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Original Research Paper

Taxifolin Potently Diminishes Levels of Reactive Oxygen Species in Living Cells Possibly by Scavenging Peroxyl Radicals

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Abstract: Taxifolin, a naturally occurring dihydroflavonol, has been reported to possess antioxidant activity, whereas there is little information regarding its antioxidant potency as a flavonoid. In the present study, the antioxidant efficacy of taxifolin in living cells was compared to that of catechin, a potent radical scavenger, by using flow cytometry with the redox-sensitive fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate. Further, diminishing efficacy of taxifolin on peroxyl radicals was assessed by using an electron spin resonance spin-trapping technique. Taxifolin significantly reduced *tert*-butyl hydroperoxide-induced increases in Reactive Oxygen Species (ROS) levels in Caco-2 cells; the effect of taxifolin was stronger than that of catechin. Taxifolin also scavenged peroxyl (ROO·) radicals with statistically significance compared to that of catechin. This study showed that taxifolin has the potential to diminish ROS levels in living cells, possibly by scavenging ROO·.

Keywords: Taxifolin, Catechin, Peroxyl Radical, Antioxidant, Oxidative Stress

Introduction

Excessive amounts of Reactive Oxygen Species (ROS) contribute to the aging process and the development of various diseases such as atherosclerosis, diabetes, cancer, neurodegenerative diseases and liver cirrhosis (Basaga, 1990). To prevent the damages caused by ROS, organisms have developed an antioxidant defense system that includes non-enzymatic antioxidants and enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Sorg, 2004). A second level of prevention against ROS-induced damage is constituted by other scavenging compounds present in the diet, especially from medicinal plants (Valko *et al.*, 2007; Xia *et al*., 2010; Jorge *et al*., 2016).

From the perspective of free radical biology, plants encounter serious oxidative stress from strong UV-Vis light, atmospheric ROS, temperature changes and the processes of oxygen consumption for photosynthesis. Flavonoids are a main class of phenolic compounds and secondary plant metabolites generally located in leaves as water soluble glycosides in the vacuoles of plant cells (Bimpilas *et al.*, 2015). Flavonoids are not only present in plants as constitutive agents but they also accumulate in plant tissues in response to microbial attack (Harborne and Williams, 2000).

Taxifolin is a dihydroflavonol that is abundant in citrus fruits and onions. Taxifolin is arguably the most interesting and promising component of dietary supplements or antioxidant-rich functional foods in the last two decades (Rice Evans *et al.*, 1996; Topal *et al.*, 2016; Li *et al*., 2017). Importantly, taxifolin exerts significant antioxidant effects that are critical in preventing the onset of apoptosis (Vladimirov *et al*., 2009). Moreover, taxifolin has been found to inhibit oxidative enzymes and the overproduction of ROS, thus ameliorating cerebral ischemia–reperfusion injury (Voulgari *et al.*, 2010).

Until now, there has been little information concerning the antioxidant potency of taxifolin as a flavonoid. Therefore, in the present study, the efficacy of taxifolin to reduce ROS levels in living cells was compared with that of catechin, which is a well-known, potent radical scavenger (Harborne and Williams, 2000;

Bimpilas *et al*., 2015), by using flow cytometry with the oxidation-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The ability of taxifolin to scavenge peroxyl radicals (ROO·) by use of an Electron Spin Resonance (ESR) spin-trapping technique were also compared with that of catechin.

Materials and Methods

Materials

(+)Taxifolin, (+)catechin, chrysin, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and *tert*-butyl hydroperoxide (t-BOOH, 70%, w/w, aqueous solution) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). DCFH-DA was obtained from Life Technologies Corporation (Carlsbad, CA, USA). α-(4-Pyridyl 1-oxide)-N-*tert*-butylnitrone (POBN) was purchased from Enzo Life Technologies (Farmingdale, NY, USA). *N*-Acetyl-*L*-Cysteine (NAC) and $Ce(SO_4)_2{\cdot}4H_2O$ were obtained from Wako Pure Chemical Industries, Limited (Osaka, Japan). All other reagents were analytical grade. The ultrapure water used in the present study was prepared with a compact ultrapure water system (Merck Millipore, Milli-Q®).

Measurement of ROS in Caco-2 Cells

Measurements of ROS in Caco-2 cells were performed by the method previously reported (Kohda *et al.*, 2016). Caco-2 cells were purchased from the European Collection of Cell Cultures (Salisbury, Wilts, UK) and cultured in Minimum Essential Medium (Life Technologies Corporation) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% non-essential amino acids (Life Technologies Corporation). The cells were maintained in a humidified atmosphere of 5% carbon dioxide at 37°C.

The measurement of ROS was performed by flow cytometry with DCFH-DA. The cells (1.0×10^6 cells/28 cm^2 dish) were incubated with the test reagents for 6 h and then DCFH-DA was added at a final concentration of 10 μM. After incubating for 30 min, the cells were collected by centrifugation (4°C and 200 × *g* for 5 min) and washed twice. The samples were filtered through a nylon mesh (37 μm) and subjected to flow cytometry (FACSAria™ III flow cytometer, Becton Dickinson, Basel, Switzerland).

ESR Measurement

An ESR spectrometer, TE-2100 (JEOL, Tokyo, Japan) and a JEOL flat quartz cell were used. The conditions were: field, 336±5 mT; power, 4 mW; field modulation, 0.200 mT; time constant, 0.1; and amplitude, 300. A manganese signal was used for the external standard.

Observation of POBN-Signal Adducts Reflecting t-BOO·

The Ce^{4+}/t-BOOH reaction was started by adding t-BOOH (final concentration, 0.4 M) to a mixture of POBN (final concentration, 10 mM) and $Ce(SO_4)_2{\cdot}4H_2O$ (final concentration, 0.2 mM) in 0.1 M sodium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. The POBN-signal adducts reflecting t-BOO· were measured 1 min after the addition of t-BOOH.

Statistical Analysis

The results are expressed as means ± standard errors of the mean. Significant differences between two groups were assessed using *t*-tests, whereas differences between multiple groups were assessed by one-way analysis of variance, followed by Scheffé's multiple comparison tests. *P*-values less than 0.05 were considered statistically significant.

Results

Taxifolin Suppresses ROS Generation Induced by t-BOOH in Caco-2 Cells

Figure 1A shows the effects of t-BOOH with or without taxifolin on the intracellular ROS generation of Caco-2 cells, measured by flow cytometry with the redox-sensitive fluorescent dye, DCFH-DA. The addition of t-BOOH (50 μM) to the Caco-2 cells shifted the Mean Fluorescence Intensity (MFI, dashed line) to the right, which indicates an increase in ROS levels measured by the DCF fluorescence. The increment in MFI induced by t-BOOH was reduced by the addition of 50 μM taxifolin. Figure 2B summarizes the MFI data measured by the method in Fig. 1A. The addition of NAC, which is often used as an antioxidant in cell experiments (Lasram *et al*., 2015), diminished the t-BOOH-induced increase in MFI (2 mM, 50% inhibition). Fifty micromolar of both taxifolin (61% inhibition) and catechin (39% inhibition), but not chrysin, reduced the t-BOOH-induced increase in MFI. The taxifolin effect was stronger than that of catechin and their difference was statistically significant. This means that taxifolin reduces the t-BOOH-induced increase in ROS levels in living cells stronger than catechin does.

Taxifolin Quenches t-BOO·

A direct method for measuring free radicals in aqueous conditions is detection by ESR spectroscopy (Venkataraman *et al.*, 2004). Figure 2A shows the ESR spectra of the spin signal adduct from t-BOO· [a(N) = 1.51 mT, a(H) = 0.23 mT] by the reaction of the Ce^{4+}/t-BOOH system with POBN. The hyperfine fit parameters are identical to those previously reported (Panasenko *et al.*, 2002; 2005). An obvious quenching by 0.5 mM Trolox, which is known as a potent peroxyl radical scavenger

(Barclay *et al.*, 1995; Stefek *et al*., 2005), supported the identity of the product. A positive correlation between the disappearance of the signal intensity of POBN signal adducts of t-BOO· and trolox concentrations was observed (Supplementary Fig. 1). Figure 2A and Supplementary Fig. 1 collectively demonstrate that this experimental condition using the ESR apparatus can assess the diminishing efficacy of specific substances against t-BOO·.

Figure 2B shows the effects of taxifolin, catechin and chrysin on the spin signal adduct of t-BOO· detected by the methods used in Fig. 2A. Both Taxifolin and catechin, but not chrysin, from 5 to 200 µM concentration-dependently diminished the signal adduct of t-BOO· [taxifolin, 34-90% inhibition; catechin, 3-82% inhibition). The scavenging effect of 200 µM taxifolin was stronger than that of 200 µM catechin and the difference was statistically significant.

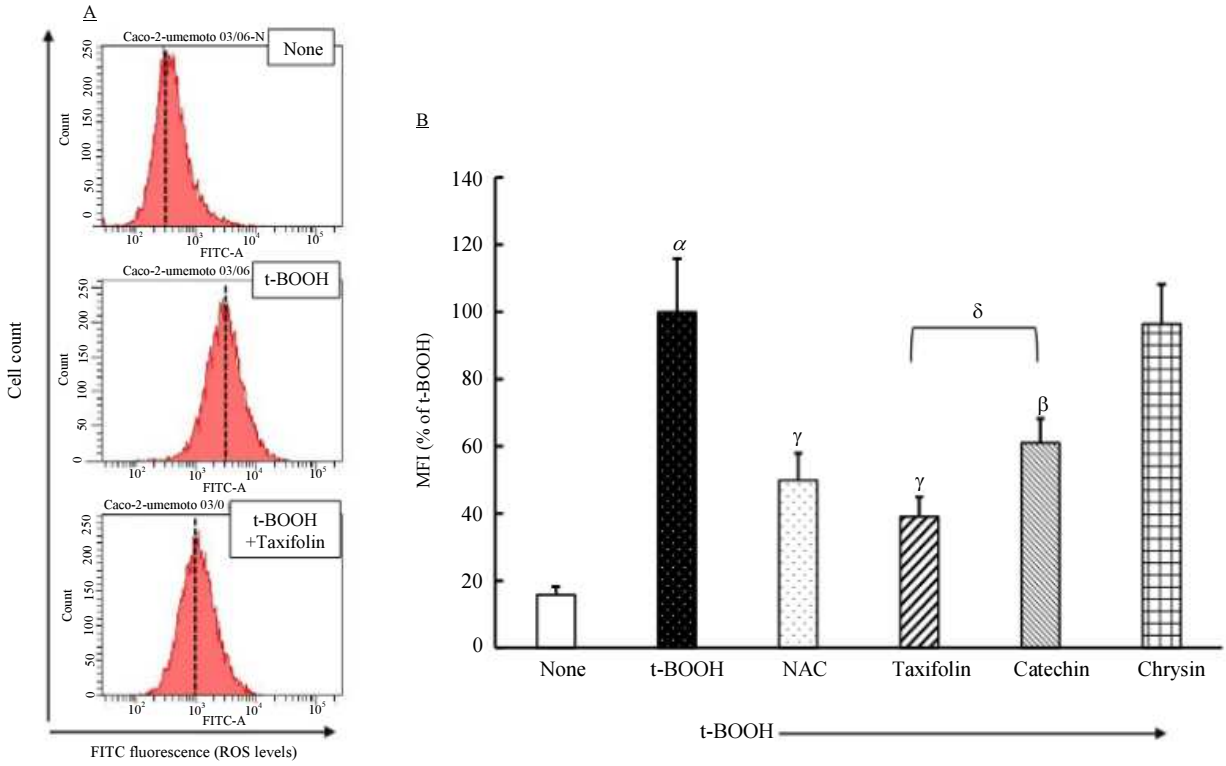
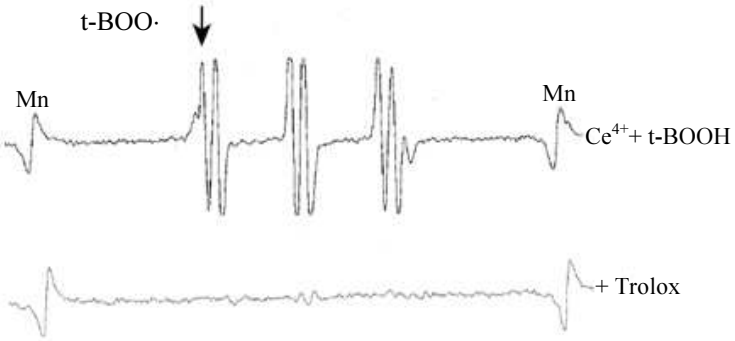
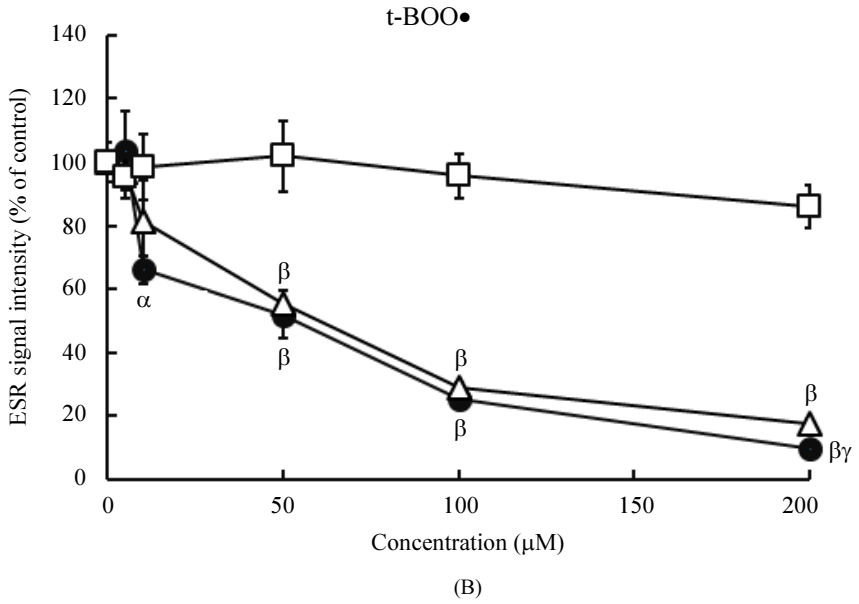


Fig. 1: Alterations in ROS levels in Caco-2 cells treated with t-BOOH in combination with taxifolin, catechin and chrysin; (A) Representative flow cytometry univariate histograms. B) Quantitative assessment of the MFI flow cytometry data. The data are presented as means ± standard errors of the mean (n = 7). $^{\alpha}p<0.01$ vs. None. $^{\beta}p<0.05$, $^{\gamma}p<0.01$ Vs. t-BOOH. $^{\delta}p<0.05$ vs. catechin. MFI, mean fluorescence intensity (shown as dashed line in part A); t-BOOH, *tert*-butyl hydroperoxide; NAC, *N*-acetyl-*L*-cysteine.t-BOOH, 50 µM; NAC, 2000 µM; taxifolin, 50 µM; catechin, 50 µM; chrysin, 50 µM



(A)



(B)

Fig. 2: Effects of taxifolin, catechin and chrysin on the amounts of spin signal adducts of t-BOO• generated by the chemical reaction system; (A) Representative ESR signal spectra of POBN spin adduct reflecting t-BOO•. Ce^{4+}, 0.2 mM; t-BOOH, 400 mM; Trolox, 0.5 mM. (B) Effects of taxifolin on ESR signal spectra. The radical intensity was defined as the ratio of the peak height of signal (indicated as an arrow in part A) to that of Manganese (Mn). The data are presented as means ± standard errors of the mean (n = 3). $^{\alpha}p<0.05$, $^{\beta}p<0.01$ vs. control. $^{\gamma}p<0.05$ vs. 200 μM catechin. t-BOO•, *tert*-butyl peroxyl radical; ESR, electron spin resonance; t-BOOH, *tert*-butyl hydroperoxide

The results of Fig. 2 show that taxifolin diminishes t-BOO· and the t-BOO· scavenging efficacy seems to be somewhat greater than that of catechin.

Discussion

ROS contributes to the development of various diseases such as atherosclerosis, diabetes, cancer, neurodegenerative diseases and liver cirrhosis. It also contributes to the aging process (Basaga, 1990). The use of antioxidant compounds, such as radical scavengers, might prevent the development and progression of these diseases to maintain health.

As shown in Fig. 1, taxifolin suppressed t-BOOH-induced increases in ROS levels in Caco-2 cells and the effect of 200 μM taxifolin was significantly stronger than that of catechin. Based on an ESR technique, Fig. 2 showed that taxifolin could diminish ROO· and 200 μM taxifolin's effect was significantly stronger than that of catechin. Chrysin was without effects on both ROS levels in Caco-2 cells and ESR radical intensity of the spin signal adduct of t-BOO·. Both taxifolin and catechin have a catechol group in ring B and taxifolin possesses an additional 4-oxo group in ring C (Supplementary Fig. 2). Thus, it is possible that the catechol group in ring B plays an important role for the ROO· scavenging activity and the 4-oxo group in ring C determines the strength of the ROO· scavenging capacity of taxifolin. The belief that the catechol group in ring B of flavonoids is the major structural feature imparting antioxidant activity has been supported by the work of Jovanovic *et al*. (1996).

Topal *et al.* (2016) and Li *et al*. (2017) have reported that taxifolin is an effective antioxidant and antiradical by using indirect *in vitro* bioanalytical methods, including 1,1-diphenyl-2-picryl-hydrazyl radical-scavenging, 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) radical-scavenging, Fe^{3+}-reducing and Cu^{2+}-reducing assays. However, to our knowledge, there is no information concerning the types of ROS scavenged by taxifolin by using an ESR method. In the present study, we showed that taxifolin scavenged ROO·. Li *et al*. (2017) has reported that taxifolin is an effective scavenger of hydroxyl radical (·OH). The present study together with the report by Li *et al*. (2017) indicates that taxifolin can be a scavenger of both ROO· and ·OH.

Determining the significance of the present finding that taxifolin quenches t-BOO· will require further study. Additional studies are also needed to clarify the mechanism by which taxifolin scavenges ROO·. However, to the best of our knowledge, the results of this study show for the first time that taxifolin diminishes ROS levels in living cells possibly by scavenging ROO·. This provides new mechanistic insight into the preventive effects of taxifolin in various disorders.

Conclusion

Taxifolin significantly reduced t-BOOH-induced increases in ROS levels in Caco-2 cells and this effect was stronger than that of another flavonoid, catechin. Taxifolin also scavenged ROO· by using an ESR method. We believe that our study makes a significant contribution to the literature because, to the best of our knowledge, this is the first time that taxifolin has the potential to diminish ROS levels in living cells possibly by scavenging ROO·.

Authors Contributions

Satoru Sakuma: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Yui Kishiwaki, Mayu Matsumura, Hiroyuki Sawada, Riko Hashimoto, Kohtaroh Gotoh, Kohhei Umemoto and Yohko Fujimoto: Participated in some experiments, coordinated the data-analysis.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and there are no ethical issues involved.

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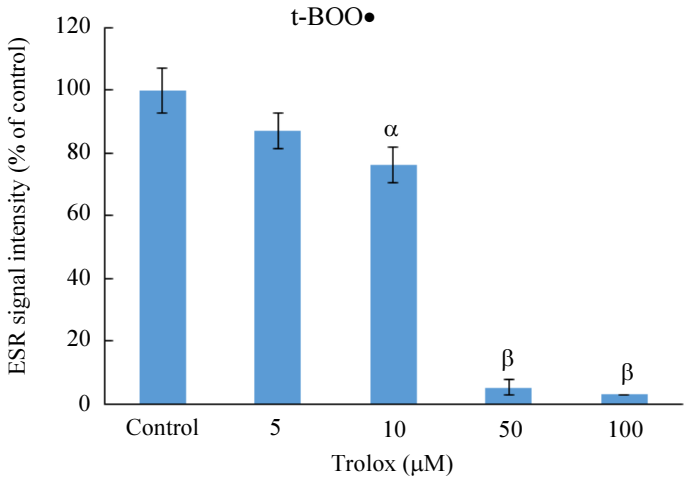
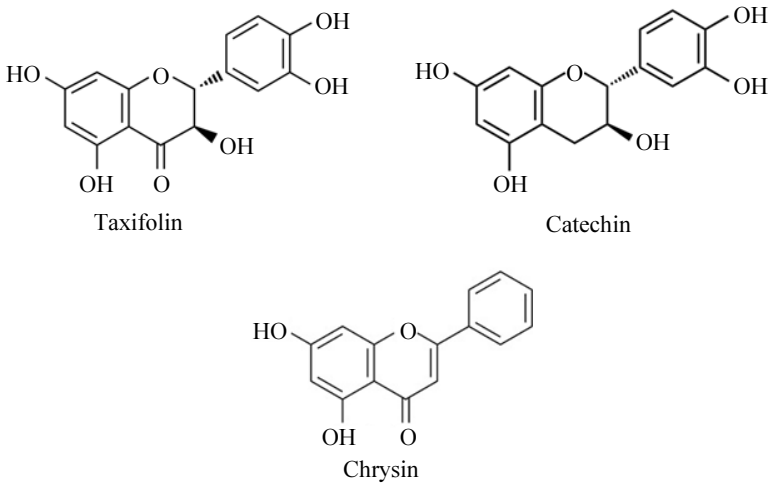
Supplementary Material



Supplementary Fig. 1: Effects of trolox on the amounts of spin signal adduct of t-BOO• generated by the chemical reaction system

The radical intensity was defined as the ratio of the peak height of signal [indicated as an arrow in Fig. 2(A)] to that of Manganese (Mn). The data are presented as means ± standard errors of the mean (n = 3). $^{\alpha}p < 0.05$, $^{\beta}p < 0.01$ vs. Control. Electron-spin resonance (ESR) coupled with spin-trapping technique used in this study is shown in Material and Methods and Results and Discussion sections in the manuscript.



Supplementary Fig. 2: Structures of taxifolin, catechin and chrysin