Arch Pharm Res Vol 28, No 10, 1122-1126, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Tungtungmadic Acid, a Novel Antioxidant, from *Salicornia herbacea*

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(Received August 28, 2005)

Tungtungmadic acid (3-caffeoyl-4-dihydrocaffeoyl quinic acid) is a new chlorogenic acid derivative that was isolated from the *Salicornia herbacea*. The structure of tungtungmadic acid was determined using chemical and spectral analysis. The antioxidant activity of tungtungmadic acid was evaluated using various antioxidant assays, including free radical scavenging, lipid peroxidation and hydroxyl radical-induced DNA strand breaks assays. Tungtungmadic acid (IC_{50} = 5.1 μM and 9.3 μM) was found to have higher antioxidant activity in the DPPH scavenging assay as well as in the iron-induced liver microsomal lipid peroxidation system. In addition, the tungtungmadic acid was also effective in protecting the plasmid DNA against strand breakage induced by hydroxyl radicals.

Key words: *Salicornia herbacea*, 3-Caffeoyl-4-dihydrocaffeoyl quinic acid, Tungtungmadic acid, Antioxidant activity

INTRODUCTION

Recently, there has been a global trend toward the use of natural phytochemicals present in natural resources as antioxidants, such as fruits, vegetables, oilseeds, and herbs (Wang *et al.*, 1997; Kitts *et al.*, 2000). Natural antioxidants can be used in the food industry, and there is evidence suggesting that these substances can exert their antioxidant effects within the human body (Rice-Evans *et al.*, 1995).

Salicomia herbacea, which is commonly known as Tungtungmadi in Korea, is one of the halophytes that can grow in salt marshes, or salt fields along the coastline in Korea (Kim and Song, 1983; Lee *et al.*, 2004). Some people living in coastal areas have used it as both a traditional medicine as well as a seasoned vegetable. This plant has previously been shown to modulate the production of cytokines and the release of nitric oxide in macrophages (Im *et al.*, 2003). However, there is little information on the active constituents in *S. herbacea* that are responsible for its antioxidant activity.

In an ongoing investigation into antioxidative compounds from natural products, it was found that an ethyl acetate soluble fraction of *S. herbacea* exhibited potent antioxidant capacity based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. A new chlorogenic acid derivative compound, tungtungmadic acid (3-caffeoyl, 4-dihydrocaffeoyl quinic acid), was isolated using a bioassay-directed chromatographic separation technique. The study reports the isolation and characterization of tungtungmadic acid from the halophyte, *S. herbacea,* collected from Korea along with its antioxidative activity.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid, L-(-)-ascorbic acid, ferrous sulfate, 1,1,3,3-tetraethoxypropan, nitrilotriacetic acid, and hydrogen peroxide were purchased from the Sigma Chemical Co. (St. Louis, MO).

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All other chemicals and solvents were of the highest grade commercially available.

Plant materials

The entire plants of *S. herbaceae* were collected from the coastal line of Busan, Korea, in August. The plant material was identified at the Pharmacognosy Laboratory, College of Pharmacy, Chungnam National University, Taejon, Korea. The plants were washed with water and dried in a dark, well-ventilated place.

Extraction and isolation

The air-dried samples (3 kg) were cut into pieces using a fodder-chopper and extracted with 80% methanol at room temperature for several days. The MeOH extract was evaporated and partitioned with EtOAC. The aqueous phase was applied to a reversed silica gel (Europrep 60-60 C18) and eluted with an aqueous acetonitrile solution (10%, 30%, 50%, and finally, 80%). The active fraction was further purified by Sephadex LH-20 using MeOH as the eluent, and HPLC on YMC-ODS-AM column under acidic conditions (30% MeOH in 0.1% acetic acid solution) afford **1** (24 mg).

General conditions

Thin-layer chromatography (TLC) was performed on Kieselgel 60 F 254 plates (20 × 20 cm, 0.25 mm; Merck) with compounds being detected by UV. The UV and IR spectra were recorded on a Shimazu UV-260 spectrophotometer and a Laser Precision Analect RFX-65S FT-IR spectrometer, respectively. The NMR spectra were obtained using a Varian spectrometer in CD_3OD with TMS as the internal standard. The chemical shifts are given in ppm values. The EIMS and HREIMS spectra were obtained using a Hewlett-Packard Model HP 5989A EIMS and a JEOL JMS-HX 110A mass spectrometer, respectively, operating at 70 eV. The TLC was performed on silica gel (Merck, Kieselgel 60 F254).

DPPH scavenging assay

An assay for the DPPH free radical scavenging potential was based on the scavenging activity of stable DPPH free radicals (Chen *et al.*, 1999). The reaction mixtures containing the test samples dissolved in methanol and 200 μM DPPH in an ethanol solution in a 96-well microtiter plate were incubated at 37°C for 30 min. After the reaction, the absorbance was measured at 520 nm, and percentage inhibition was calculated. The IC_{50} values denote the concentration of a sample required to scavenge 50% of the DPPH free radicals.

Lipid peroxidation inhibition assay

A murine liver homogenate from the young male ICR mice weighing 20-25 g was used. The reaction mixture was composed of 0.25 mL of the liver homogenate, 0.1 mL of a Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of a solution containing various concentrations of the test samples. Malondialdehyde (MDA), which is the lipid peroxidation product in the cells, was assayed according to a thiobarbituric acid fluorometric method using 1,1,3,3-tetramethoxypropane as the standard (Giinther *et al.*, 1995). The appropriate controls were performed in an attempt to eliminate any possible interference with the thiobarbituric acid assay. The percent inhibition of lipid peroxidation by the sample was calculated. The IC_{50} values indictaes the sample concentration required to inhibit 50% of microsomal lipid peroxidation.

Assay of oxidative DNA single strand breaks

The DNA single strand breaks in supercoiled DNA were examined after agarose gel electrophoresis. In order to prepare the Fe^{+3}-nitrilotriacetic acid (NTA), $FeCl_3$ was dissolved in a 20 mM phosphate buffer to a Fe^{+3} to a NTA molar ratio of 1:1, and the pH was adjusted to 7.4. The PCMV-β plasmid DNA (0.5 μg) was treated with 100 μM H_2O_2, 100 μM Fe^{3+}-NTA in a 50 mM KH_2PO_4-KOH buffer at pH 7.4 with or without the test samples. The reaction solution (30 μL) was mixed with 3 μL of an electrophoresis-loading buffer and placed onto a 1.0% agarose gel prepared in a TAE buffer (40 mM Tris acetate, 2 mM EDTA). After electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide for 10 min, destained for 30 min, visualized under UV light, and then photographed. The DNA single strand breaks were quantitatively analyzed by capturing the gel images on a Gel Doc Image Analysis System (Kodak) and measuring the density of the supercoiled (sc) DNA band and the open circle (oc) DNA band using NIH Image software (Bethesda, MD). The level of DNA cleavage activity is expressed in terms of the percentage of scDNA to ocDNA conversion according to the following equation: DNA cleavage activity = ([% of $scDNA]_{control}$ [% of $scDNA]_{sample}$ × 100)/[% of $scDNA]_{control}$.

Statistical analysis

All experiments were performed at least three times. The significance of the difference was calculated using a Student's *t* test, and values <0.05 were considered significant.

RESULTS AND DISCUSSION

The aim of this study was to isolate and characterize the new antioxidant compounds from *S. herbacea*, which is commonly known as Tungtungmadi in Korea, as well as to evaluate their antioxidant activity. Compound **1** was

isolated for the first time using antioxidant activity-directed purification including the DPPH method.

HRMS showed that compound **1** had a molecular formula of $C_{25}H_{26}O_{12}$. The ^{1}H-NMR spectrum of compound **1** showed an olefinic proton at 6.26 and 7.50 ppm with coupling constants of 15.9 Hz, indicating that the pair of protons was *trans* to each other. A pair of tri-substituted, and an aromatic moiety, which was indicated by two pairs of three ABX protons at 6.48 to7.04 ppm and an adjacent two methylene protons at 2.55 and 2.75, were the characteristic signals of the ^{1}H-NMR spectrum (Table I). The UV absorption at 330 nm is consistent with an unsaturated carbonyl chromophore, conjugated with an aromatic residue. These results indicated that compound **1** contained a trans-hydroxycinnamoyl moiety and a dihydrohydroxycinnamoyl moiety. There was a signal at 7.04 ppm, which is typical for the H-2 in the caffeic acid moiety. This confirmed that compound **1** contained a caffeoyl moiety, a dihydrocaffeoyl moiety, and a quinic acid. The ^{1}H-NMR data of the quinic acid moiety (Table I) also demonstrated that two of the three CHOH moieties in the cyclohexane ring were acylated, as evidenced by the pronounced downfield shifts of the geminal proton signals [δ_H = 5.57 ppm (H-3) and 4.88 (H-4), respectively] while one of these groups was unsubstituted [δ_H = 4.28 ppm (H-5)]. The HHCOSY spectrum showed a crosspeak from the signal assignable to the proton of the unsubstituted CHOH moiety to the H4 signal. Therefore, the acylation of positions 3 and 4 was established. The HMBC showed a crosspeak from the signal that was assigned to the H4 proton to the C9" of the dihydrocaffeoyl moiety. Conclusively, the structure of compound **1** was established as 3-caffeoyl, 4-dihydrocaffeoyl quinic acid (Fig. 1), which is a new natural chlorogenic acid derivative, and was given the name, tungtungmadic acid.

Table I. NMR data of tungtungmadic acid (**1**)

		δ_H	δ_C
Quinic acid moiety	1	-	75.1
	2	2.08 1H, m 2.30 1H, dd (*J* 15, 3.9 Hz)	36.9
	3	5.57 1H, q (*J* 3.9 Hz)	69.9
	4	4.88 1H, m	76.6
	5	4.281H dt (*J* 9.3, 3.9 Hz)	65.7
	6	2.06 1H, m 2.17 1H, m	41.9
	7 (carboxyl)	-	177.8
Caffeoyl moiety	1'	-	127.0
	2'	7.04 1H, d (*J* 2.1 Hz)	115.1
	3'	-	149.5
	4'	-	146.7
	5'	6.77 1H, d (*J* 8.1 Hz)	116.5
	6'	6.94 1H, dd (*J* 8.1, 2.1 Hz)	123.0
	7'	6.26 1H, d (*J* 15.9 Hz)	115.2
	8'	7.50 1H, d (*J* 15.9 Hz)	147.3
	9'	-	168.6
Dihydrocaffeoyl moiety	1"	-	133.5
	2"	6.65 1H, d (*J* 2.1 Hz)	116.3
	3"	-	146.1
	4"	-	144.5
	5"	6.64 1H, d (*J* 8.1 Hz)	116.3
	6"	6.48 1H, d (*J* 8.1, 2.1 Hz)	120.4
	7"	2.75 2H, m	31.2
	8"	2.55 2H, m	37.2
	9"	-	174.0

Free radical scavengers are believed to be prospects as protective or therapeutic agents against these diseases. In the course of our preliminary screening program, it was found that compound **1** exhibited strong free radical scavenging activity in the DPPH assay. In order to determine the antioxidant potential of tungtungmadic acid, its DPPH free radical scavenging and lipid peroxidation inhibitory activities were compared with those of α-tocopherol, and chlorogenic acid as well as other structurally related hydroxycinnamates, such as caffeic and ferulic acids (Table II). In the DPPH scavenging assay and the iron-induced liver microsomal lipid peroxidation system revealed that tungtungmadic acid (IC_{50} = 5.1 μM and 9.3 μM) had higher antioxidant activity than chlorogenic acid, caffeic acid, ferulic acid, and α-tocopherol. This shows that tungtungmadic acid can be a better antioxidant than the commercially available α-tocopherol. It has been reported that the ability to scavenge the DPPH radicals is related to the inhibition of lipid peroxidation (Ratty *et al.*, 1988; Rekka *et al.*, 1991).

The induction of a single strand break into supercoiled plasmid DNA leads to the formation of open circular DNA.

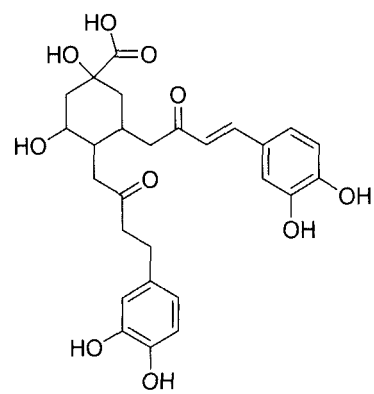

Fig. 1. Structure of tungtungmadic acid (3-caffeoyl-4-dihydrocaffeoyl quinic acid)

Table II. Antioxidant activity of tungtungmadic acid from *Salicornia herbacea* along with its related phenolics and α-tocopherol

Compounds	Antioxidant activity (IC_{50}, μM)	
	DPPH [a]	MDA [b]
Tuntungmadic acid	5.1 ± 0.37	9.3 ± 0.43
Chlorogenic acid	11.3 ± 0.22	22.3 ± 0.46
Caffeic acid	12.4 ± 0.64	21.4 ± 0.52
Ferulic acid	38.3 ± 0.35	45.5 ± 0.78
α-Tocopherol	49.7 ± 0.27	41.2 ± 0.37

[a] DPPH free radical scavenging activity.
[b] Inhibition of liver microsomal lipid peroxidation. Each value is the mean ± SD.

DNA is a sensitive target of hydroxyl radicals and Fe^{3+}-NTA/H_2O_2 can cause strand breaks in isolated DNA (Cai *et al.*, 1998). Although the precise nature of the reactive species is unclear, a bound hydroxyl radical or its equivalent derived from a reaction between Fe^{3+}-NTA/H_2O_2 has been suggested to mediate the DNA strand single breaks (Meneghini, 1997; Cai *et al.*, 1998). Therefore, in this study, the pMCV-β plasmid DNA cleavage by hydroxyl radicals generated by Fe^{3+}-NTA/H_2O_2 was measured using agarose gel electrophoresis in order to illustrate the protective effect of tungtungmadic acid on DNA strand breaks. As shown in Fig. 2, the incubation of plasmid DNA with Fe^{3+}-NTA/H_2O_2 resulted in the increased formation of open circle DNA, which indicates that Fe^{3+}-NTA/H_2O_2 can induce DNA single strand breaks. The addition of tungtungmadic acid to Fe^{3+}-NTA/H_2O_2 inhibited the conversion of super-coiled DNA to the open circle form in a dose-dependent manner. Moreover, almost complete protection by tungtungmadic acid was found at a dose of 20 μM. This indicates that tungtungmadic acid is a potent hydroxyl radical scavenger and can protect against Fe^{3+}-NTA/H_2O_2-mediated DNA single strand brakes.

Chlorogenic acid (5-*O*-caffeoylquinic acid; an ester of caffeic acid with quinic acid) is found in many plants such as coffee beans, apples, and blueberries. Chlorogenic acid is recognized as an antioxidant, because it inhibits hydroxyl radical formation and lipid peroxidation (Rice-Evans *et al.*, 1997; Jung *et al.*, 1999; Nakatani *et al.*, 2000; Zang *et al.*, 2003; Daglia *et al.*, 2004). There are reports showing that some chlorogenic acid isomers, such as neochlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid) from plums and prunes (Kasai *et al.*, 2000; Nakatani *et al.*, 2000), and the chlorogenic acid isomers have antioxidant activities (Nakatani *et al.*, 2000). The antioxidant mechanism of phenolics (chlorogenic acid, caffeic, and ferulic acids) has been suggested to occur *via* direct inhibition related to the H-donating ability of phenol (Rice-Evans *et al.*, 1997). The

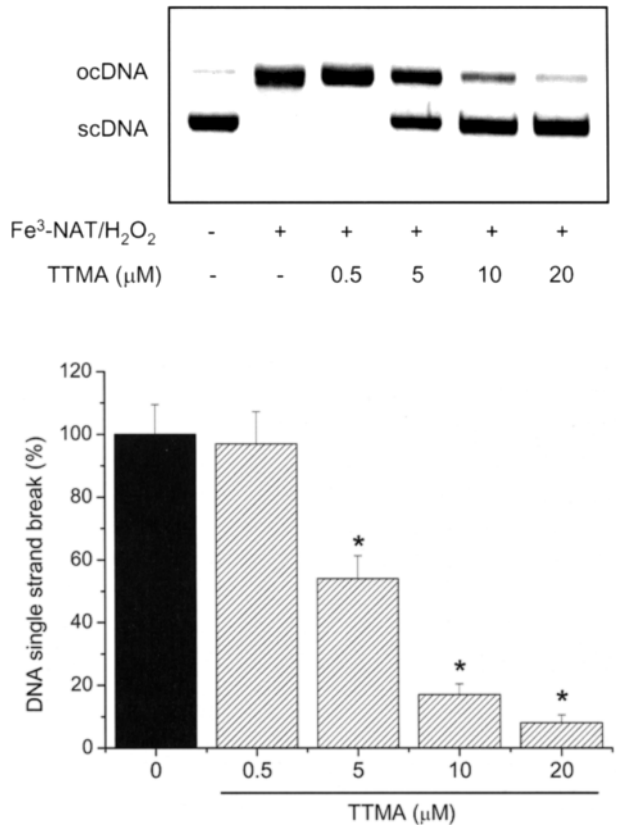

Fig. 2. Protective effect of tungtungmadic acid on Fe^{3+}-NTA/H_2O_2-induced DNA strand breaks. The pMCV-β plasmid DNA (0.5 μg/lane) was incubated with 100 μM Fe^{3+}-NTA and 100 μM H_2O_2 in the presence or absence of tungtungmadic acid at 37°C for 1 h. The DNA single strand breaks were determined as described in materials and methods. One of three representative experiments is shown. Each bar shows the mean ± SD of three independent experiments. *Significantly different from the Fe^{3+}-NTA/H_2O_2 group.

anti-radical activity of the phenolic compounds depends on their molecular structure, i.e. the availability of phenolic hydrogen as well as the possibility for stabilizing the resulting phenoxyl radicals formed by hydrogen donation (Silva *et al.*, 2000). Generally, the antioxidant activity was shown to increase with increasing number of hydroxyl groups. It is well known that free radicals cause several diseases, such as degenerative neuronal disease, coronary heart disease, and cancer. Furthermore, many biochemical and clinical studies suggest that natural and synthetic antioxidant compounds can help treat diseases mediated by oxidative stresses. This study demonstrated that tungtungmadic acid has excellent antioxidant activity. However, further investigation of the potential efficacy of tungtungmadic acid in preventing diseases caused by the overproduction of free radicals will be needed.

In summary, a new natural chlorogenic acid derivative from *S. herbacea*, which is commonly known as Tungtungmadi in Korea, was isolated and identified to be 3-caffeoyl, 4-dihydrocaffeoyl quinic acid, which was assigned

the name tungtungmadic acid. Tungtungmadic acid exhibited strong free radical scavenging activity and inhibited lipid peroxidation. Additional assay models investigating the antioxidant and inhibition mechanism in addition to *in vivo* studies of the antioxidant activities will be needed to address the bioavailability of tungtungmadic acid.

ACKNOWLEDGEMENT

This work was supported by Korea Research Foundation Grant (KRF-2002-041- F20068).

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