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Inhibitory Effects of *Orostachys japonicus* Extracts on the Formation of *N*-Nitrosodimethylamine

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In Korea, *Orostachys japonicus* has been used traditionally as a drug and health food. The aim of this study was to investigate possible inhibitory effects of *O. japonicus* extracts on the formation of *N*-nitrosodimethylamines (NDMA). Chloroform extraction was the most effective method for recovering the highest number of phenolic compounds and flavonoids; in these extracts the greatest nitrite-scavenging activity and inhibition of NDMA formation occurred at pH 2.5. The chloroform extract was separated into 10 fractions (J1−J10); fraction J4 inhibited NDMA formation by 90.1 ± 0.4%. This fraction was then separated into five subfractions (J4-1−J4-5) using a silica gel column. Subfractions J4-2 [(+)-catechin] and J4-4 (3,4-dihdroxybenzoic acid) inhibited NDMA formation by 89.5 ± 0.9 and 77.6 ± 0.8%, respectively.

KEYWORDS: NDMA; nitrite-scavenging ability; *Orostachys japonicus*; phenolics

INTRODUCTION

Exposure to carcinogenic *N*-nitroso compounds (NOC) can occur exogenously via consumption of food or endogenously by the reaction of secondary amines with nitrite under acidic conditions (*1*). NOCs are potential mutagens and carcinogens in animals and humans, even at low concentrations. Volatile *N*-nitrosamines (NA) are found in various foods, and thiocyanate is known to catalyze their formation, especially under acidic conditions (*2*−*5*). However, the level of nitrite in food or drinking water is generally very low (*6*). Nitrate itself is relatively nontoxic, but ≈5% of all ingested nitrate is converted to the more toxic nitrite in the oral cavity (*7*, *8*). There, nitrite is converted to nitrous acid and subsequently to N_2O_3 at low pH; in its deprotonated state, it reacts with secondary amines to form NAs. The formation of these carcinogenic substances may be inhibited by dietary antioxidants such as ascorbic acid, tocopherols, polyphenols, and allyl sulfur compounds (*9*, *10*). Consumption of whole strawberries, kale juice, garlic juice, Korean green tea, and Maesil (*Prunus mume* Sieb. et Zacc.), which contain antioxidants such as ascorbic acid, polyphenols, and allyl sulfur compounds, has been reported to be effective in inhibiting the formation of *N*-nitrosodimethylamine (NDMA) (*2*, *3*). These antioxidants inhibit NOC formation via rapidly reducing nitrosating species such as N_2O_3 into nitric oxide (NO) (*9*). Recently, the potency and absorption of natural antioxidants other than L-ascorbic acid, α-tocopherol, and carotenoids have been investigated, and neutralization of free radicals by these compounds may be a key factor in the prevention of cancer (*11*−*13*).

Orostachys japonicus A Berger (Crassulaceae) is a perennial herb that contains many medicinal compounds and is an ingredient in traditional oriental medicine. In Korea, it is referred to as Wa-song and is used traditionally as a general anti-inflammatory agent to treat hepatitis, boils, and hemorrhoids, as a hemostatic agent to treat hematemesis, epistaxis, and hematochezia, and as an anticancer agent (*14*).

The activity of *O. japonicus* against oxidative stress has been demonstrated both in vivo and in vitro, and the extract from this plant may be a natural source of free radical scavengers (*14*, *15*). We studied the ability of various *O. japonicus* extracts to scavenge nitrite and inhibit the formation of the carcinogen NDMA. In addition, we separated the extract that exhibited the greatest degree of inhibition of NDMA formation using a silica gel column and characterized the reactive substances using mass spectrometry (MS), ^{1}H nuclear magnetic resonance (^{1}H NMR), and ^{13}C nuclear magnetic resonance (^{13}C NMR).

MATERIALS AND METHODS

Reagents. Folin−Ciocalteu reagent, caffeic acid, quercetin, and dimethylamine (DMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium carbonate, aluminum nitrate, potassium acetate, sodium nitrite, sulfanilic acid, naphthylamine, and ammonium sulfamate

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Table 1. Total Phenolic and Flavonoid Contents of *O. japonicus* Extracts

extract	total phenols[a] (mg/100 g)	total flavonoids[b] (mg/100 g)
crude extract	909.2 ± 0.6d	77.3 ± 0.9c
hexane	721.7 ± 0.7c	99.8 ± 0.8e
chloroform	2003.3 ± 0.6f	624.2 ± 0.7f
ethyl acetate	1315.2 ± 0.9e	88.9 ± 0.6d
butanol	523.1 ± 0.9b	10.1 ± 0.1b
water	28.6 ± 0.3a	3.4 ± 0.1a

[a] Total phenolic content based on a standard curve generated from caffeic acid. Values (mean ± SD, $n = 3$) followed by the same letter are not significantly different at $p < 0.05$. [b] Total flavonoid content based on a standard curve generated from quercetin. Values (mean ± SD, $n = 3$) followed by the same letter are not significantly different at $p < 0.05$.

were purchased from Yakuri Pure Chemical Co. (Osaka, Japan). All other chemical reagents were of analytical grade.

Extraction and Isolation of *O. japonicus*. In November 2004, *O. japonicus* samples were collected from local medicinal herb cultivators in Jinju, Korea. Dried and powdered *O. japonicus* (2.5 kg) was extracted three times in methanol (1 L), and the organic solutions were combined, filtered, and concentrated using a rotary vacuum evaporator. The crude extract (60.3 g) was dissolved in 1 L of dH_2O and serially fractionated using 3 × 1 L volumes of *n*-hexane, chloroform, ethyl acetate, and butanol, followed by evaporation of each fraction in a vacuum to yield 10.4, 8.9, 13.8, 11.4, and 5.7 g of extract, respectively. The chloroform extract was chromatographed on a silica gel (800 g, 70−230 mesh; Merck) column and eluted with a hexane/ethyl acetate gradient (100:10 → 20:1), yielding 10 fractions (J1−J10). The active fraction J4 was refractionated by silica gel (700 g, 70−230 mesh) column chromatography, using a step-gradient elution of hexane/acetone (50:1 → 1:2), resulting in five subfractions (J4-1−J4-5). The subfractions that inhibited NDMA formation (J4-2 and J4-4) were subjected to column chromatography using silica gel (500 g, 70−230 mesh) with hexane/ethyl acetate (25:1 → 1:1), followed by crystallization and analyses using MS (JEOL) as well as ^{1}H NMR and ^{13}C NMR (Bruker DRX 500 MHz) spectra. Melting points were measured on a Thomas Scientific capillary melting point apparatus.

Determination of Total Phenolics and Flavonoids. The total phenolic content of the extracts was determined using the Folin−Denis method (*16*). Folin−Ciocalteu reagent (0.5 mL) and 10% sodium carbonate solution (0.5 mL) were added to each extract (1 mL) and mixed thoroughly; after 3 min, the absorbance was measured at 700 nm and compared to a caffeic acid calibration curve.

The flavonoid content of the extract was measured using the Moreno et al. method (*17*) against the dietary flavonoid quercetin. Half a milliliter of extract was mixed with 0.1 mL of 10% aluminum nitrate and 0.1 mL of 1 M potassium acetate and then diluted to 5 mL with 80% ethanol; absorbance at 415 nm was measured immediately.

Nitrite-Scavenging Ability of Extracts. The nitrite-scavenging ability of each extract was determined using the methods of Kato et al. (*18*) and Kim et al. (*19*) by measuring the absorbance at 520 nm to determine residual nitrite capacity. Extract (1 mL) was added to the 1 mL of 1 mM sodium nitrite at pH 2.5, 4.2, or 6.0 (prepared using a 0.2 M citric acid buffer), after which the reaction mixture was incubated in a water bath at 37 °C for 1 h. Five milliliters of 2% acetic acid and 0.4 mL of Griess reagent (a 1:1 ratio of 1% sulfanilic acid in 30% acetic acid and 1% naphthylamine in 30% acetic acid) were then added to the reaction mixture (1 mL) and incubated at room temperature for 15 min. Spectrophotometric analysis was performed at 520 nm to determine residual nitrite levels.

Inhibition of NDMA Formation by Extracts, Fractions, and Subfractions. For extracts, fractions (J1−J10), and subfractions (J4-1−J4-5), NDMA formation was monitored in 0.2 M citrate buffer (pH 2.5) containing 200 mM DMA and 100 mM sodium nitrite. The NDMA-generating solution was incubated at 37 °C for 1 h with gentle shaking in the presence or absence of extracts, fractions, and subfractions, respectively. The reactions were halted by the addition of 500 mg of ammonium sulfamate, and the NDMA was extracted with 1 mL of dichloromethane. The NDMA concentration in the extracts was determined using gas chromatography (GC; model 5890A, Hewlett-Packard, Avondale, PA) with a thermal energy analyzer (TEA; model 543, Thermo Electron Corp., Waltham, MA).

GC-TEA conditions were as follows: DB-5 fused silica capillary column, 30 m long, 0.53 mm i.d.; 5 mL/min flow rate; oven temperature programmed for 50−80 °C at 3 °C/min and for 80−180 °C at 10 °C/min; injection port temperature, 180 °C; pyrolyzer temperature, 550 °C; interface temperature, 200 °C; and analyzer pressure, 1.9 Torr. The detection limit of the equipment was 0.05 μg/kg. NDMA was confirmed by the disappearance of the peak after the sample was subjected to ultraviolet light. The inhibitory effect on NDMA formation was calculated by comparing the peak values from before and after the addition of sample.

Statistical Analyses. Values are expressed as means of three replicate determinations ± standard deviation (SD). All statistical analyses were

Table 2. Nitrite-Scavenging Abilities[a] (Percent) of *O. japonicus* Extracts at pH 2.5, 4.2, and 6.0

	concn of *O. japonicus* extract at pH 2.5			concn of *O. japonicus* extract at pH 4.2			concn of *O. japonicus* extract at pH 6.0		
extract	100 μg/mL	250 μg/mL	500 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL
crude extract	53.9 ± 0.4c	61.4 ± 0.8c	73.6 ± 0.7c	22.7 ± 0.4d	31.1 ± 0.7c	43.7 ± 0.2c	7.7 ± 0.3e	8.9 ± 0.1e	11.5 ± 0.3d
hexane	22.5 ± 0.6a	32.4 ± 0.5a	39.2 ± 0.7a	7.6 ± 0.2a	12.3 ± 0.5a	16.0 ± 0.6a	3.4 ± 0.4a	4.7 ± 0.1a	6.4 ± 0.4a
chloroform	74.8 ± 1.3e	85.9 ± 1.0e	91.6 ± 1.0e	52.8 ± 0.3f	62.6 ± 1.2e	78.9 ± 0.8e	13.1 ± 0.1f	22.1 ± 0.5f	35.4 ± 0.5f
ethyl acetate	59.4 ± 1.0d	69.7 ± 0.5d	79.8 ± 1.3d	34.3 ± 0.2e	42.4 ± 0.8d	51.6 ± 0.6d	4.8 ± 0.1c	9. 3± 0.2d	12.7 ± 0.1e
butanol	33.7 ± 0.6b	43.0 ± 0.6b	52.5 ± 0.7b	11.8 ± 0.6c	29.1 ± 0.3b	33.5 ± 0.7b	6.5 ± 0.1d	8.5 ± 0.1c	9.8 ± 0.1c
water	21.9 ± 0.5a	31.2 ± 1.1a	37.4 ± 0.6a	8.3 ± 0.3b	12.0 ± 0.5a	15.9 ± 0.4a	4.2 ± 0.1b	5.7 ± 0.1b	7.5 ± 0.1b

[a] Nitrite-scavenging abilities mean inhibition percent of extracts to control. Values (mean ± SD, $n = 3$) in the same column followed by the same letter are not significantly different at $p < 0.05$.

Table 3. Inhibition (Percent) of NDMA Formation[a] in *O. japonicus* Extracts at pH 2.5, 4.2, and 6.0

	concn of *O. japonicus* extract at pH 2.5			concn of *O. japonicus* extract at pH 4.2			concn of *O. japonicus* extract at pH 6.0		
extract	100 μg/mL	250 μg/mL	500 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL
crude extract	58.2 ± 0.2d	63.9 ± 0.9d	71.9 ± 0.5d	11.8 ± 0.8d	21.8 ± 0.8d	27.3 ± 0.4c	2.8 ± 0.1c	3.5 ± 0.1b	4.6 ± 0.1d
hexane	23.5 ± 0.1a	32.9 ± 0.6b	39.4 ± 0.5b	1.2 ± 0.1a	3.3 ± 0.1a	4.8 ± 0.1a	0.9 ± 0.1a	1.3 ± 0.1a	1.8 ± 0.1a
chloroform	75.3 ± 0.4f	85.5 ± 0.9f	90.3 ± 0.5f	32.5 ± 0.9f	43.4 ± 0.4f	49.6 ± 1.0e	10.1 ± 0.1e	12.7± 0.6d	19.0 ± 0.1f
ethyl acetate	59.7 ± 0.7e	69.1 ± 0.5e	78.3 ± 0.8e	17.5 ± 0.3e	25.2 ± 0.7e	29.8 ± 0.8d	3.4 ± 0.5d	4.3 ± 0.1c	5.0 ± 0.1e
butanol	41.8 ± 0.5c	48.9 ± 0.3c	51.4 ± 0.9c	4.4 ± 0.1c	8.5 ± 0.2c	12.2 ± 0.9b	2.1 ± 0.1b	3.5 ± 0.1b	4.3 ± 0.1c
water	26.5 ± 0.5b	28.5 ± 0.4a	32.9 ± 0.2a	2.2 ± 0.1b	4.2 ± 0.1b	5.6 ± 0.3a	1.2 ± 0.1a	1.7 ± 0.1a	2.2 ± 0.2b

[a] Inhibition of NDMA formation means inhibition percent of extracts to control. Values (mean ± SD, $n = 3$) in the same column followed by the same letter are not significantly different at $p < 0.05$.

Table 4. Inhibition of NDMA Formation in Chloroform Fractions and Subfractions at pH 2.5

chloroform fraction	inhibition of NDMA formation[a] (%)
fraction J1	22.4 ± 0.7d
fraction J2	42.1 ± 0.7f
fraction J3	11.5 ± 0.4b
fraction J4	90.1 ± 0.4j
fraction J5	54.8 ± 0.7i
fraction J6	44.3 ± 1.3g
fraction J7	34.3 ± 0.5e
fraction J8	52.3 ± 0.9h
fraction J9	13.0 ± 0.3c
fraction J10	8.6 ± 0.1a
after subfractionation by silica gel chromatography	
subfraction J4-1	37.5 ± 1.0c
subfraction J4-2	89.5 ± 0.9e
subfraction J4-3	26.2 ± 1.1b
subfraction J4-4	77.6 ± 0.8d
subfraction J4-5	17.4 ± 1.2a

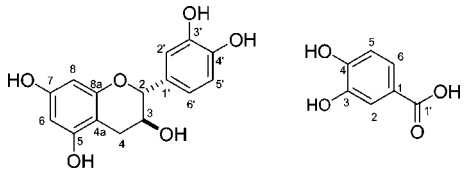
[a] Inhibition of NDMA formation means inhibition percent of extracts to control. Values (mean ± SD, $n = 3$) in the same column followed by the same letter are not significantly different at $p < 0.05$.

carried out using SPSS 12.0 for Windows. Values of $p < 0.05$ were considered to be significantly different.

RESULTS AND DISCUSSION

The highest total phenol and flavonoid contents were obtained in the chloroform extract (**Table 1**). Phenolic compounds are very important to plants due to the scavenging ability of their hydroxyl groups (*20*). They have many health-related properties such as anticancer, antiviral, and anti-inflammatory activities, as well as effects on capillary fragility and inhibition of platelet aggregation in humans (*21*).

All of the *O. japonicus* extracts exhibited a concentration-dependent increase in the ability to scavenge nitrite (**Table 2**) and inhibit NDMA formation (**Table 3**); these activities were greatest at pH 2.5 and reduced at higher pH values (4.2 and 6.0). At 500 μg/mL and pH 2.5, the chloroform extract demonstrated the highest nitrite-scavenging ability (91.6 ± 1.0%) and inhibition of NDMA formation (90.3 ± 0.5%; **Tables 2** and **3**). Thus, it is likely that both phenols and flavonoids of *O. japonicus* inhibited NDMA formation through their nitrite-scavenging abilities, and they may inhibit NA formation through the rapid reduction of N_2O_3 to NO. In agreement with the observations in this study, in vitro studies have shown that the optimum pH for the nitrosation reaction is between 2.0 and 3.4 (*22*), and Byun et al. (*23*) have reported that nitrite scavenging at pH 1.2 was greater than that at pH 4.2 or 6.0. At low pH, nitrite is converted to nitrous acid and subsequently to N_2O_3, which in its deprotonated form reacts with secondary amines to produce NAs (*24*). N_2O_3 is a powerful electrophilic nitrosating agent and has been shown to cause deamination of primary amines, as well as triggering the formation of NOCs (*25*).



(1) (2)

Figure 1. Structures of (+)-catechin (**1**) and 3,4-dihydroxybenzoic acid (**2**) isolated from *O. japonicus*.

In addition, our results concur with studies demonstrating that phenolic compounds inhibit the nitrosation of secondary amines by competing for the nitrosating agent (*26*). For example, Choi et al. (*2*) and Chung et al. (*3*) reported that Korean green tea and kale effectively reduced the exogenous and endogenous formation of NAs and suggested that phenolic compounds might act as nitrite scavengers; in addition, Lee et al. (*27*) demonstrated that the reaction of flavonols with nitrous acid protects against the formation of NA.

Fractions isolated from chloroform extract were assayed for their ability to inhibit formation of NDMA at pH 2.5, and fraction J4 exhibited the greatest inhibitory effect, followed by fractions J5 and J8 (90.1 ± 0.4, 54.8 ± 0.7, and 52.3 ± 0.9%, respectively; **Table 4**). Fraction J4 was separated into five subfractions (J4-1−J4-5), of which J4-2 demonstrated the greatest inhibition of NDMA formation, followed by subfrac-

Table 5. Spectral Data for the Compounds Isolated from *O. japonicus* in CD_3OD

compound	^{1}H NMR		^{13}C NMR		HREIMS (*m*/*z*)
(+)-catechin[a]	H-4β	2.51 (dd, J = 16.1, 8.1 Hz, 1H)	C-4	28.9	290.0791
	H-4α	2.85 (dd, J = 16.1, 5.4 Hz, 1H)	C-3	69.2	
	H-3	3.98 (m, H)	C-2	83.3	
	H-2	4.57 (d, J = 7.5 Hz, 1H)	C-8	96.0	
	H-6	5.86 (d, J = 2.3 Hz, 1H)	C-6	96.8	
	H-8	5.93 (d, J = 8.3, 1.9 Hz, 1H)	C-4a	101.3	
	H-6′	6.72 (dd, J = 8.3, 1.9 Hz, 1H)	C-2′	115.7	
	H-5′	6.76 (d, J = 1.5 Hz, 1H)	C-5′	116.5	
	H-2′	6.91 (d, J = 1.5 Hz, 1H)	C-6′	120.5	
			C-1′	132.7	
			C-3′	146.6	
			C-4′	146.7	
			C-8a	157.3	
			C-5	158.0	
			C-7	158.2	
3,4-dihydroxybenzoic acid[b]	H-5	6.88 (m, 1H)	C-2	114.5	154.0266
	H-2	7.50 (m, 1H)	C-5	116.4	
	H-6	7.53 (m, 1H)	C-1	121.7	
			C-6	122.8	
			C-3	144.6	
			C-4	150.1	
			C-1′	169.2	

[a] ^{1}H and ^{13}C NMR spectra recorded at 500 and 125 MHz, respectively. [b] ^{1}H and ^{13}C NMR spectra recorded at 300 and 75.462 MHz, respectively.

tions J4-4 and J4-1 (89.5 ± 0.9, 77.6 ± 0.8, and 37.9 ± 1.0%, respectively; **Table 4**).

Confirmation of the chemical structure in subfractions J4-2 and J4-4 was accomplished by comparing MS, ^{1}H NMR, and ^{13}C NMR data (**Figure 1**; **Table 5**). Subfraction J4-2 (1) formed a light orange powder with a mp of 172−175 °C and was identified as (+)-catechin using MS,^{1}H NMR, and ^{13}C NMR (**Table 5**). Subfraction J4-4 (2) formed a white powder with a mp of 199−202 °C that was identified as 3,4-dihydroxybenzoic acid (**Table 5**).

Carcinogenic NAs are produced by electrophilic reactions between nitrite or nitrous acid and secondary or tertiary amines under acidic conditions, and most phenolic compounds function as inhibitors of amine nitrosation (*28*). In this study we identified *O. japonicus* as a natural source of bioactive compounds such as phenols and flavonoids and found that a chloroform extract of this plant exhibited both a high nitrite-scavenging activity and inhibition of NDMA formation. Further separation of the chloroform extract indicated that fraction 4 and subfractions J4-2 and J4-4 demonstrated high levels of these activities and contained (+)-catechin and 3,4-dihydroxybenzoic acid, respectively. Catechol is known to be particularly effective in inhibiting nitrosation, as it reacts competitively with nitrite under acidic conditions, and catechin is one of the most widely consumed flavonoids (*28*, *29*). Thus, the intake of *O. japonicus* may lead to a reduced risk of cancer, and extracts may be beneficial as a natural source of nutritional additives.

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