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ORIGINAL ARTICLE

Antimicrobial activities of active component isolated from *Lawsonia inermis* leaves and structure-activity relationships of its analogues against food-borne bacteria

Ji-Yeon Yang · Hoi-Seon Lee

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Abstract The antimicrobial activities of *Lawsonia inermis* leaf extract and 2-hydroxy-1,4-naphthoquinone analogues against food-borne bacteria. The antimicrobial activities of five fractions derived from the methanol extract of *Lawsonia inermis* leaves were evaluated against 7 food-borne bacteria. 2-Hydroxy-1,4-naphthoquinone was isolated by chromatographic analyses. 2-Hydroxy-1,4-naphthoquinone showed the strong activities against *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella enterica*, *Shigella sonnei*, *Staphylococcus epidermidis*, and *S. intermedius*, but exerted no growth-inhibitory activities against *S. typhimurium*. The antimicrobial activities of the 2-hydroxy-1,4-naphthoquinone analogues were tested against 7 food-borne bacteria to establish structure-activity relationships. Hydroxyl (2-hydroxy-1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone), methoxy (2-methoxy-1,4-naphthoquinone), and methyl (2-methyl-1,4-naphthoquinone, and 5-hydroxy-2-methyl-1,4-naphthoquinone) functional groups on the 1,4-naphthoquinone skeleton possessed potent activities, whereas bromo (2-bromo-1,4-naphthoquinone and 2,3-dibromo-1,4-naphthoquione) and chloro (2,3-dichloro-1,4-naphthoquinone) exhibited no activity against 7 food-borne bacteria. The *L. inermis* leaf extract and 2-hydroxy-1,4-naphthoquinone analogues should be useful as natural antimicrobial agents against food-borne bacteria.

Keywords Antimicrobial activity · Food-borne bacteria · Functional group · 2-hydroxy-1,4-naphthoquinone · *Lawsonia inermis*

J.-Y. Yang · H.-S. Lee (✉)
College of Agriculture & Life Sciences, Chonbuk National University, Jeonju, Chonbuk, Republic of Korea
e-mail: hoiseon@chonbuk.ac.kr

Introduction

Antimicrobial activity is the primary mode of food deterioration which is often accompanied by the loss of economic profit, safety, and quality (Rahman and Kang 2009). Synthetic food preservatives are widely used to achieve sufficient expiration dates (Rahman and Kang 2009; Xiong et al. 2013). Food consumers are concerned about the safety of chemical preservatives and increasingly avoid the use of synthetic food preservatives (Hannuksela and Haahtela 1987). Chemical food additives are harmful to humans because some cause allergic reactions, cancer, poisoning, and teratogenicity (Hannuksela and Haahtela 1987; Safford et al. 1990). These problems indicate the need to develop new preservative systems with fewer side-effects to humans. For this reason, natural food preservatives have various advantages. Medicinal plants containing antimicrobial activity are widely used to prolong the expiration date and improve food safety (Kim et al. 2004; Lou et al. 2010). In the interim, plants with antimicrobial compounds are abundant because various plants have antimicrobial and bacteriostatic toxicity (Lee and Ahn 1998). Consequently, the potential to use natural antimicrobial agents from plants as food additives is considerable.

Medicinal plants play a significant role improving the quality of human life (Yang et al. 2013; Kim et al. 2013). *Lawsonia inermis* L. (Lythraceae) is commonly known as "henna" and is used as a traditional medicinal plant in Asia and the Middle East (Charisty et al. 2012). Many studies have demonstrates the anthelmintic, antimicrobial, antihyperglycemic, antioxidative, and antiulcer activities of *L. inermis* (Philip et al. 2011; Eguale and Giday 2009; Habbal et al. 2011). Although some researchers have studied the bacteriostatic activities of flower, fruit, and leaf extracts of *L. inermis*, only a few have identified antimicrobial activities and bioactive compounds. The present study was conducted to isolate antimicrobial constituents from *L. inermis* leaves

against food poisoning bacteria. Furthermore, the structure-activity relationships of its analogues are discussed in relation to antimicrobial activity.

Materials and methods

Chemicals

2-Bromo-1,4-naphthoquinone, 2,3-dibromo-1,4-naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, and 1,4-naphthoquinone were purchased from Aldrich (Milwaukee, WI, USA). 2-Amino-3-chloro-1,4-naphthoquinone, 5-hydroxy-2-methyl-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone were purchased from Sigma (St. Louis, MO, USA). All chemicals were reagent grade.

Preparation of plant material and extraction

Air-dried leaves of *L. inermis* were purchased from a local market (Jecheon, South Korea). A voucher specimen was authenticated by Prof. Jeong-Moon Kim and deposited at the herbarium of the Department of Landscape Architecture, College of Agriculture, Chonbuk National University. The dried samples (200 g) were ground in a blender and extracted with 100 % methanol (MeOH, 2,500 ml×2) in a shaking incubator at 30ºC for 48 h. The extract of *L. inermis* leaves was then filtered and concentrated *in vacuo* at 40ºC using a rotary vacuum evaporator (EYELA auto jack NAJ-100, Tokyo, Japan), which resulted in 64.26 g (yield, 32.13 %) of methanol extract. The extract of *L. inermis* leaves (20 g) was dissolved in water (800 ml) and was successively partitioned with hexane (800 ml×2), chloroform (800 ml×2), ethyl acetate (800 ml×2), and butanol (800 ml×2), to give hexane (2.1 g), chloroform (1.2 g), ethyl acetate (1.7 g), butanol (3.4 g), and water fractions (8.7 g). These fractions were concentrated and refrigerated (4ºC) prior to use.

Isolation and identification

The chloroform fraction (10 g) partitioned from the methanol extract was subjected to silica gel (Merck, Rahway, NJ, USA) column chromatography (5.5×70 cm) and was eluted with chloroform: methanol (10:0 to 0:10, gradient, v/v). The separated fractions were analyzed via thin layer chromatography (TLC), and fractions showing similar patterns were pooled, resulting in four fractions (LI 1-LI 4). The LI 2 fraction (2.1 g) was subjected to silica gel column chromatography (5.5×70 cm) using chloroform: methanol (10:1, v/v) as the mobile phase to provide three fractions (LI 21-LI 23). The LI 23 fraction (1.3 g) was isolated by preparative high performance liquid chromatography (preparative HPLC, LC-908, Japan Analytical Industry Co., Ltd., Tokyo, Japan) using a Jai gel GS series column (GS 310 50 cm+GS 310 30 cm) with methanol (100 %) as the mobile phase at a flow rate of 3.5 ml min^{-1} and produced three fractions (LI 231-LI 233). Finally, LI 233 (430 mg) was isolated as a single peak. The structure of LI 233 was identified by various spectroscopic methods. The electron ionization mass spectra (EI-MS) were obtained on a JEOL GSX 400 mass spectrometer (Jeol Ltd., Tokyo, Japan). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured using a JNM-EX 600 (Jeol Ltd., Tokyo, Japan) spectrometer in deuterated chloroform with tetramethylsilane as the internal standard at 600 and 150 MHz. Correlation spectroscopy, distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple-quantum correlation were used to determine the association between carbons and protons. Chemical shifts were expressed in δ (ppm).

Table 1 Antimicrobial activities of materials derived from *Lawsonia inermis* leaves against food-borne bacteria

Compound	Bacteria strains						
	B. cereus	*L. monocytogenes*	*S. enterica*	*S. typhimurium*	*S. sonnei*	*S. epidermidis*	*S. intermedius*
MeOH[a]	+[c]	++	+++	++	+	+	+
Hexane[b]	–	–	–	–	–	–	–
$CHCl_3$[b]	++	+++	+++	++	+	+	+
EtOAc[b]	–	–	+	–	–	–	–
BuOH[b]	–	–	–	–	–	–	–
Water[b]	–	–	–	–	–	–	–

[a] Dose: 20.0 mg/disc

[b] Dose: 10.0 mg/disc

[c] Inhibitory zone diameter (mm)>30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –

Table 2 ^{1}H NMR, ^{13}C NMR, and DEPT data[a] for LI-233[a]

Carbon	Partial structure	δ_c (ppm)	δ_H (ppm)
1	C = O	180.9247	-
2	COH	155.3026	7.3443 (s[b])
3	CH	109.6885	6.2999 (s)
4	C = O	183.9599	-
5	C	131.8828	-
6	CH	125.4964	8.0452–8.0578 (d, *J*=7.56 Hz)
7	CH	132.1413	7.6444–7.6707 (t, *J*=15.78 Hz)
8	CH	134.2860	7.7200–7.7452 (t, *J*=15.12 Hz)
9	CH	125.6975	8.0452–8.0578 (d, *J*=7.56 Hz)
10	C	128.3976	-

[a] ^{1}H NMR (600 MHz), ^{13}C NMR and DEPT (150 MHz), TMS, δ ppm, *J* in Hz

[b] *s* singlet, *d* doublet, *t* triplet

Test microorganisms

The growth-inhibitory activities of the samples were evaluated against food-borne bacteria, including the Gram-positive bacteria *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 15313, *Staphylococcus epidermidis* ATCC 12228, and *Staphylococcus intermedius* ATCC 29663 and the Gram negative bacteria *Salmonella enterica* ATCC 25931, *Salmonella typhimurium* IFO 14193, and *Shigella sonnei* ATCC 25931. The bacterial strains were prepared from stocks obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). Bacterial strains were aerobically cultured at 37ºC for 24 h in nutrient broth (NB, Difco, Detroit, MI, USA).

Agar diffusion method

The agar diffusion method was used to determine the antimicrobial activities of the five fractions partitioned from the methanol extract of *L. inermis* leaves and the 2-hydroxy-1,4-naphthoquinone analogues (Yang and Lee 2012). Briefly, microorganisms were incubated in NB at 37ºC for 24 h to yield 1.0×10^{7} CFU ml^{-1} compared to the turbidity of the McFarland turbidity standard. A suspension of the incubated microorganisms (0.1 ml of 1.0×10^{7} CFU ml^{-1}) was spread on Mueller Hinton agar (MHA, Difco, USA) plates. Each concentration of samples (20, 10, 1.0, 0.5, 0.25, 0.1 mg/disc) was dissolved in methanol, and sterilized paper discs (8 mm in diameter) were impregnated with 40 μl of sample. Methanol served as the negative control and 40 μl was injected onto a sterilized paper disc. After drying in a fume hood, the injected paper discs were placed on inoculated MHA plates. These plates were incubated under aerobic conditions at 37ºC for 24 h. Treatments were performed in triplicate, and antimicrobial activity was expressed as the diameter of the inhibition zone (mm).

Results and discussion

The yield of the methanol extract of the *L. inermis* leaves was 32.13 %. The five fractions were partitioned from the methanol extract of the *L. inermis* leaves. The highest yield was obtained from the water fraction (43.5 %), followed by the butanol fraction (17.0 %), hexane fraction (10.5 %), ethyl

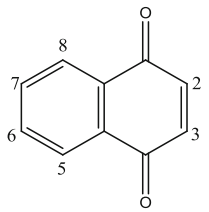

Fig. 1 Structure-activity relationships of the 2-hydroxy-1,4-naphthoquinone analogues

Compounds	Position					
	2	3	5	6	7	8
1,4-Naphthoquinone	H	H	H	H	H	H
2-Bromo-1,4-naphthoquinone	Br	H	H	H	H	H
2,3-Dibromo-1,4-naphthoquinone	Br	Br	H	H	H	H
2,3-Dichloro-1,4-naphthoquinone	Cl	Cl	H	H	H	H
2-Amino-3-chloro-1,4-naphthoquinone	NH_2	Cl	H	H	H	H
2-Methyl-1,4-naphthoquinone	CH_3	H	H	H	H	H
2-Methoxy-1,4-naphthoquinone	OCH_3	H	H	H	H	H
2-Hydroxy-1,4-naphthoquinone	OH	H	H	H	H	H
5-Hydroxy-1,4-naphthoquinone	H	H	OH	H	H	H
5-Hydroxy-2-methyl-1,4-naphthoquinone	CH_3	H	OH	H	H	H

acetate fraction (8.5 %), and chloroform fraction (6.0 %). The antimicrobial activities of the methanol extract and the five fractions derived from the *L. inermis* leaves are given in Table 1. The methanol extracts and chloroform fractions exhibited antimicrobial activities against 7 food-borne bacteria at 20 and 10 mg/disc, respectively. However, hexane, butanol, ethyl acetate, and water fractions had no antimicrobial activity against any of the tested microorganisms. The negative control did not exhibit an antimicrobial effect against 7 food poisoning bacteria. Therefore, the chloroform fraction was selected to isolate the active compound of *L. inermis* leaves.

To isolate the active compound of the *L. inermis* leaves, silica gel column chromatography, TLC, and preparative HPLC were performed with mixed organic solvents. LI 233 was isolated and identified by various spectroscopic analyses, including EI-MS, ^{1}H, ^{13}C, and DEPT NMR (Table 2). The isolated compound was identified as 2-hydroxy-1,4-naphthoquinone (Fig. 1). 2-Hydroxy-1,4-naphthoquinone ($C_{10}H_6O_3$, MW: 174.15): EI-MS (70 eV) *m*/z M^+ 174 (100), 146 (20), 129 (5), 118 (15), 105 (80), 89 (15), 77 (24), 69 (5), 50 (8), 40 (4); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.0452-8.0578 (d, *J*=7.56 Hz), 7.7200-7.7452 (t, *J*=15.12 Hz), 7.6444-7.6707 (t, *J*=15.78 Hz), 7.3443 (s), 6.2999 (s); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 183.9599 (C), 180.9247 (C), 155.3026 (C), 134.2860 (CH), 132.1413 (CH), 131.8828 (C), 128.3976 (C), 125.6975 (CH), 125.4964 (CH), 109.6885 (CH). These findings were similar to those of a previous study (Malamidou-Xenikaki et al. 2003).

The antimicrobial activity of 2-hydroxy-1,4-naphthoquinone isolated from *L. inermis* leaves was evaluated

Table 3 Antimicrobial activities of 2-hydroxy-1,4-naphthoquinone analogues against food-borne bacteria.

Compound	Dose (mg/disc)	*B. cereus*	*L. monocytogenes*	*S. enterica*	*S. typhimurium*	*S. sonnei*	*S. epidermidis*	*S. intermedius*
2-Hydroxy-1,4-naphthoquinone	1.0	++++	++++	++++	−	+++	+++	+++
	0.5	+++	++	+++	−	++	++	++
	0.25	++	++	+++	−	+	+	++
	0.10	+	+	++	−	+	+	+
1,4-Naphthoquinone	1.0	+++[a]	+++	++++	−	+++	++	+++
	0.5	+++	++	+++	−	+++	++	+++
	0.25	++	++	+++	−	++	+	+++
	0.10	+	+	++	−	+	+	++
2-Bromo-1,4-naphthoquinone	1.0	−	−	+	−	−	−	−
2,3-Dibromo-1,4-naphthoquinone	1.0	−	−	−	−	−	−	−
2,3-Dichloro-1,4-naphthoquinone	1.0	−	−	−	−	−	−	−
2-Amino-3-chloro-1, 4-naphthoquinone	1.0	−	−	−	−	+++	−	−
	0.5	−	−	−	−	+++	−	−
	0.25	−	−	−	−	++	−	−
	0.10	−	−	−	−	+	−	−
2-Methyl-1,4-naphthoquinone	1.0	++++	++++	++++	−	+++	+++	++++
	0.5	+++	+++	+++	−	+++	+++	+++
	0.25	++	+++	+++	−	+	++	+++
	0.10	+	+++	+++	−	−	+	+++
2-Methoxy-1,4-naphthoquinone	1.0	−	+++	++++	−	−	−	−
	0.5	−	+++	++++	−	−	−	−
	0.25	−	++	+++	−	−	−	−
	0.10	−	++	+++	−	−	−	−
5-Hydroxy-1,4-naphthoquinone	1.0	++++	++++	++++	++	+++	+++	+++
	0.5	+++	++	+++	+	+++	++	+++
	0.25	++	++	++	+	+	+	++
	0.10	+	+	++	+	+	+	++
5-Hydroxy-2-methyl-1, 4-naphthoquinone	1.0	++++	++++	++++	+++	+++	+++	++++
	0.5	++++	+++	+++	++	+++	+++	+++
	0.25	++	+++	+++	+	++	++	+++
	0.10	++	++	++	+	+	+	+++

[a] Inhibitory zone diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; <10 mm, −

using the agar diffusion method against 7 food-borne bacteria (Table 3). Based on the inhibitory zone diameter (mm), 2-hydroxy-1,4-naphthoquinone had potent antimicrobial activity against 6 food-borne bacteria except *S. typhimurium.* To establish the structure-activity relationships of the 2-hydroxy-1,4-naphthoquinone analogues, the antimicrobial activities of 2-hydroxy-1,4-naphthoquinone and 11 structural analogues (2-amino-3-chloro-1,4-naphthoquinone, 2-bromo-1,4-naphthoquinone, 2-chloro-3-morpholino-1,4-naphthoquinone, 2-chloro-3-pyrrolidino-1,4-naphthoquinone, 2,3-dibromo-1,4-naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-2-methyl-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 1,4-naphthoquinone) were evaluated by the agar diffusion method at 1.0 mg/disc (Fig. 1) (Table 3). As a result, 1,4-naphthoquinone, which is the skeleton for the 2-hydroxy-1,4-naphthoquinone analogues had strong antimicrobial effects against 6 food-borne bacteria except *S. typhimurium* at 1.0 and 0.5 mg/disc. Based on the 1,4-naphthoquinone skeleton, 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone exhibited excellent growth-inhibitory activities against all test microorganisms. 2-Hydroxy-1,4-naphthoquinone and 2-metyl-1,4-naphthoquione had strong antimicrobial activities against 6 food-borne bacteria except *S. typhimurium.* 2-Methoxy-1,4-naphthoquinone showed outstanding antimicrobial toxicity against *L. monocytogenes* and *S. enterica.* 2-Amino-3-chloro-1,4-naphthoquinone possessed moderate antimicrobial effects against *S. sonnei*. However, 2-bromo-1,4-naphthoquinone, 2,3-dibromo-1,4-naphthoquinone, and 2,3-dichloro-1,4-naphthoquinone had weak or no activity against 7 food-borne bacteria. Thus, hydroxyl (2-hydroxy-1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone), methoxy (2-methoxy-1,4-naphthoquinone), and methyl (2-methyl-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone) functional groups on the 1,4-naphthoquinone skeleton possessed potent bacteriostatic activities, whereas bromo (2-bromo-1,4-naphthoquinone and 2,3-dibromo-1,4-naphthoquione) and chloro (2,3-dichloro-1,4-naphthoquinone) exhibited no activity against 7 food-borne bacteria except for an analogue containing an amino group (2-amino-3-chloro-1,4-naphthoquinone). These findings were similar to those of previous studies reporting that 1,4-naphthoquione derivatives containing hydroxyl and methyl functional groups exhibit selective bacteriostatic effects against harmful intestinal bacteria. 5-Hydroxy-2-methyl-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, and 2-methyl-1,4-naphtoquinone including hydroxyl and methyl functional groups on the 1,4-naphthoquinone skeleton play a role selective growth regulatory role against intestinal bacteria (Kim et al. 2009; Lim et al. 2007).

Based on the Material Safety Data Sheet provided by Sigma-Aldrich (MSDS 2012), the oral LD_{50} values of 2-methyl-1,4-naphthoquinone (500 mg/kg), 2-methoxy-1,4-naphthoquinone (320 mg/kg), and 1,4-naphthoquinone (320 mg/kg) indicated moderate acute toxicity to mammals. The acute toxicity of *L. inermis* in animals has been reported (Mudi et al. 2011). The acute lethal dose of the aqueous extract derived from *L. inermis* leaves is 800-1,600 mg/kg (Mudi et al. 2011). These results indicate that *L. inermis* leaves and 1,4-naphthoquinone analogues could be useful natural bactericides for food additives and the pharmaceutical industries and are potentially suitable as alternative chemical preservatives.

Conclusion

These results were encouraging, as *L. inermis* leaves showed potential antimicrobial activity. It suggested that *L. inermis* leaves could be of use as a source of natural antimicrobial components for food supplementation, and pharmaceutical industries, potentially suitable for the replacement of synthetic preservatives. Owing to their excellent protective features exhibited in antimicrobial tests, *L. inermis* leaves could be utilized as a natural source of antimicrobial agent. In this regard, current and previous results suggest that the *L. inermis* leaves and its active compound analogues should be useful for the development of eco-friendly food supplemental agents and pharmaceutics.

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