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Antioxidant and cytotoxic activities of naphthalene derivatives from *Diospyros kaki*

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Abstract 4,8-Dihydroxy-5-methoxy-2-naphthaldehyde (Compound I) was isolated from blackened heartwood of *Diospyros kaki* and was methylated with diazomethane. Antioxidant and cytotoxic activities of Compound I and two methylated derivatives [4-hydroxy-5,8-dimethoxy-2-(2-oxopropyl)-naphthalene (D-1) and 2-glycidyl-4-hydroxy-5,8-dimethoxy naphthalene (D-2)] were evaluated. Compound I showed higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and reducing power than D-1 and D-2. However, D-1 and D-2 exhibited slightly stronger 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)$^+$ ($ABTS^+$) radical scavenging activity than Compound I. Compound I also exhibited stronger cytotoxic activity than D-1 and D-2 against the growth of HT-29 colon cancer cells. The results supported the hypothesis that methylation of naphthalene derivatives slightly increased $ABTS^+$ radical scavenging activity, but significantly decreased DPPH radical scavenging activity, reducing power, and cytotoxic activity.

Key words Naphthalene derivatives · Persimmon wood · Antioxidant activity · Cytotoxic activity

Introduction

Diospyros is a deciduous genus that belongs to the Ebenaceae family. *Diospyros* species are widely distributed in the tropical to temperate regions of the world. Many of them have black heartwood, which is often used in high-quality furniture, sculptures, and musical instruments. In addition, their extractives have been studied for their medicinal importance. Especially, *Diospyros kaki* (persimmon) has been cultivated in Eastern Asia, and its fruit has been traditionally used for many medicinal purposes because it contains many bioactive compounds such as polyphenols, carotenoids, and ascorbic acid.[1]

A unique feature of the *Diospyros* genus is the presence of a large number of naphthalene derivatives. Several naphthaldehydes have been isolated from *Diospyros spp.*,[2,3] and some naphthoquinones were isolated from six species of the genus *Diospyros* in Japan and their antifungal activities were investigated.[4] Ganapaty et al.[5] showed that naphthalene derivatives obtained from *Diospyros assimilis* exhibited moderate inhibitory effects against protozoan parasites. Noda et al.[6] investigated the difference in biodegradation properties between sapwood and blackened heartwood of *Diospyros kaki* and reported that the blackened portion was more resistant to fungal and termite attacks.

In our previous work,[7] three new naphthalene derivatives, 4-hydroxy-5,6-dimethoxy-2-naphthaldehyde; 5,6,8-trimethoxy-3-methyl-1-naphthol; and 4,8-dihydroxy-5-methoxy-2-naphthaldehyde (Compound I), were isolated from blackened heartwood of *Diospyros kaki*. Compound I was a major extractive (0.1% by dry weight of the wood sample) of the blackened heartwood of *Diospyros kaki* and it has two phenolic hydroxyl groups, which were expected to exhibit high bioactivities. In this study, we investigated the antioxidant and cytotoxic activities of Compound I and its two derivatives synthesized by methylation, 4-hydroxy-5,8-dimethoxy-2-(2-oxopropyl)-naphthalene (D-1) and 2-glycidyl-4-hydroxy-5,8-dimethoxy naphthalene (D-2).

Materials and methods

Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH); 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); dimethyl sulfoxide (DMSO); ferric chloride; l-ascorbic acid (vitamin C); butylated hydroxytoluene

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(BHT); trichloroacetic acid (TCA); peroxidase; hydrogen peroxide; potassium ferricyanide; sodium phosphate; potassium phosphate; and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All the other organic solvents and chemicals used in this study were of analytical grade.

Isolation of Compound I

The wood sample of *Diospyros kaki* was obtained from Kannra-gun, Gunma prefecture, Japan. The blackened heartwood of *D. kaki* (430.8 g) was extracted three times for 3 days, using methanol (2 l) each time at room temperature. The combined methanol solution (6 l) was evaporated under reduced pressure to give 5.65 g (1.3% of wood) of methanol extract. The methanol extract was chromatographed on silica gel with ethyl acetate and *n*-hexane (1:2 v/v) to give Compound I (372.1 mg). ^{1}H and ^{13}C NMR [$(CD_3)_2CO$] spectra were run on a Bruker Avance 400 spectrometer (Bruker, Billerica, MA, USA), and the results are shown in Table 1.

Methylation of Compound I

Two hundred milligrams of Compound I was dissolved in 10 ml of methanol and reacted with diazomethane for 1 day at room temperature. The reaction products were chromatographed on silica gel with mixed solvents of chloroform and *n*-hexane to isolate 4-hydroxy-5,8-dimethoxy-2-(2-oxopropyl)-naphthalene (D-1) (57.8 mg) and 2-glycidyl-4-hydroxy-5,8-dimethoxy naphthalene (D-2) (20.5 mg). The ^{1}H and ^{13}C NMR ($CDCl_3$) spectra are shown in Table 1. The purified compounds were dissolved in DMSO with a concentration of 50 mg/ml for the experiments and were diluted with DMSO when needed.

DPPH radical scavenging activity

The DPPH radical scavenging activity (RSA) of each compound was determined according to the method of Lee et al.[8] After a 0.1-ml aliquot of the compounds in DMSO was mixed with 0.9 ml of 0.041 mM DPPH in ethanol for 30 min, the optical density (OD) of the sample was measured at 517 nm using a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). RSA was expressed as a percentage inhibition and it was calculated using the following formula:

$$\text{DPPH RSA (\%)} = [1 - (\text{sample OD}/\text{control OD})] \times 100$$

ABTS radical scavenging activity

ABTS RSA was evaluated using the method of Muller.[9] Each compound (in 0.1 ml of DMSO), potassium phosphate buffer (0.1 ml, 0.1 M, pH 5.0), and hydrogen peroxide (20 µl, 10 mM) were mixed and preincubated at 37° for 5 min.

Table 1. ^{1}H and ^{13}C NMR spectral data for 4,8-dihydroxy-5-methoxy-2-naphthaldehyde (Compound I) and its two methylated derivatives (D-1 and D-2)

Position	Compound I			D-1			D-2		
	δ_H	δ_C	Observed HMBC	δ_H	δ_C	Observed HMBC	δ_H	δ_C	Observed HMBC
1	8.26 *d* (1.5)	120.0	C-3, C-4a, C-8, C-9	7.59 *d* (1.6)	113.6	C-3, C-4a, C-8a, C-8, C-9	7.60 *d* (1.6)	112.7	C-3, C-4a, C-8, C-8a, C-9
2		135.1			133.7			136.6	
3	7.16 *d* (1.5)	105.5	C-1, C-4 C-4a, C-9	6.80 *d* (1.6)	112.6	C-1, C-4, C-4a, C-9	6.86 *d* (1.6)	112.5	C-1, C-4 C-4a, C-9
4		155.6			157.3			154.5	
4a		118.5			114.7			114.5	
5		149.2			150.0[b]			150.0[f]	
6	7.03 *d* (8.4)	108.5	C-4a, C-5, C-8	6.67 *s*	103.4[c]	C-4a, C-5, C-8	6.66 *s*	103.0[g]	C-4a, C-5, C-8
7	6.95 *d* (8.6)	108.9	C-5, C-8, C-8a	6.67 *s*	103.3[c]	C-5, C-8, C-8a	6.66 *s*	103.2[g]	C-5, C-8, C-8a
8		148.8			150.0[b]			150.0[f]	
8a		127.0			128.4			128.4	
9	10.08 *s*	191.8	C-2, C-3	3.78 *s*	51.5	C-1, C-2, C-3, C-10, C-11	2.86 *dd* (14.4, 5.2)	39.2	C-1, C-2, C-3, C-10, C-11
							3.09 *dd* (14.4, 5.2)		
10					206.5		3.24 *m*	52.4	
11				2.18 *s*	29.2	C-9, C-10	2.64 *dd* (5.2, 2.4)	47.1	C-9, C-10
							2.84 *dd* (5.2, 2.4)		
4-OH	9.66 *s*		C-3, C-4, C-4a	9.49 *s*		C-3, C-4, C-4a	9.46 *s*		C-3, C-4, C-4a
5-OMe	4.09 *s*	56.3	C-5	3.96 *s*[a]	55.7[d]	C-5	3.96 *s*[e]	55.7[h]	C-5
8-OH	9.12 *s*								
8-OMe				4.02 *s*[a]	56.3[d]	C-8	4.02 *s*[e]	56.3[h]	C-8

[a–h] Signals within a column are interchangeable
Coupling constants *J* (parentheses) in Hertz
δ_H and δ_C in ppm
HMBC, heteronuclear multiple bond correlation

After preincubation, ABTS (30 µl, 1.25 mM, in 0.05 M phosphate-citrate buffer, pH 5.0) and peroxidase (30 µl, 1 unit/ml) were added to the mixture, and it was then incubated at 37°C for 10 min. The OD level was obtained with a multiplate reader (Sunrise RC/TS /TS Color-TC/TW/BC/6Filter, Tecan Austria, Grödig, Austria) at 405 nm, and the ABTS RSA was calculated by the following formula:

$$\text{ABTS RSA (\%)} = [1 - (\text{sample OD}/\text{control OD})] \times 100$$

Reducing power

The reducing power of each extract was determined according to the method of Oyaizu.[10] The compounds (in 1.0 ml of DMSO), sodium phosphate buffer (1.0 ml, 0.2 M, pH 6.6), and potassium ferricyanide (1.0 ml, 10 mg/ml) were mixed and incubated at 50°C for 20 min. Trichloroacetic acid (1.0 ml, 100 mg/ml) was added to the mixture and then the mixture was centrifuged at 13 400 *g* for 5 min. The supernatant (1.0 ml) was mixed with distilled water (1.0 ml) and ferric chloride (0.1 ml, 1.0 mg/ml), and then its absorbance was measured at 700 nm.

Cell culture

HT-29 human colon cancer cells were obtained from the Korean Cell Line Bank (KCLB). HT-29 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FSB), penicillin (100 unit/ml), streptomycin (100 mg/ml), and 2 mg/ml $NaHCO_3$ in an incubator at 37°C with 5% CO_2.

Cytotoxic activity

Cell viability was measured with blue formazan that was metabolized from MTT by mitochondrial dehydrogenase, which is active only in live cells. HT-29 cells were preincubated in a 96-well plate at a density of 1.0×10^5 cells/well for 24 h; cells were pretreated with various concentrations of Compound I, D-1, and D-2 (10, 50, and 100 µg/ml). After incubation for 24 h, the MTT reagent (5 mg/ml) was added to each well, and the plate was incubated for an additional 1 h at 37°C. At the end of incubation, the medium was removed and the intracellular formazan product was dissolved in 100 µl of DMSO. The absorbency of each well was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioRad, Model 680, USA), and the percentage viability was calculated.

Statistical analysis

All measurements were performed in triplicate, and analysis of variance was conducted by the general linear model using SAS software; Student-Newman-Keul's multiple range tests were used to compare the significant differences of the mean values among treatments ($P < 0.05$).

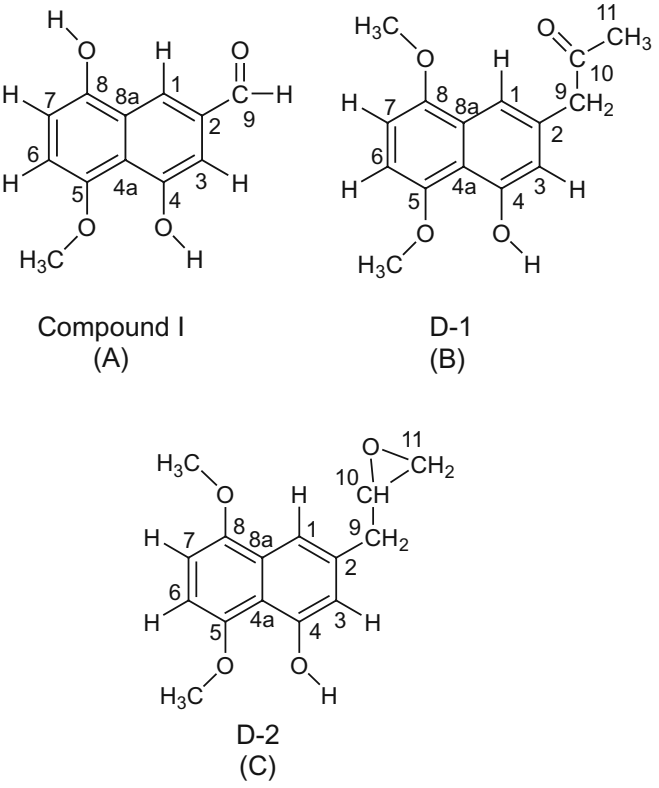

Fig. 1. Structures of *A* 4,8-dihydroxy-5-methoxy-2-naphthaldehyde (Compound I) and its two methylated derivatives, *B* 4-hydroxy-5,8-dimethoxy-2-(2-oxopropyl)-naphthalene (D-1) and *C* 2-glycidyl-4-hydroxy-5,8-dimethoxy naphthalene (D-2)

Results and discussion

Identification of the derivatives

Compound I is a major extractive of the blackened heartwood of *Diospyros kaki* (Fig. 1A). It has two phenolic hydroxyl groups, which were expected to exhibit high bioactivities. To investigate the effect of the phenolic hydroxyl groups on antioxidant and cytotoxic activities, Compound I was methylated in this study. We expected that all hydroxyl groups would be methylated to yield only 4,5,8-trimethoxy-2-naphthaldehyde if dimethyl sulfate was used. Many researchers have reported that phenolic hydroxyl groups affect the antioxidant activity, and so 4,5,8-trimethoxy-2-naphthaldehyde was expected to have little antioxidant activity. Therefore, we carried out the methylation using diazomethane to produce multiple methylated compounds. The methylation was carried out in methanol because Compound I could not be dissolved in diethyl ether. In this reaction, it was expected that one or both hydroxyl groups would be changed to a methoxy group and that 2-aldehyde would react to yield a ketone group or an epoxy group. However, the isolated compounds D-1 (Fig. 1B) and D-2 (Fig. 1C) were methylated at only one hydroxyl group. This fact implies that the acid dissociation constant of the hydroxyl group at position 4 is very low. Unfortunately,

Table 2. DPPH Radical scavenging activity (RSA), ABTS radical scavenging activity (RSA), and reducing power of 4,8-dihydroxy-5-methoxy-2-naphthaldehyde (Compound I) and its two methylated derivatives, (D-1 and D-2)

	DPPH RSA (IC_{50})[1] (μg/ml)	ABTS RSA (IC_{50})[1] (μg/ml)	Reducing power (IC_{50})[2]
Compound I	16.49 ± 0.07^{az}	25.34 ± 1.41^{by}	40.16 ± 0.12^{ax}
D-1	350.46 ± 7.35^{bx}	17.89 ± 1.17^{az}	231.73 ± 3.76^{by}
D-2	338.39 ± 7.24^{bx}	18.78 ± 0.80^{az}	227.68 ± 0.75^{by}
Ascorbic acid	26.90 ± 0.15	18.81 ± 0.57	24.01 ± 0.15
BHT	310.16 ± 3.98	15.75 ± 1.12	52.32 ± 0.22

All measurements were done in triplicate, and all values are means ± SD
Different letters within a column (a,b) or a row (x,z) are significantly different ($P < 0.05$)
DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene
[1] IC_{50}, concentration for scavenging 50% of DPPH radicals
[2] IC_{50}, concentration for increasing 0.500 in optical density

neither 4,5,8-trimethoxy-2-naphthaldehyde nor 4-hydroxy-5,8-dimethoxy-2-naphthaldehyde could be obtained; however, it was interesting to investigate the bioactivities of Compound I, D-1 and D-2 to understand the effect of hydroxyl, ketone, and epoxy groups on their bioactivities.

Antioxidant activity

Radical scavengers were evaluated by their reactivity toward stable free radicals DPPH and $ABTS^+$. The DPPH and $ABTS^+$ systems are both commonly used to measure the total antioxidative status of various biological specimens because of their good reproducibility and easy quality control.[11,12] The results for DPPH and $ABTS^+$ RSAs are summarized in Table 2. The IC_{50} values of the three compounds for DPPH and $ABTS^+$ RSA were compared with those of ascorbic acid and BHT, which were used as positive controls in this study. The DPPH RSA of Compound I was significantly higher than that of D-1 and D-2, whereas the $ABTS^+$ RSA of Compound I was slightly lower than that of D-1 and D-2. The $ABTS^+$ method is appropriate for lipophilic systems in which the antioxidant is added to the preformed radical cation produced on the one-electron oxidation of $ABTS^+$ and is applied to the monitoring of lipophilic antioxidants such as carotenoids and lipophilic extracts of nutritional components.[13] In the present study, the methoxy groups (by methylation) attached to D-1 and D-2 derived from Compound I constitute a hydrophobic alkyl functional group. The effect of antioxidant on $ABTS^+$ is thought to be due to the presence of methoxy groups. Also, the synthetic compounds D-1 and D-2 exhibited similar IC_{50} values for DPPH and $ABTS^+$ RSA to those of BHT, which is a well-known synthetic antioxidant. On the other hand, the scavenging of the $ABTS^+$ radical by the compounds was mainly higher than that of the DPPH radical. Factors like stereoselectivity of the radicals or the solubility of the compounds in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals.[14] Wang et al.[15] reported that some compounds which have $ABTS^+$ RSA did not show DPPH RSA.

Reducing power is generally associated with the presence of reducing substances, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[16] The reducing power of the compounds was also evaluated and is listed in Table 2. The reducing power of Compound I was significantly higher than that of D-1 and D-2, and the IC_{50} value of Compound I was similar to that of BHT. These results showed similar trends to those for DPPH RSA.

Cytotoxic activity

The cytotoxic activities of Compound I, D-1, and D-2 were evaluated with respect to HT-29 human colon cancer cells. HT-29 cells were originally derived from a human colon cancer, and are representative of a vascularized solid tumor. The effect of the compounds on the growth and proliferation of HT-29 cells were determined via MTT reduction assay. The cells were exposed for 24 h to various concentrations (10, 50, and 100 μg/ml) of the compounds. As shown in Fig. 2, among the three naphthaldehyde derivatives, the cytotoxic activity of Compound I was significantly higher than those of D-1 and D-2. Compound I effected a 15%–45% reduction in cell viability compared to the control, and this was determined to occur in a dose-dependent manner. The results suggested that the phenolic hydroxyl moiety of naphthalene plays important role in cytotoxic activity. Li et al.[17] reported that methylation decreased cytotoxicity of flavonoid. The cytotoxicity of phenolic derivatives was significantly affected by the methylation position of the phenolics.[18] The present results suggested that the hydroxyl groups at positions 4 and 8 of Compound I might be important to cytotoxic activity.

There is little difference in the antioxidant and cytotoxic activities of D-1 and D-2, which have a ketone structure and an epoxy structure, respectively. Therefore, it was concluded that the structural change did not have a significant effect on these activities. In order to obtain more information on the antioxidant and cytotoxic activities of naphthalene derivatives in detail, we need to conduct further research.

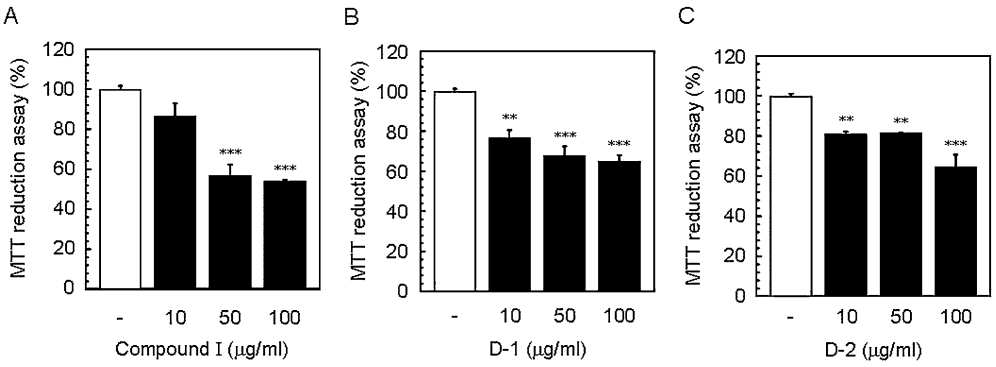

Fig. 2. Cytotoxic effects of Compound I (**A**) and its two methylated derivatives, D-1 (**B**) and D-2 (**C**), on HT-29 cells. The cells were exposed to the indicated concentrations of the compounds for 24 h. After 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (*MTT*) reduction assay, the MTT reduction rate was calculated by setting each of the control survivals equal to 100%. The data (mean ± SD) are representative of at least three independent experiments. Significance versus control untreated cells: $^{**}P < 0.01$, $^{***}P < 0.001$

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