} value was 16 ㎍/㎖, which was lower than that of the reference substance, and compound **6** had a very low antioxidative activity as the IC_{50} value is greater than 100 ㎍/㎖.

Also, Fig. 2 indicates that the antioxidation activity of each isolate was shown to be concentration dependent, and the radical scavenging activity tended to increase with increasing concentration. In particular, the compounds **1**, **2** and **4** exhibited antioxidative activity corresponding to ascorbic acid as the reference substance at concentrations of 0.1 and 0.05 mg/mL, and the compounds **3** and **5**, which are glycoside compounds, also had antioxidative effects. However, compound **6** showed low antioxidation activity at all concentrations.

3.2 Antiviral activity test

The antiviral activity measurement for the separated compounds was performed according to the SRB assay. Three viruses were used: Influenza PR8 (Influenza virus A/PR/8), HRV 1B (Rhinovirus 1B) and EV 71 (Enterovirus 71). And the concentrations of each compound were adjusted to 0.4, 2, 10 and 50 ㎍/㎖, respectively. According to the experimental results, in the case of HRV 1B, the cell viability was lower than 70% in all six compounds, and was insignificant activity compared to the reference substance, rupintrivir as shown in Fig. 3. On the other hand, in the case of EV 71, the cell viability of compound **4** was close to 100% at a concentration of 50 ㎍/㎖, which was higher than that of the reference substance, Rupintrivir as shown in Fig. 4. Finally, as shown in Fig. 5, in the case of Influenza PR8, compound **4** also exhibited 100% activity at a concentration of 10 ㎍/㎖, showing higher activity than the reference substance, oseltamivir. Compound **4**, it has been reported that antiviral activity is excellent in various previous researches, and the same result can be confirmed in this study (Silva *et al*. [15], Wu *et al*. [16]).

4. Conclusions

In this study, we conducted antioxidation and antiviral activity tests on the phenolic compounds isolated from EtOAc soluble fractions and H_2O soluble fractions of Japanese anise leaves and twigs. According to the antioxidant activity test results, the antioxidant activity was found to be concentration dependent in all compounds, and the radical scavenging ability tended to increase with increasing concentration. Compounds **1**, **2** and **4** exhibited antioxidative activities corresponding to ascorbic acid as the standard substance at concentrations of 0.1 and 0.05 mg/mL. And compounds **3** and **5**, which are glycoside compounds, also have antioxidative effects. But compound **6** showed low antioxidant activity at all concentrations. Also, we evaluated the antiviral activity for the six isolated compounds. According to the results, we found that most of the compounds had less activity than the reference material in the three viruses used in the experiment. However, in the case of virus EV 71 and Influenza PR8, compound **4** was the only effective. In the case of compound **4**, it has been reported that antiviral activity is excellent in various previous researches, and the same result can be confirmed in this study.

As a result, we confirmed that the phenolic compounds isolated from Japanese anise leaves and twigs have potential for natural biomass resources which can be used as natural antioxidants and antiviral agents. Especially, it could be applied as a natural antioxidant that can replace synthetic sulfation agent rather than antiviral agent.

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