



Title	In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid.
Author(s)	Sato, Yuki; Itagaki, Shirou; Kurokawa, Toshimitsu et al.
Citation	International journal of pharmaceutics, 403(1-2), 136-138 <a href="https://doi.org/10.1016/j.ijpharm.2010.09.035">https://doi.org/10.1016/j.ijpharm.2010.09.035</a>
Issue Date	2011-01-17
Doc URL	<a href="https://hdl.handle.net/2115/78780">https://hdl.handle.net/2115/78780</a>
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Type	journal article
File Information	IJP403_Sato.pdf



**International Journal of Pharmaceutics**

**Note**

**In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid**

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## **Abstract**

Dietary polyphenols are thought to be beneficial for human health as antioxidants. Coffee beans contain a common polyphenol, chlorogenic acid. Chlorogenic acid is the ester of caffeic acid and quinic acid. Although these polyphenols have received much attention, there is little evidence indicating a relationship between the effect and the rate of absorption. In this study, we focused on the beneficial effects of chlorogenic acid and caffeic acid, a major metabolite of chlorogenic acid. We carried out *in vitro* and *in vivo* experiments. In the *in vitro* study, caffeic acid had stronger antioxidant activity than that of chlorogenic acid. The uptake of chlorogenic acid by Caco-2 cells was much less than that of caffeic acid. The physiological importance of an orally administered compound depends on its availability for intestinal absorption and subsequent interaction with target tissues. We then used an intestinal ischemia-reperfusion model to evaluate antioxidant activities *in vivo*. We found that both chlorogenic acid and caffeic acid had effects on intestinal ischemia-reperfusion injury. Since caffeic acid has a stronger antioxidant activity than that of chlorogenic acid and chlorogenic acid is hydrolyzed into caffeic acid in the intestine, it is possible that caffeic acid plays a major role in the protective effect of chlorogenic acid against ischemia-reperfusion injury.

**Keywords** *intestine; antioxidant; chlorogenic acid; caffeic acid; absorption; ischemia-reperfusion;*

## 1. Introduction

Dietary polyphenols are thought to be beneficial for human health by exerting various biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, and alteration of signal transduction pathways (Stocker, 1999). Epidemiological studies have shown relationships between consumption of polyphenol-rich foods and prevention of diseases such as cancer, coronary heart disease and osteoporosis, and results of those studies have promoted interest in polyphenols (Steinmetz et al., 1996). Polyphenols are mainly classified into phenolic acids and flavonoids. A major class of the former is hydroxycinnamic acids, and chlorogenic acid is the major representative of hydroxycinnamic acids. Chemically, chlorogenic acid is an ester formed between caffeic acid and quinic acid. Chlorogenic acid is hydrolyzed by intestinal microflora into various aromatic acid metabolites including caffeic acid and quinic acid (Gonthier et al., 2003). It is widespread in plants, fruits and vegetables (Clifford, 1999). Coffee beans are a major source of chlorogenic acid in the diet, daily intake of chlorogenic acid in coffee drinkers being about 1 g (Konishi et al., 2004). Chlorogenic acid and caffeic acid have vicinal hydroxyl groups on an aromatic residue, and they exhibit antimutagenic, carcinogenic and antioxidant activities *in vitro*, which is to scavenge reactive oxygen species (ROS) (Rice-Evans et al., 1996).

It is well known that ROS are responsible for ischemia/reperfusion (I/R) injury and

that the intestine is highly sensitive to I/R injury (Kong et al., 1998). An intestinal I/R model has been frequently used as that of oxidative injury (Megison et al., 1990). Protective effects of antioxidants administered before ischemia on I/R injury have also been reported (Guneli et al., 2007; Guven et al., 2008). Administration of antioxidants may therefore help to remove ROS and thus improve the clinical outcome. It is thought that dietary antioxidants can enhance cellular defense and help to prevent oxidation damage to cellular components. However, there are little reports assessed antioxidant activities against oxidative injury including absorption mechanism of antioxidants.

In this study, we investigated the antioxidant activities of chlorogenic acid and caffeic acid in *in vitro* and *in vivo* studies. We also focused on the uptake of chlorogenic acid and its metabolite caffeic acid in the intestine.

## 2. Materials and Methods

For measurement of superoxide anion, we used 2-methyl-6-p-methoxyphenylethynylimidazopyrazynone (MPEC) (ATTO Corp., Osaka, Japan) to emit oxidation as described previously (Itagaki et al., 2009). The 50% inhibitory concentration (IC<sub>50</sub>) value was determined from the concentration-inhibition of light emission

curve calculated by using Origin ® (version 6.1J). A formula fitting logistically was expressed as follows:

$$Y=(A_1-A_2)/\{1+(x+x_0)^P\}+A_2,$$

where  $A_1$  is the initial value,  $A_2$  is the final value,  $x$  is concentration,  $x_0$  is the  $IC_{50}$  value,  $P$  is power.

A radical chain-breaking activity assay was carried out as described previously (Itagaki et al., 2009). The results are expressed as total antioxidant performance (TAP) values, representing the percentage of inhibition of 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a,-diazas-indacene-3-propionic acid saccinimidyl ester (BODIPY) (Invitrogen, Carlsbad, CA) oxidation in rat plasma with respect to that occurring in a control sample:

$$TAP=[(AUC_{control}-AUC_{plasma})/AUC_{control}]\times 100,$$

where  $AUC_{control}$  and  $AUC_{sample}$  represent the area under the curve (AUC) of BODIPY oxidation kinetics in the control and plasma samples, respectively. Control samples were prepared using phosphate buffer saline (PBS) (pH 7.4) (137 mM NaCl, 2.68 mM KCl, 8.10 mM  $Na_2HPO_4$ , 1.47 mM  $KH_2PO_4$ ).

Caco-2 cells, widely used as an intestinal model, obtained from RIKEN (Ibaraki, Japan) were maintained in plastic culture flasks (Corning Costar Corp., Cambridge, MA) as described previously (Saito et al., 2005). For uptake studies, Caco-2 cells were seeded at a

cell density of  $1.0\text{-}3.0 \times 10^5$  cells/cm<sup>2</sup> on 12-well plastic plates (Corning Costar Corp.) and the experiment was performed as described previously (Itagaki et al., 2005). Concentration of chlorogenic acid and caffeic acid (both from Sigma, St Louis, MO) were determined using an HPLC system. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Male Wistar rats (7-9 weeks, 200-300 g) were fasted for 14-16 h before the experiments. They were anaesthetized by an intraperitoneal (*i.p.*) injection of 50 mg/kg sodium pentobarbital. Surgical procedures were carried out as described previously (Itagaki et al., 2009). A 5-cm-long loop of the jejunum was identified and ligated at both ends. Five hundred  $\mu\text{l}$  of chlorogenic acid or caffeic acid (1 mM) was administered directly into the jejunum loops 1 h before the induction of ischemia.

Evans blue (20 mg/kg, Sigma, St. Louis, MO) was administered intravenously 5 min before reperfusion. After 60 min of intestinal reperfusion, rats were sacrificed by exsanguination from the superior mesenteric artery. The 5-cm-long portion of the jejunum was rapidly resected from the intestine. Measurement of Evans blue was performed as described previously (Itagaki et al., 2009). The content of each intestinal sample was expressed as Evans blue  $\mu\text{g}/\text{mg}$  dry weight of tissue.

Student's t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined

by one-way analysis of variance (ANOVA). Statistical significance was defined as  $p < 0.05$ .

### 3. Results and Discussion

I/R is a condition that restores the blood stream after temporary hypoxia. It has been reported that the hypoxanthine-xanthine oxidase system seems to be an important and probably the initial source of free radical production after hypoxia and reperfusion of the gut (Schoenberg et al., 1985). After reperfusion, much oxygen inflowing with nutritive blood activates xanthine oxydase and produces superoxide anion (Schoenberg et al., 1990). Moreover, the intestinal mucosa is one of the richest sources of xanthine oxidase, and the inhibitory activity of xanthine oxidase also contributes to the protective effect against oxidation damage (Granger et al., 1981). It is difficult to quantitate superoxide anion *in vivo* because of its reactive nature and short life. The MPEC assay is a simple, reproducible and suitable assay for measuring superoxide anion-scavenging activities of various compounds.  $IC_{50}$  values of chlorogenic acid and caffeic acid were  $41.0 \pm 12.1 \mu\text{M}$  and  $10.1 \pm 9.32 \mu\text{M}$  ( $n=3$ , mean  $\pm$  S.D.), respectively. We have already reported the  $IC_{50}$  values of other antioxidants and that of allopurinol was about  $15.0 \mu\text{M}$  (Takano et al., 2009). Allopurinol is a frequently prescribed agent for gout and is the most commonly used xanthine oxidase inhibitor (Pacher et al., 2006). These facts indicate that caffeic acid has superoxide

anion-scavenging activity almost the same as that of allopurinol. We then investigated the chain-breaking activities of caffeic acid and chlorogenic acid using BODIPY. The TAP value of caffeic acid and that of chlorogenic acid increased in a concentration-dependent manner (Fig. 1). The results of two studies suggested that caffeic acid has almost the same antioxidant activity as chlorogenic acid in the *in vitro* study (TAP assay) although it showed stronger antioxidant activity than that of chlorogenic acid in the reaction between reagents (MPEC assay).

Chlorogenic acid is hydrolyzed to caffeic acid in the small intestine, and it is known to be poorly absorbed (Dupas et al., 2006). Next, we investigated the effects of extracellular pH on each uptake of chlorogenic acid and caffeic acid. We found that uptake of caffeic acid by Caco-2 cells was dependent on extracellular pH, while extracellular pH did not affect chlorogenic acid uptake (supplemental file). These findings suggest that caffeic acid plays a more important role in protective effects on I/R injury. From the point of view that extracellular pH in the intestine in a physiological condition would be about 6.0, we hypothesized that hydrolyzation of chlorogenic acid is an important factor associated with its protective effect on I/R injury.

Many studies have focused on dietary antioxidant activities against oxidative stress. We have reported that antioxidants have protective effects on intestinal I/R injury (Kurokawa et al., 2006; Itagaki et al., 2010). The same as these reports, we investigated protective effects

of chlorogenic acid and caffeic acid on intestinal I/R injury. We considered that the appropriate concentrations of chlorogenic acid and caffeic acid would be 1 mM since both sufficient antioxidant activities were demonstrated more over 1 mM in the results of MPEC assay and TAP values described above. Extravasation of Evans blue was used as a marker of changes in capillary permeability and tissue edema (Cavriani et al., 2004). Vascular permeability has been shown to be significantly higher in rats with I/R than in sham-operated rats, and elevated vascular permeability is also an indicator of I/R injury. The elevation of vascular permeability by intestinal I/R was attenuated by treatment with chlorogenic acid and caffeic acid (Fig. 2). Their protective effects on I/R injury were almost the same. These results suggested that the effect of chlorogenic acid is derived from caffeic acid hydrolyzed in the intestine, though chlorogenic acid itself showed a protective effect almost the same as that of caffeic acid. These findings indicate that daily intake of antioxidants is important for protection against oxidative stress.

In summary, we found that chlorogenic acid and caffeic acid exhibit protective effects against I/R injury in the rat small intestine. However, uptake of chlorogenic acid by Caco-2 cells was much less than that of caffeic acid, which is a metabolite of chlorogenic acid. Since chlorogenic acid is hydrolyzed into caffeic acid in the intestine and caffeic acid has a stronger antioxidant activity than that of chlorogenic acid, it is possible that caffeic acid plays an important role in the protective effect of chlorogenic acid against I/R injury. Further studies

are needed to obtain evidence of health benefits of chlorogenic acid and caffeic acid.

## **Acknowledgments**

This work was supported in part by The SKYLARK Food Science Institute.

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## Figure legends

Fig. 1. Antioxidant activities of caffeic acid and chlorogenic acid determined by the TAP assay.

Each value represents the mean with S.D. of 3 independent experiments.

Fig. 2. Effects of chlorogenic acid and caffeic acid on changes in vascular permeability in the small intestine.

Each point represents the mean  $\pm$  S.D. of 4-5 measurements. Rats in the I/R+chlorogenic acid group and I/R+caffeic acid group were administered chlorogenic acid and caffeic acid, respectively, into the loop 1 h before ischemia. Rats in the sham-operated and ischemia-reperfusion groups were administered a vehicle 1 h before ischemia. \*; significantly different from the sham-operated group at  $P < 0.05$ . †; significantly different from the ischemia-reperfusion group at  $P < 0.05$ .

Supplemental figure. Time course of chlorogenic acid (A) and caffeic acid (B) uptake into Caco-2 cells.

Each point represents the mean with S.D.  $\pm$  4 measurements. The uptake of chlorogenic acid or caffeic acid (500  $\mu$ M) was measured at pH 6.0 (closed circles) or 7.4 (open circles) at 37°C.