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Inhibition of Low-Density Lipoprotein Oxidation and Oxidative Burst in Polymorphonuclear Neutrophils by Caffeic Acid and Hispidin Derivatives Isolated from Sword Brake Fern (*Pteris ensiformis* Burm.)

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Several antioxidant compounds have been previously identified from sword brake fern (*Pteris ensiformis* Burm.) by DPPH bleaching and Trolox equivalent antioxidant capacity (TEAC) analyses. Among the isolates, 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside and hispidin 4-*O*-β-D-glucopyranoside [6-(3,4-dihydroxystyryl)-4-*O*-β-D-glucopyranoside-2-pyrone] were two new compounds. The aim of this study is to elucidate the possible effect of the aqueous extract of sword brake fern (SBF) and these two compounds in preventing atherosclerosis. The results demonstrated that SBF and these two compounds strongly inhibited Cu^{2+}-mediated low-density lipoprotein (LDL) oxidation measured by thiobarbituric acid-reactive substances assay (TBARS), conjugated diene production, and relative electrophoretic mobility. The commercial antioxidant DL-α-tocopherol showed lower antioxidant activity than these two compounds at the same molecular concentration. SBF and these two compounds also suppressed *N*-formylmethionyl-leucylphenylalanine (*f* MLP)-stimulated reactive oxygen species (ROS) production in human polymorphonuclear neutrophils (PMN). These findings indicate that sword brake fern may prevent atherosclerosis via inhibition of both LDL oxidation and ROS production.

KEYWORDS: ROS; oxLDL; caffeic acid; hispidin; oxidative burst

INTRODUCTION

Atherosclerosis is a complex and chronic inflammatory disease (*1*). Proinflammatory cells that infiltrate into the vascular wall secrete large amounts of reactive oxygen species (ROS) and oxidative enzymes and lead to oxidative modification of low-density lipoprotein (oxLDL). OxLDL was taken up through the scavenger receptors, resulting in massive accumulation of lipids and further foam cell formation (*2*), the hallmark of early atherogenesis. Thus, attenuation of oxidative stress and inflammation is one of the major issues in the prevention of the initiation and progression of atherosclerosis.

To find the crucial component of functional foods for the prevention or treatment of atherosclerosis, many polyphenols having the LDL-antioxidant activity were isolated from various fruits, vegetables, and beverage plants (*3–7*). *Pteris ensiformis* Burm. (sword brake fern) is one of the most popular herbs used in beverages in Taiwan for hundreds of years. Due to its bitter flavor, the whole plant of sword brake fern is traditionally blended with other herbs, such as *Mentha arvensis*, *Scoparia dulcis*, or *Wedelia chinensis*, and cooked in water for a long time to remove its unpleasant taste. After the addition of brown sugar to the aqueous filtrate, the resulting beverage is ready to serve cold as a common soft drink. Although there are no official data regarding annual consumption, it is relatively easy to spot vendors of traditional herbal drinks in traditional markets.

It has been previously demonstrated that the aqueous extract of sword brake fern (SBF) exerts immunomodulatory effect by inhibiting the release of TNF-α, IL-1β, IL-6, NO, and PGE_2 in lipopolysaccharide (LPS)-activated murine macrophages, RAW 264.7 cells (*8*). Recently it was found that caffeic acid and two novel compounds, 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside and hispidin 4-*O*-β-D-glucopyranoside (**Figure 1**), were among the major polyphenol compounds in SBF, with contents of 2.64 ± 0.23, 9.99 ± 0.17, and 4.11 ± 0.08 mg/g of dry weight, respectively. They were the antioxidant principals analyzed by DPPH bleaching and Trolox equivalent antioxidant capacity (TEAC) analyses (*9*).

Although we have published papers regarding the free radical scavenging activity and phenolic constituents of SBF, little is known about its activity on the inhibition of oxidative burst in human polymorphonuclear neutrophils (PMN) or human LDL oxidation, which is the compelling issue in pinpointing new

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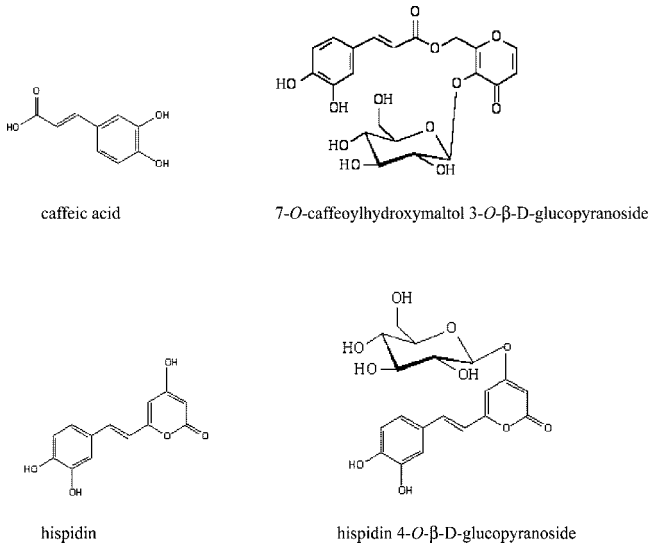

Figure 1. Structures of caffeic acid, 7-*O*-caffeoylhydroxymaltol 3-*O*-*β*-D-glucopyranoside, hispidin, and hispidin 4-*O*-*β*-D-glucopyranoside.

potential cardioprotective uses for a traditional remedy. In addition, the antioxidant activity of these two identified compounds has never been compared with aglycone in a lipid or primary leukocyte system. In this regard, the aim of the present study is to address the hypothesis that SBF and its newly isolated antioxidant components are inhibitors of LDL oxidation and oxidative burst and to compare the potency of these two new compounds with their aglycones, caffeic acid and hispidin.

MATERIALS AND METHODS

Chemicals. Luminol, DL-α-tocopherol, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (*f*MLP), hispidin, caffeic acid, and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated. Solvents were from Merck (Darmstadt, Germany).

Plant. Sword brake fern (*P. ensiformis* Burm.) was obtained from the Taitung District Agricultural Research and Extension Station, Taitung, Taiwan, and was identified by Dr. Jenq-Jer Yang of the Department of Pharmacy, Chia-Nan University of Pharmacy and Science. Voucher specimens were deposited as PE-0104 in the herbarium of the Department of Biotechnology, Chia-Nan University of Pharmacy and Science.

Aqueous Extraction of Sword Brake Fern and Purification of 7-*O*-Caffeoylhydroxymaltol 3-*O*-*β*-D-Glucopyranoside and Hispidin 4-*O*-*β*-D-Glucopyranoside. Fresh whole plants (8.1 kg wet weight) of sword brake fern were cut into small pieces and extracted with boiling water (3 × 20 L). One liter of the combined aqueous extract was lyophilized to yield a dark brown powder (ca. 5.7 g), which was redissolved in deionized water prior to use. It was denoted as the crude aqueous extract, SBF.

The rest of the aqueous extract was concentrated to a small volume and partitioned with chloroform to yield the chloroform and aqueous layers. The resulting aqueous layer was further partitioned with n-butanol to give n-butanol and aqueous layers. The n-butanol layer (30.0 g) was then passed through Diaion HP20SS chromatography and was eluted with water and methanol (1:0 V0:1) to give five fractions (A1 VA5).

7-*O*-Caffeoylhydroxymaltol 3-*O*-*β*-D-glucopyranoside (ca. 100.0 mg) was obtained by recrystallization of the marc of fraction A3 (3.6 g) with methanol and water (1:1). The rest of fraction A3 was then passed through preparative reverse-phase HPLC (Purospner, 20 × 250 mm, methanol/water = 1:1, flow rate = 3 mL/min) to yield hispidin 4-*O*-*β*-D-glucopyranoside (ca. 100.0 mg). The structural identification of these two compounds was determined and published as described previously (*9*).

Preparation and Oxidation of LDL. LDL (d = 1.019–1.063) was prepared from the plasma of anonymous donors from the Tainan Blood Center (Tainan, Taiwan) by sequential ultracentrifugation (*10*). Lipoprotein was desalted and concentrated by filtration (Amicon Ultra-4, Millipore, Beverly, MA) against PBS at 450*g* and 4 °C for 120 min. The protein concentration was measured according to the method of Bradford (*11*), using bovine serum albumin as a standard. Oxidation of LDL was carried out by incubating EDTA-free LDL (0.1 mg/mL) with 10 μM Cu^{2+} in PBS in the presence of vehicle or different concentrations of test reagents, at 37 °C.

Analysis of LDL Oxidation. Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (*12*). The quantity of conjugated dienes in LDL was assessed by monitoring the change at 234 nm (ΔA_{234}) (*13*). To measure the electrophoretic mobility, the oxLDL was concentrated by filtration (Microcon YM-3, Millipore) at 450*g* and 4 °C for 120 min. About 1–2 μL of each concentrated sample was loaded onto Titan lipoprotein gel (Helena Laboratories, Beaumont, TX) and run at 80 V for 45 min. The electrophoretic mobility of LDL was determined by Fat Red 7B staining according to the manufacturer's instructions.

Preparation of Human Polymorphonuclear Neutrophils (PMN). PMN were isolated from heparinized blood donated by healthy volunteers using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation according to the manufacturer's instructions. The isolated PMN were resuspended in RPMI-1640 medium containing 2 mM glutamine and 2.5% autologous plasma.

Measurement of *f*MLP-Induced ROS Production in Human PMN. ROS production was measured using modified luminol-enhanced chemical luminescence (CL) (*14*). The CL response of PMN was measured using a microtiter plate luminometer within 5 h after blood collection. Each well contained 3 × 10^5 PMN, 1 mM luminol, and vehicle (ddH_2O for SBF and 0.5% DMSO for compounds) or tested substance and was incubated at 37 °C in 5% CO_2 for 15 min. Activator, 10 μM *f*MLP (Sigma), was then added, and the light emission, expressed as relative light units (RLU), was monitored every 1 min for 5 s during a 10 min observation period, and the kinetic curve was obtained.

Statistical Analysis. All experiments were repeated at least three times. The results were analyzed by Student's unpaired t test, and a p value of <0.05 was taken to be significant.

RESULTS

Inhibition of TBARS Formation. It is recognized that oxidatively modified oxLDL plays an important role in the generation and progression of atherosclerotic plaque (*15*). Three different approaches were employed to measure changes in parameters associated with LDL oxidation to evaluate the effect of SBF in preventing copper-induced oxidation of LDL. These included formation of TBARS and conjugated dienes during lipid peroxidation and increase in the electrophoretic mobility of LDL due to apolipoprotein B100 modification (*16*). Incubation of LDL (0.1 mg/mL) with Cu^{2+} (10 μM) at 37 °C for 3 h increased the malondialdehyde (MDA) formation from 1.95 ± 0.20 to 59.04 ± 2.07 nmol/mg of LDL. Addition of SBF, 7-*O*-caffeoylhydroxymaltol 3-*O*-*β*-D-glucopyranoside, hispidin 4-*O*-*β*-D-glucopyranoside, and their aglycones dose-dependently inhibited MDA formation, and the IC_{50} values are shown in **Table 1**. The result showed that SBF was a strong antioxidant with IC_{50} of 1.77 ± 0.5 μg/mL. The inhibitory effects of 7-*O*-caffeoylhydroxymaltol 3-*O*-*β*-D-glucopyranoside and hispidin 4-*O*-*β*-D-glucopyranoside were less potent than those of their aglycones, caffeic acid and hispidin, respectively; nevertheless, they were markedly more active than the positive control, DL-α-tocopherol.

Inhibition of Conjugated Diene Formation. Oxidation of LDL is accompanied by an increase in absorbance at 234 nm,

Table 1. Inhibition of MDA Formation by the Aqueous Extract of Sword Brake Fern (SBF) as well as Caffeic Acid and Hispidin Derivatives

test reagent	IC_{50}[a]
SBF	1.77 ± 0.05 μg/mL
7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside	5.76 ± 0.56 μM
caffeic acid	3.08 ± 0.38 μM
hispidin 4-*O*-β-D-glucopyranoside	2.63 ± 0.27 μM
hispidin	1.78 ± 0.03 μM
α-tocopherol	14.17 ± 0.71 μM

[a] IC_{50} was the concentration necessary for a 50% reduction of MDA formation in Cu^{2+}-mediated LDL oxidation as described under Materials and Methods. Values represent means ± SEM ($n = 3$).

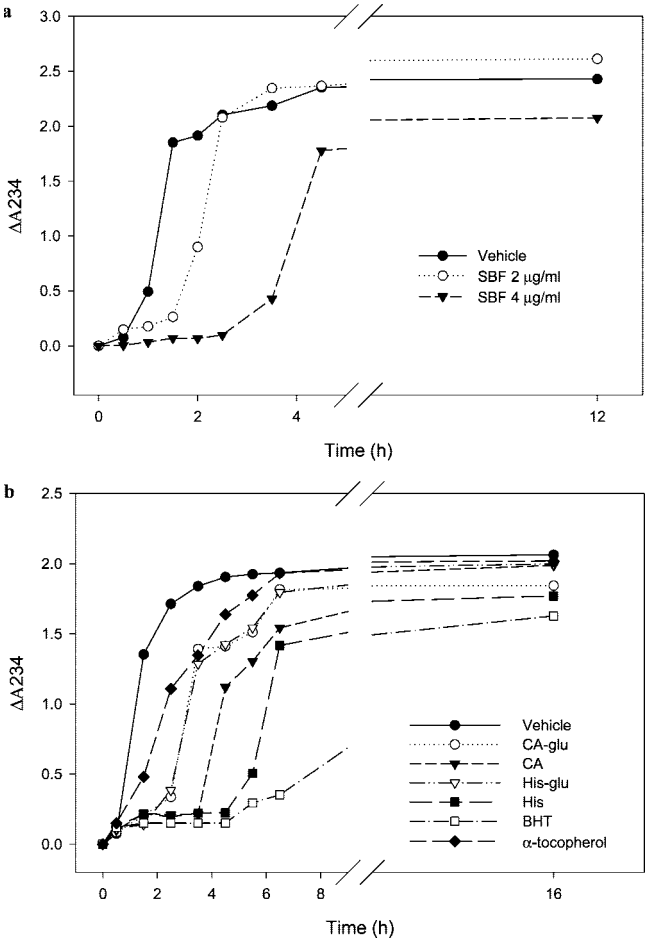

Figure 2. Aqueous extracts of sword brake fern (SBF), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, hispidin 4-*O*-β-D-glucopyranoside, and their aglycones inhibit conjugated diene formation in copper-induced LDL: (**a**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle (ddH_2O) or SBF (2 or 4 μg/mL) at 37 °C for indicated period; (**b**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle (DMSO), 10 μM of caffeic acid (CA), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (CA-glu), hispidin (His), hispidin 4-*O*-β-D-glucopyranoside (His-glu) or references, DL-α-tocopherol and BTH, at 37 °C for indicated period. The formation of conjugated diene was measured by change in absorbance at 234 nm (ΔA_{234}). This experiment was repeated three times with similar results.

due to the formation of conjugated dienes in constituent polyenoic fatty acids (*17*). As shown in **Figure 2A**, the lag phase of diene formation in vehicle control was 0.5 h. SBF-treated LDL showed a longer resistance to copper-initiated LDL oxidation. The lag phases prolonged to 1.5 and 2.5 h for 2 and 4 μg/mL of SBF-treated LDL, respectively.

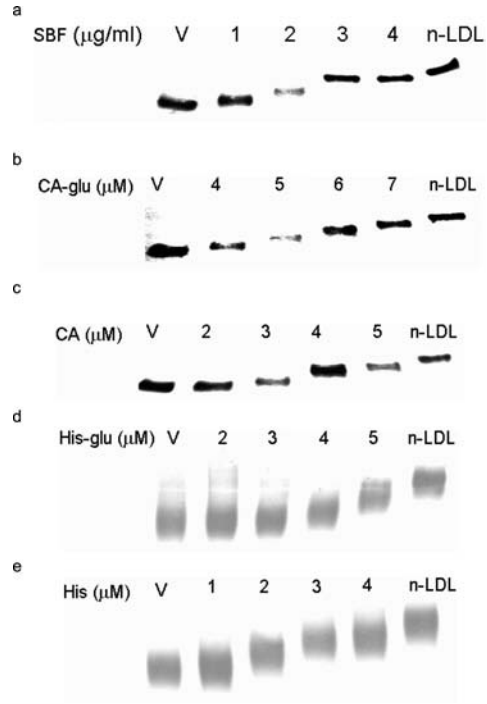

Figure 3. Aqueous extract of sword brake fern (SBF), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, hispidin 4-*O*-β-D-glucopyranoside, and their aglycones inhibits electrophoretic mobility in copper-induced LDL. Native LDL (n-LDL) (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle (V) or indicated concentrations of (**a**) SBF, (**b**) 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (CA-glu), (**c**) caffeic acid (CA), (**d**) hispidin 4-*O*-β-D-glucopyranoside (His-glu), or (**e**) hispidin (His) for 3 h. Concentrated LDL (1−2 μL) was separated on Titan Lipoprotein Gel and stained with Fat Red 7B.

Figure 2B also demonstrated that the lag stage of diene formation was prolonged when 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, caffeic acid, hispidin 4-*O*-β-D-glucopyranoside, or hispidin (10 μM) was added during LDL oxidation. The order of inhibitory potency of the tested substance was BHT > hispidin > caffeic acid >7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside ≈ hispidin 4-*O*-β-D-glucopyranoside > DL-α-tocopherol.

Inhibition of LDL Electrophoretic Mobility Change. The ability of SBF to inhibit the alteration in the surface charge of the apolipoprotein B100 when LDL was incubated with copper ions was monitored by electrophoretic mobility in agarose gel electrophoresis. **Figure 3A** showed that treatment of native LDL (n-LDL, 0.1 mg/mL) with Cu^{2+} (10 μM) in vehicle at 37 °C for 3 h increased electrophoretic mobility. LDL treated with SBF (1−4 μg/mL) reduced copper-induced LDL net charge change dose-dependently as indicated by decreased mobility shift. SBF (2 μg/mL) reduced the electrophoretic mobility by about 50%, as compared with the vehicle control (V, water only).

Panels **b** and **c** of **Figure 3** show that 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside and cafffeic acid significantly reduced copper-induced LDL net charge change. 7-*O*-Caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside and cafffeic acid (5 μM) reduced the electrophoretic mobility by about 50 and 75%, respectively, as compared with the vehicle control (V, DMSO). Panels **d** and **e** of **Figure 3** show that hispidin 4-O-β-D-glucopyranoside and its aglycone, hispidin, also significantly reduced electrophoretic mobility shift as compared with the vehicle group (V, DMSO), and the estimated IC_{50} values were about 5 and 2.5 μM, respectively. Their patterns were generally

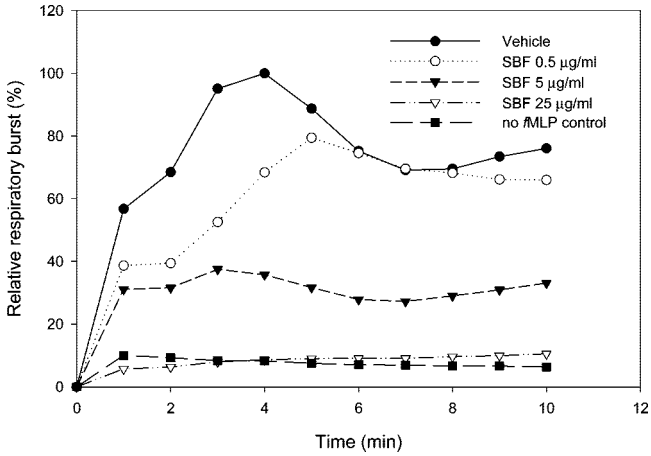

Figure 4. Aqueous extract of sword brake fern (SBF) inhibits *f*MLP-induced ROS production in human PMN. Each well contained 3×10^5 PMN, 1 mM luminol, and vehicle (ddH_2O) or indicated concentration of SBF and was incubated at 37 °C in 5% CO_2 for 15 min. Activator, 10 μM *f*MLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 1 min for 5 s during a 10 min observation period, and the kinetic curve was obtained. This experiment was repeated three times with similar results.

Table 2. Effect of Caffeic Acid and Hispidin Derivatives on the Inhibition of *f*MLP Induced ROS Production in PMN

test reagent	IC_{50}[a]
7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside	11.24 ± 2.37 μM
caffeic acid	3.06 ± 0.56 μM
hispidin 4-*O*-β-D-glucopyranoside	9.88 ± 0.15 μM
hispidin	5.04 ± 1.87 μM

[a] IC_{50} was the concentration necessary for a reduction 50% of luminol-enhanced chemical luminescence in *f*MLP-induced PMN as described under Materials and Methods. Values represent means ± SEM ($n = 3$).

consistent with those seen for the lag-phase measurements. DL-α-Tocopherol showed a lower antioxidant activity as compared to the tested substances at equimolar concentrations (data not shown).

Inhibition of Neutrophil ROS Production. The chemoattractant *f*MLP interacts with PMN, generating signals that induce activation of NADPH oxidase, which in turn produces the ROS. Both extra- and intracellular production of ROS can be detected by luminol-enhanced CL as shown in **Figure 4**. Treatment of PMN with SBF reduced *f*MLP-induced production of ROS in a dose-dependent manner. A complete inhibition was observed in 25 μg/mL of SBF-treated PMN. Likewise, a dose-dependent effect was observed in 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, hispidin 4-*O*-β-D-glucopyranoside or their aglycone-treated PMN. The IC_{50} values are shown in **Table 2**. 7-*O*-Caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside and hispidin 4-*O*-β-D-glucopyranoside suppressed *f*MLP-stimulated oxidative burst in PMN less potently than their aglycones.

DISCUSSION

The underlying pathophysiology of atherosclerosis is attributed to endothelial injury induced by oxidative stress, which subsequently promotes atherogenesis via oxidation of LDL (*18*). This process is perpetuated by infiltration of inflammatory cells, such as neutrophils and macrophages, into the site of injury (*18*). The present studies indicate that SBF, 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, hispidin 4-*O*-β-D-glucopyranoside, and their aglycones can prevent atherogenesis via inhibiting copper-catalyzed oxidation of LDL and neutrophil ROS production in vitro. In the LDL oxidation model, the protective property was evidenced by the antioxidant activity of these substances toward both the apolipoprotein moiety of LDL, with decrease oin the electrophoretic mobility shift, and the lipid part of LDL, with delayed diene formation and less MDA formation. In the case of oxidative burst in PMN, the protective property was confirmed by the decreased luminol-enhanced CL.

Caffeic acid (3,4-dihydroxycinnamic acid) is among the major hydroxycinnamic acids widely present in nature and is a potent antioxidant (*19*, *20*). In plants, hydroxycinnamic acids can also be covalently linked to cell wall polysaccharides or esterified with lignin (*21*), but little is known about the precise positions, abundance, or chemical reactivity of these bound derivatives in fruits and other plant foods. It has been shown that the electron-donating or -withdrawing properties of the phenolic acids are related to their antioxidant effect (*22*). Esterification with alkyl group may not decrease the H-donating ability of the phenolic ring, and the reducing capacity was not different from that of the acids (*23*). We have previously found that caffeic acid and 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside exhibited compatible reducing properties, as assessed by DPPH bleaching and TEAC experiments previously (*9*), indicating that esterification of caffeic acid with hydroxymaltol 3-*O*-β-D-glucopyranoside had little or no effect on its reducing property.

On the other hand, it has been demonstrated that esterification of polyphenolic acids had various effects upon oxidation experienced in a lipid medium, depending on both the acid and the nature of the ester (*24*). This was supported by the fact that decreased antioxidant activity in lipid systems was found when caffeic acid was esterified with quinic acid (*25*). The high efficiency of caffeic acid inhibiting Cu^{2+}-mediated LDL has also been attributed to its copper ion chelating and alkoxyl radical scavenging activities (*26*). Because the catechol moiety for copper ion chelating was preserved in 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside, the decreased antioxidant activity may be due to changes in solubility and partition of caffeic acid in the LDL system.

Caffeic acid is an inhibitor of 5-lipoxygenase activity (*27*), which is required for neutrophil adherence and chemotaxis (*28*). Therefore, the inhibition of ROS production by caffeic acid and its derivative may be due to blockage of neutrophil reactivity to the chemoattractant, *f*MLP.

Hispidin [6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone] was first found in the basidiomycete *Inonotus hispidus* (formerly *Polyporus hispidus*). It is a protein kinase C (PKC) inhibitor and is preferentially cytotoxic to cancer cells (*29*). Hispidin and its derivatives have also been shown to be potent free radical scavengers (*30*, *31*). We have previously shown that both hispidin and hispidin 4-*O*-β-D-glucopyranoside possessed compatible DPPH and ABTS radical scavenging activities (*9*). Herein, we found that both hispidin and hispidin 4-*O*-β-D-glucopyranoside strongly inhibited Cu^{2+}-mediated LDL oxidation and that the former was the stronger. Similar to those for caffeic acid and its derivative, the antioxidant potential may also be ascribed to copper chelating activity due to the existence of a catechol moiety, whereas the decreased activity of hispidin 4-*O*-β-D-glucopyranoside as compared with hispidin may be also due to lower solubility in the LDL system.

It is well-known that PKC promotes cytoskeletal and membrane association of cytosolic NADPH oxidase components, and,

as a result, treatment of phagocytes with PKC inhibitor can block oxidative burst (*32*). This may explain why hispidin and hispidin 4-*O*-*β*-D-glucopyranoside inhibited *f*MLP-stimulated neutrophil oxidative burst.

Apparently, the antioxidant activities of SBF and phenolic components tested here are distinct from those of other commercial antioxidants, such as α-tocopherol, in that these substances at least have three distinct antioxidant properties. First, they are chemical antioxidants and are able to bind to and scavenge ROS. Second, they are copper chelators. Finally, they have biological activity that suppresses ROS generation in *f*MLP-stimulated PMN. The last is of greater importance because it suppresses the enzymatic generation of ROS and would induce a marked reduction in ROS.

ABBREVIATIONS USED

CL, chemiluminescence; *f*MLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; LDL, low-density lipoprotein; MDA, malondialdehyde; PBS, phosphate buffered saline; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; SBF, aqueous extract of sword brake fern; TBARS, thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant capacity.

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