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Flavonoids and chlorogenic acid from *Eriobotrya japonica* scavenge peroxynitrite

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Abstract – Peroxynitrite is a cytotoxic intermediate produced by the reaction between the superoxide anion radical and nitric oxide. Flavonoids (afzelin, quercitrin and quercetin 3-*O*-sambubioside), and chlorogenic acid and its methyl ester obtained from leaves of loquat (*Eriobotrya japonica*) have recently been shown to scavenge 1,1-diphenyl-2-picrylhydrazyl radical and to inhibit lipid peroxidation in mouse liver homogenate. The aim of this study is to investigate the inhibitory effects of the above components on peroxynitrite produced stimulated by 3-morpholinosydnonimine (SIN-1) to produce superoxide anion radical and nitric oxide at the same time. In addition, the present study tests whether or not the components directly scavenge peroxynitrite itself. The results showed that the components with the aromatic *ortho*-dihydroxyl groups (catechol) were more potent inhibitors of peroxynitrite formation by SIN-1. In particular, the methyl ester form of chlorogenic acid showed the most potent inhibition. At 5 μM concentration, the order of minimizing peroxynitrite formation were : methyl chlorogenic acid > quercitrin > quercetin 3-*O*-sambubioside > chlorogenic acid > afzelin. Authentic peroxynitrite was directly scavenged by the components in a manner similar to peroxynitrite formation by SIN-1. In particular, when compared with penicillamine as a positive control, methyl chlorogenate was as effective in inhibiting peroxynitrite formation and approximately 2 times more effective in scavenging an authentic peroxynitrite. These results demonstrate therefore, that components extracted from the leaves of *Eriobotrya japonica* effectively scavenged peroxynitrite.
Key words: *Eriobotrya japonica,* peroxynitrite, scavenging, flavonoids, methyl chlorogenic acid, 3-morpholinosydnonimine (SIN-1), dihydrorhodamine 123 (DHR 123).

Introduction

Peroxynitrite ($ONOO^-$) produced by the prompt reaction of the superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$) (Blough and Zafiriou, 1985 and Malinski *et al.*, 1993) is a potent oxidant for the SH group, and peroxynitrous acid (ONOOH) (pKa = 6.8); that is, proton-linked peroxynitrite, induces lipid peroxidation as it passes through an activated state with reactivity equivalent to the hydroxyl radical ($^{\bullet}OH$) in the process of epimerization to nitric acid (Radi *et al.*, 1991a,b). In addition, in the presence of transition metal ions in superoxide dismutase (SOD), peroxynitrite produces the nitronium ion (NO_2^+), which has a potent nitrating activity and attacks aromatic amino acid residues such as tyrosine (Beckmann *et al.*, 1994).

Peroxynitrite ($ONOO^-$) has been reported to be a potent suppressor of mitochondrial respiration because it inhibits the activity of aconitase (Hausladen and Fridovich, 1994, Castro *et al.*, 1994, and Yoshie and Ohshima, 1997), a generator of DNA single strand breaks (Szabo and Salzman, 1995), and is an inducer of the nitration of surfactant protein A (SP-A), resulting in decreasing mannose binding ability and lipid aggregation (Zhu *et al.*, 1996). In these tests, peroxynitrite has shown a stronger toxic effect than has nitric oxide. In recent years, the importance of the physiological, biochemical, biological and cytotoxic properties of biologically produced peroxynitrite has been recognized, and some natural products and synthetic compounds have been widely studied as peroxynitrite scavengers. Since flavonoids and phenolic non-flavonoid compounds extracted from natural

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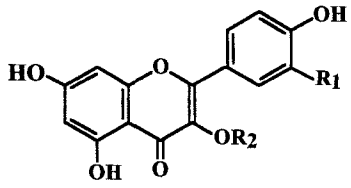
products have been reported to scavenge peroxynitrite and inhibit DNA damage (Chung *et al.*, 1998, Haenen *et al.*, 1997, Oshima *et al.*, 1998, Kono *et al.*, 1997, Pannala *et al.*, 1998, and Grace *et al.*, 1998), we decided to examine the effect of the *Eriobotrya japonica* components which include flavonoids (afzelin, quercitrin, quercetin 3-*O*-sambubioside), and chlorogenic acid and its methyl ester form on inhibitory and scavenging activities induced by peroxynitrite formation by SIN-1 and authentic peroxynitrite *in vitro*. Further studies are planned which will attempt out to identify the active sites of the *Eriobotrya japonica* components.

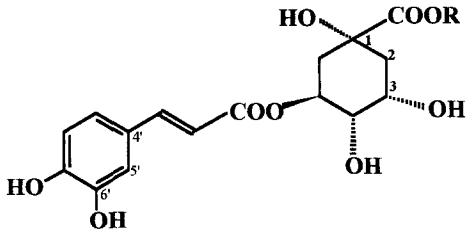
Materials and Methods

Chemicals – 3-Morpholinosydnonimine (SIN-1), dihydrorhodamine 123 (DHR 123), and peroxynitrite were of high quality and were purchased from Sigma Chemical Company (St. Louis, MO, USA), Molecular Probes (Eugene, Oregon, USA), and Cayman (Ann Arbor, MI, USA), respectively.

Plant materials – Fresh leaves of *Eriobotrya japonica* were collected near Sunchon in Spring, 1996. A voucher specimen was deposited as No. 960421 in the Herbarium of the Botanical Gardens in Sunchon National University, Korea.



1 (afzelin): R_1=H, R_2= rhamnoside
2 (quercitrin): R_1=OH, R_2= rhamnoside
3(quercetin 3-*O*-sambubioside): R_1=OH, R_2=sambubioside



4 (chlorogenic acid) : R=H
5 (methyl chlorogenic acid) : R=CH_3

Fig. 1. The chemical structures of the *Eriobotrya japonica* components.

Isolation of compounds – *Eriobotrya japonica* components was isolated as described previously (Jung *et al.*, 1999). The chemical structures of these components are illustrated in Fig. 1.

Measurement of peroxynitrite – DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored at −80°C as a stock solution. DHR 123 diluted from the stock was placed in ice without exposure to light prior to the study. The buffer used was the mixture of 90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride at pH 7.4, including 100 μM diethylenetriaminepentaacetic acid (DTPA), each prepared with high quality deionized water and purged with nitrogen. The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured after 5 min (1 hr) with or without treatment of authentic peroxynitrite (SIN-1). Oxidation of DHR 123 by SIN-1 gradually increased. However, DHR 123 was oxidized rapidly by authentic peroxynitrite, and its final fluorescent intensity was unchanged over time. The fluorescence intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500, Bio-Tex instruments) at the excitation wavelength of 480nm and the emission wavelength of 525nm respectively. The effects were expressed as the % inhibition of oxidation of DHR 123 (Kooy *et al.*, 1994).

Results

The ability of the *Eriobotrya japonica* components to decrease peroxynitrite formation by SIN-1 were determined. SIN-1, the concomitant product of both the superoxide anion radical and nitric oxide, raises the possibility that a significant amount of peroxynitrite may be formed from the combination of these two radicals. Because of this, SIN-1 is frequently used as a standard compound for the continuous formation of peroxynitrite (Hogg *et al.*, 1992, and Muller *et al.*, 1997). The results (Fig. 2) indicate that the aromatic *ortho*-dihydroxylated *Eriobotrya japonica* components were potent inhibitors of peroxynitrite formation by SIN-1. However, kaempferol 3-*O*-rhamnoside (afzelin) without aromatic *ortho*-dihydroxylated catechol group showed a low contribution. In particular, methyl chlorogenate, which includes on aromatic *ortho*-dihydroxyl group and a methyl group at the carboxylic acid of chlorogenic acid, showed the most potent inhibition. At 5 μM concentration, the

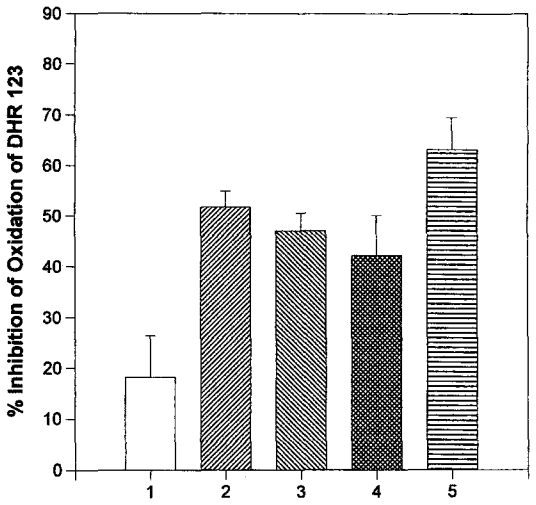


Fig. 2. The effect of the *Eriobotrya japonica* components on peroxynitrite formation by SIN-1. In each space in 96 well, The mixture of 90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride, pH 7.4, including 100 μM DTPA and 5 μM DHR 123 were added to 5 μM *Eriobotrya japonica* components and 10 μM SIN-1. The fluorescence of oxidation of DHR 123 was measured at the excitation wavelength of 480 nm and the emission wavelength of 525 nm for 1hr. Each value is the mean ± S.E. of three samples. kaempferol 3-*O*-rhamnoside (afzelin) (**1**), quercetin 3-*O*-rhamnoside (quercitrin) (**2**), quercetin 3-*O*-sambubioside (**3**), chlorogenic acid (4), methyl chlorogenate (5)

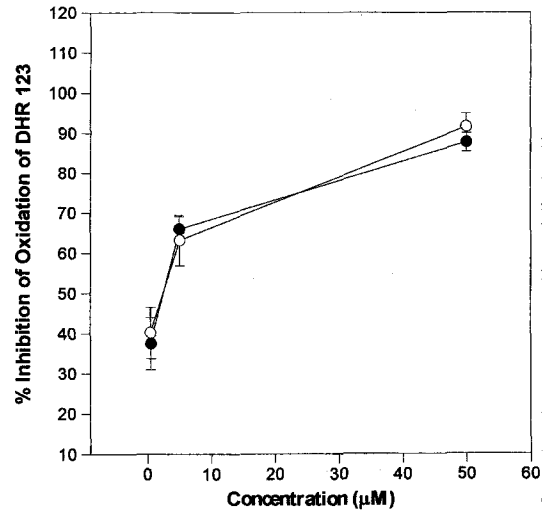


Fig. 3. Dose-response curve of methyl chlorogenate (○) and penicillamine (●) on peroxynitrite formation by SIN-1. In each space in 96 well, The mixture of 90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride, pH 7.4, including 100 M DTPA and 5 μM DHR 123 were added to methyl chlorogenate (0.5 μM, 5 μM, and 50 μM) or penicillamine (0.5 μM, 5 μM, and 50 μM) and 10 μM SIN-1. The fluorescence of oxidation of DHR 123 was measured at the excitation wavelength of 480 nm and the emission wavelength of 525 nm for 1 hr. Each value is the mean ±S.E. of three samples.

ability of *Eriobotrya japonica* components to minimize peroxynitrite formation were: methyl chlorogenate>quercetin 3-*O*-rhamnoside (quercitrin)>quercetin 3-*O*-sambubioside>chlorogenic acid >kaempferol 3-*O*-rhamnoside (afzelin). The most specific inhibitory effect of the methyl chlorogenate was further investigated by comparison with penicillamine as an effective scavenger of peroxynitrite *in vitro* (Chung *et al.*, 1998), and the results are shown in Figure 3. Both of these compounds efficiently scavenged peroxynitrite, the concentration necessary for 50% inhibition being 1.56 ± 0.61 μM and 1.25 ± 0.30 μM respectively. Thus, methyl chlorogenate was as efficient as penicillamine in inhibiting peroxynitrite formation.

The ability of the *Eriobotrya japonica* components to scavenge authentic peroxynitrite was determined. The obtained results (Figs. 4 & 5) are similar to those of their inhibitory effects on peroxynitrite formation by SIN-1. The most specific inhibitory effect of methyl chlorogenate was further investigated by comparison with penicillamine, and the results are shown in Figure 5. Both of these compounds efficiently scavenged peroxynitrite. Methyl chlorogenate and penicillamine were needed for 1.44 ± 0.05 μM and 2.77 ± 0.22 μM to scavenge 50% authentic peroxynitrite, indicating that methyl chlorogenate was approximately 2 times more effective.

Discussion

The inhibitory and scavenging abilities of the *Eriobotrya japonica* components (**1-5**) on peroxynitrite formation by SIN-1 and authentic peroxynitrite were closely related to aromatic *ortho*-dihydroxyl groups (catechol). Compounds **2-5** with aromatic *ortho*-dihydroxyl groups had a relatively strong peroxynitrite scavenging activity. However, afzelin (**1**), a compound without a catechol group, had the lowest activity. Since flavonoids **1-3** have the common hydroxyl groups at positions 5 and 7, the hydroxyl groups at these positions seemed to have little effect. Methyl chlorogenate (**5**) was much more active than the flavonoids and free chlorogenate tested. A possible explanation for this is

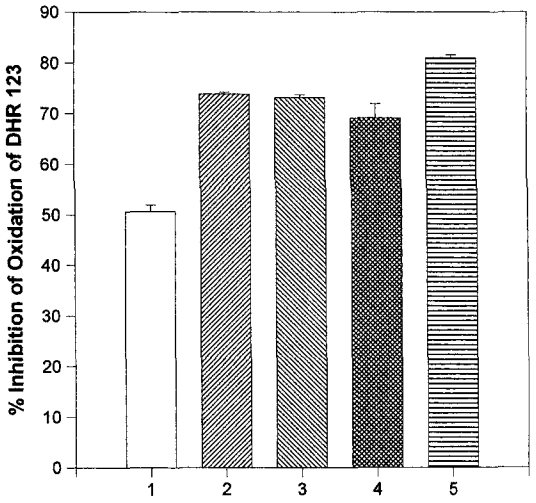


Fig. 4. The effect of the *Eriobotrya japonica* components on authentic peroxynitrite-scavenging activity. In each space in 96 well, The mixture of 90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride, pH 7.4, including 100 μM DTPA and 5 μM DHR 123 were added to 5 μM *Eriobotrya japonica* components and 10 μM authentic peroxynitrite. The fluorescence of oxidation of DHR 123 was measured at the excitation wavelength of 480 m and the emission wavelength of 525 nm for 5 mins. Each value is the mean ± S.E. of three samples. kaempferol 3-*O*-rhamnoside (afzelin) (**1**), quercetin 3-*O*-rhamnoside (quercitrin) (**2**), quercetin 3-*O*-sambubioside (**3**), chlorogenic acid (**4**), methyl chlorogenate (**5**).

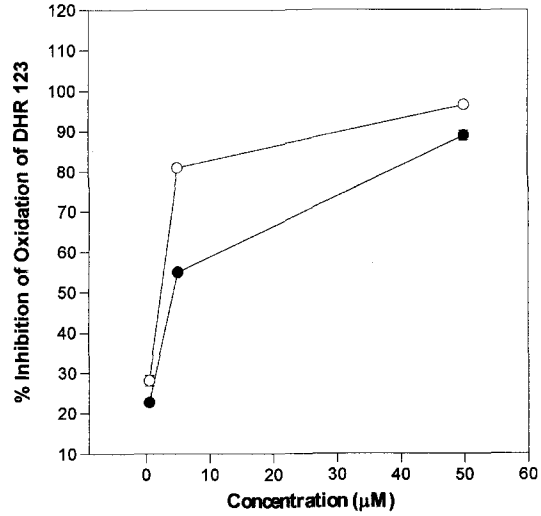


Fig. 5. Dose-response curve of methyl chlorogenate (○) and penicillamine (●) on authenic peroxynitrite scavenging activity. In each space in 96 well, The mixture of 90 mM sodium chloride, 50 mM sodium phosphate, and 5 mM potassium chloride, pH 7.4, including 100 μM DTPA and 5 μM DHR 123 were added to methyl chlorogenate (0.5 μM, 5 μM, and 50 μM) or penicillamine (0.5 μM, 5 μM, and 50 μM) and 10 μM authentic peroxynitrite. The fluorescence of oxidation of DHR 123 was measured at the excitation wavelength of 480 nm and the emission wavelength of 525 nm for 5mins. Each value is the mean ± S.E. of three samples.

that the flavonoids and free chlorogenate could form inter-molecular hydrogen bonding between the hydroxyl groups in the flavonoids or between a carboxylic acid and a hydroxyl group in the aromatic ring of chlorogenic acid. However, the rate of formation of inter-molecular hydrogen bonding in methyl chlorogenate seemed to decrease due to the presence of a methyl group. Therefore, methyl chlorogenate, which contains more hydrogen tends to scavenge peroxynitrite more easily when compared to the flavonoids and free chlorogenate tested.

It has been reported that the hydroxyl groups of flavonoids were responsible for peroxynitrite scavenging. In particular the catechol group with 3',4'-dihydroxyl groups made the primary contribution. Indeed, the substitution of ethyl groups for one or both hydroxyl groups in the catechol group drastically reduced the activity. Flavonoids and related phenolic compounds have been observed to stimulate the conversion of the covalently closed circular double-stranded supercoiled DNA to a relaxed open circle in the presence of NO. Among these, however, caffeic acid, rutin, and quercetin did not induce. Furthermore, the compounds decreased the peroxynitrite-mediated single strand breakages dose dependently (Haenen *et al.*, 1997, and Oshima *et al.*, 1998). In a phenolic non-flavonoid compound in diet, chlorogenic acid has also been reported to be rapidly oxidized by peroxynitrite in concentration and pH-dependent manner and to inhibit the formation of single strand breaks and nitration by peroxynitrite (Kono *et al.*, 1997, Pannala *et al.*, 1998, and Grace *et al.*, 1998).

There are two possible mechanisms for scavenging peroxynitrite: (1) nitration and (2) electron donation (Pannala *et al.*, 1998). When exposed to peroxynitrite, chlorogenic acid showed maximal activity, preventing peroxynitrite-induced tyrosine nitration. However, it did not show any significant increase in absorbance at approximately 430 nm, indicating that the chlorogenic acid acted as an antioxidant. This was not due, in other words, to nitration itself. Further

research should be done to investigate the effects of the *Eriobotrya japonica* components on the nitration of peroxynitrite.

The present study has shown that in the inhibition and scavenging activities of *Eriobotrya japonica* components on peroxynitrite formation by SIN-1 and authentic peroxynitrite, respectively, the aromatic *ortho*-dihydroxylated *Eriobotrya japonica* components have more potent effects. In particular, the methyl chlorogenate with aromatic *ortho*-dihydroxyl groups and a methyl group at the carboxylic acid of chlorogenic acid had the most potent effect. The *Eriobotrya japonica* components, therefore, will mediate luxury peroxynitrite produced in the condition of atherosclerosis, ischemia/reperfusion, and inflammation.

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