

Antimicrobial agents of 4-methoxysalicylaldehyde isolated from *Periploca sepium* oil against foodborne bacteria: structure–activity relationship

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Received: 17 April 2018 / Accepted: 3 May 2018 / Published online: 15 May 2018
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Abstract This study was designed to evaluate the antimicrobial activities of 4-methoxysalicylaldehyde isolated from *Periploca sepium* and its derivatives against six foodborne bacteria (*Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *S. sonnei*, *Staphylococcus intermedius* and *S. aureus*). Essential oil extracted from *P. sepium* roots exhibits strong antimicrobial activity against foodborne bacteria. The antimicrobial compound of *P. sepium* isolated by chromatographic techniques was identified as 4-methoxysalicylaldehyde. To compare the antimicrobial activities of 4-methoxysalicylaldehyde and its derivatives (4-hydroxysalicylaldehyde, salicylaldehyde, 3-methoxysalicylaldehyde, 5-methoxysalicylaldehyde, 3-methylsalicylaldehyde, and 5-methylsalicylaldehyde), the MIC test was performed. These activities were exhibited by 4-methoxysalicylaldehyde (MIC 30.1–67.3 µg/mL) followed by 4-hydroxysalicylaldehyde (MIC 41.1–61.5 µg/mL) and 4-methoxysalicylaldehyde (MIC 41.3–92.1 µg/mL) against all tested microorganisms. The results indicate that 4-methoxysalicylaldehyde and its derivatives represent natural antimicrobial alternatives.

Keywords Antimicrobial agent · Foodborne bacteria · 4-Methoxysalicylaldehyde · *Periploca sepium*

Introduction

Microbial contamination and food spoilage are one of the causes of foodborne disease, which appears to increase the focus of attention on food safety [1–3]. Infectious diseases including foodborne disease are one of the main causes of global health problems in developing countries [1, 2, 4]. Synthetic preservatives are used for food processing in order to prolong the expiration date and safety of foods. However, the side effects associated with synthetic preservatives and their impact on human health question the stability of synthetic preservatives [2, 5]. In recent years, the role of natural products and growing apprehension about the safety of synthetic antibiotics has stimulated various studies of plant materials [2, 4].

Plant-derived products have been used to manufacture acaricides, insecticides, traditional medicines, and for protection against foodborne pathogens [1, 2, 6, 7]. Plant essential oils and their constituents are potential antimicrobial agents and food preservatives [2, 5, 8, 9]. Such oils represent attractive antimicrobial agents as they reduce the risk of pathogenic bacterial resistance and generally display low toxicities in mammals, unlike synthetic antibiotics or food preservatives [2, 4, 5, 10]. *Periploca sepium* Bunge (Asclepiadaceae), widespread throughout northeastern and southwestern China, has been traditionally used as a Chinese herbal medicine. *P. sepium* roots are also used for treatment of rheumatoid arthritis, cardiac palpitation, shortness of breath and wounds, which is attributed to its oligosaccharides, pregnane glycosides, flavonoids and triterpenoids [11–13]. This study was undertaken with the main objective of identifying active component in *P. sepium* roots, and to investigate the antimicrobial activity of its constituents against six foodborne pathogens.

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Materials and methods

Chemicals

4-Hydroxysalicylaldehyde, 3-methoxysalicylaldehyde, 5-methoxysalicylaldehyde, 3-methylsalicylaldehyde, 5-methylsalicylaldehyde and salicylaldehyde were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Isolation and identification

Roots of *P. sepium* were purchased from Jeonju market (Korea). Extracted oil of *P. sepium* roots was obtained through steam distillation for 4 h. The oil yield was 0.24% and stored at 4 °C in a refrigerator. Essential oil (4 g) was loaded to silica gel chromatography (70–230 mesh; diameter, 8 × 90 cm; 600 g; Merck, Rahway, NJ, USA) for purification. Essential oil was eluted with ethyl acetate:hexane (0:10–10:0, v/v) to obtain five fractions (PS1 to PS5). Five fractions were bioassayed against six foodborne bacteria. The PS3 fraction exhibited strong antimicrobial activity. Active PS3 was re-chromatographed and eluted by multi-step solvent gradients as follows: ethyl acetate:hexane (2:8–5:5, v/v). A Jaigel GS series column (GS-310 500 mm plus GS-310 300 mm) was connected to a recycling prep HPLC (Japan Analytical Industry Co. Ltd., Tokyo, Japan) and equilibrated with acetone (100%). Active PS32 (3.8 g) was injected into HPLC and eluted at 1 mL/min (flow rate) and UV was determined out at 294 nm. PS321 and PS322 were obtained and bioassayed at 5.0 mg/disc. The PS321 fraction showed the strongest activity against six foodborne bacteria at 5.0 mg/disc. Finally, PS321 (3.5 g, yield 68.9%), was isolated by prep HPLC.

The PS321 structure was demonstrated by spectroscopic techniques. ¹H- and ¹³C-NMR spectra were studied using a JNM-ECA600 spectrometer (JEOL Ltd., Tokyo, Japan) instrument with CHCl₃ as the solvent and C₄H₁₂Si as the internal standard. The UV–visible light absorption spectrum was also obtained with CHCl₃ using a UV spectrometer (DR/4000 spectrophotometer, HACH, Seoul, Korea). In addition, EI-MS was determined using a JEOL JMS-DX 30 mass spectrometer.

Bacterial culture

Six foodborne bacteria were tested in this study, including three Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 15313, and *Staphylococcus intermedius* ATCC 29663; three Gram-negative bacteria: *Shigella sonnei* ATCC 25931,

Salmonella typhimurium IFO 14193, and *Shigella flexneri* ATCC 29903. The pure bacterial strains were obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). All bacteria were grown aerobically at 37 °C in nutrient broth (NB; Difco, USA) while *S. aureus* were grown in Tryptic Soy broth (TSB; Difco, USA).

Disc diffusion

Antimicrobial activity of *P. sepium* oil, 4-hydroxysalicylaldehyde and its derivatives were determined via disc diffusion method, as suggested by Kim et al. [14], against six foodborne bacteria. First, the bacterial suspensions were cultured in NB and then diluted to a turbidity adjusted to that of 0.5 McFarland (containing approximately 1.0×10^7 CFU/mL). The Muller Hinton agar (MHA; Difco, USA) plate was inoculated with bacterial suspensions (100 µL) containing 10^7 CFU/mL. The sterilized paper discs were soaked with 40 µL of each dilution (*P. sepium* oil, 4-hydroxysalicylaldehyde and its derivatives) (20 to 0.125 mg/disc) and placed on each MHA plate [15]. Methanol as negative control was also injected into the discs. The plates were left in an incubator at 37 °C for 24 h. Analytical experiments were conducted in triplicate.

Minimum inhibitory concentrations

Antimicrobial activities of 4-hydroxysalicylaldehyde and its derivatives were investigated via the minimum inhibitory concentration (MIC) using the broth dilution method. The MIC test was conducted by dissolving *P. sepium* oil, 4-hydroxysalicylaldehyde and its derivative (10 mg) and serially diluted two-fold in methanol (10 mL) in order to obtain 100–1 µg/mL concentrations; 100 µL of Muller Hinton broth (MHB) containing tested samples (50 µL) was dispensed into a 96-well microtiter plates using a micro-pipette followed by the addition of 50 µL bacterial suspension (10^7 CFU/mL). All plates without tested samples were used as a control. After all plates were incubated at 37 °C for 24 h, the MIC values were determined based on turbidity at 600 nm.

Results and discussion

Antimicrobial activity of the oil extracted from *P. sepium* roots was evaluated via the disc diffusion method (Table 1). Measurements of inhibition zone values of the extracted oil of *P. sepium* roots showed potent antimicrobial activities against *S. aureus*, *L. monocytogenes*, *S. sonnei*, and *S. typhimurium*. By contrast, the essential oil of *P. sepium* was less active against *S. intermedius* and *S. flexneri*. The negative control lacked antimicrobial activity

Table 1 Antimicrobial activities of essential oil of *P. sepium* seeds against foodborne bacteria

Sample	Conc. (mg/disc)	Bacterial species					
		Gram-positive bacteria			Gram-negative bacteria		
		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
<i>P. sepium</i> oil	20	++++ ^a	++++	++	++++	++	+++
	10	+++	+++	+	+++	+	++
	5	++	+++	–	+++	–	++
	2	++	+	–	++	–	+
Negative control (only solvent)	20	–	–	–	–	–	–

Cultured on Mueller–Hinton agar at 37 °C for 24 h in an incubator

^aDiameter of inhibition zone > 30 mm; ++++ ; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and < 10 mm, –

against the microorganisms tested. Wang et al. [16] reported that the *P. sepium* oil showed antimicrobial and antioxidant activities against a wide range of bacterial strains. In addition, *P. sepium* also exhibited insecticidal [12, 17] and acaricidal activities [18]. In addition, similar studies have been demonstrated for species such as *Periploca laevigata* [3].

The antimicrobial compound was isolated by bioassay-guided separation and identified spectroscopically. Spectroscopic analysis verified PS321 as 4-methoxysalicylaldehyde (Tables 2, 3). Spectroscopic data for 4-methoxysalicylaldehyde (4-methoxy-2-hydroxybenzaldehyde) (C₈H₈O₃, molecular weight 151.0) were as follows: EI-MS (70 eV) m/z M⁺ 151, 134, 108, 95, 69, 53, 32; ¹H NMR (CD₃OD, 600 MHz) δ 11.42 (1H, t, *J* = 7.2 Hz), 7.30 (1H, t, *J* = 10.1 Hz), 6.47 (1H, t, *J* = 0.9 Hz), 6.38 (1H, t, *J* = 8.1 Hz), 4.60 (1H, s), 3.80 (1H, m, *J* = 14.5 Hz); and ¹³C NMR (CDCl₃, 150 MHz) δ 194.5 (CH), 166.9 (C–O), 164.9 (C–OH), 135.3 (CH), 115.2 (C), 108.5 (CH), 100.7 (CH), 55.7 (CH₃). Antimicrobial effects of the oil derived from *P. sepium* roots

Table 2 ¹H NMR, ¹³C NMR, and DEPT spectral data^a of PS321

Carbon	Partial structure	δ _C (ppm)	δ _H (ppm)
1	CH	194.5	11.42 (1H, t ^b , <i>J</i> = 7.2 Hz)
2	C–O	166.9	
3	C–OH	164.9	4.60 (1H, s)
4	CH	135.3	7.30 (1H, t, <i>J</i> = 10.1 Hz)
5	C	115.2	
6	CH	108.5	6.47 (1H, t, <i>J</i> = 0.9 Hz)
7	CH	100.7	6.38 (1H, t, <i>J</i> = 8.1 Hz)
8	CH ₃	55.7	3.80 (1H, m, <i>J</i> = 14.5 Hz)

^a¹H NMR (600 MHz), ¹³C NMR and DEPT (150 MHz), TMS, δ ppm, *J* in Hz

^bs: singlet, d: doublet, t: triplet, m: multiplet

Table 3 Structures of 4-methoxysalicylaldehyde analogues

Chemicals	Position			
	R ₁	R ₂	R ₃	R ₄
4-Methoxysalicylaldehyde	H	CH ₃ O	H	H
4-Hydroxysalicylaldehyde	H	OH	H	H
Salicylaldehyde	H	H	H	H
3-Methoxysalicylaldehyde	CH ₃ O	H	H	H
5-Methoxysalicylaldehyde	H	H	CH ₃ O	H
3-Methylsalicylaldehyde	CH ₃	H	H	H
5-Methylsalicylaldehyde	H	H	CH ₃	H

depend on the presence of high content of 4-methoxysalicylaldehyde [16]. Analytical data of 4-methoxysalicylaldehyde were consistent with reported studies [12, 16, 19]. *Decalepis arayalpathra*, *Decalepis hamiltonii*, and *Hemidesmus indicus* roots contain 4-methoxysalicylaldehyde [20, 21], which has been widely utilized in soft drinks and bakery products as a flavoring agent and for extending the shelf-life of food [16, 22].

To investigate the structure–activity relationships of 4-methoxysalicylaldehyde and its derivatives, 4-hydroxysalicylaldehyde, salicylaldehyde, 3-methoxysalicylaldehyde, 5-methoxysalicylaldehyde, 3-methylsalicylaldehyde,

and 5-methylsalicylaldehyde were selected (Table 3). Antimicrobial activities of 4-methoxysalicylaldehyde and its analogues were examined against six foodborne bacteria via disc diffusion (Table 4). Among compounds with methoxy group in the C-4 position of the salicylaldehyde, 4-methoxysalicylaldehyde exhibited strong-to-moderate antimicrobial activities against six foodborne bacteria at 2.0–1.0 mg/disc. However, 4-methoxysalicylaldehyde exhibited weak or no antimicrobial activity against the microorganisms tested at 0.5 mg/disc. Similarly, among compounds with methoxy group in the C-5 position of the salicylaldehyde, 5-methoxysalicylaldehyde showed strong-to-weak antimicrobial activities against all the tested bacteria at 2.0 mg/disc. In contrast, 3-methoxysalicylaldehyde, which carries a methoxy group in the C-3 position of the salicylaldehyde, did not show antimicrobial activity against six foodborne bacteria. In this regard, the altered position of methoxy group on salicylaldehyde appeared to affect the antimicrobial activities against six foodborne bacteria. At a dose of 2.0–1.0 mg/disc, 4-hydroxysalicylaldehyde, which has a salicylaldehyde conjugated with the hydroxy group in the C-4 position, also demonstrated enormous antimicrobial activity against six foodborne bacteria. Especially, *S. aureus* was the most susceptible to 4-hydroxysalicylaldehyde at 0.125 mg/disc. The difference between 4-hydroxysalicylaldehyde and others relates to a single additional

hydroxyl group in the position of salicylaldehyde. Studies demonstrated that the addition of an extra hydroxyl group in the benzaldehyde derivatives resulted in enhanced antimicrobial activity [2, 23]. The observations were similar to a previous study conducted by Stojković et al. [24] who reported that caffeic acid containing two hydroxyl groups exhibited a higher antimicrobial activity than *p*-coumaric acid, containing a single hydroxyl group. Salicylaldehyde displayed limited antimicrobial activity against *S. typhimurium*, *S. aureus*, *L. monocytogenes*, and *S. flexneri* except for *S. intermedius* and *S. sonnei*. However, 3-methylsalicylaldehyde and 5-methylsalicylaldehyde, which contain a methyl group in the C-3 and C-5 positions of the salicylaldehyde, showed no antimicrobial activities against six foodborne bacteria at exposure levels of 2.0 mg/disc.

The MIC values of 4-methoxysalicylaldehyde and its derivatives required for inhibitory activity against six foodborne bacteria are listed in Table 5. In addition, not only 4-methoxysalicylaldehyde (MIC 30.1–67.3 µg/mL) but also 4-hydroxysalicylaldehyde (MIC 41.1–61.5 µg/mL) inhibited the growth of six foodborne bacteria. 5-Methoxysalicylaldehyde showed a moderate inhibitory activity (MIC 41.3–92.1 µg/mL), whereas salicylaldehyde exhibited inhibitory activity (MIC 62.8–81.5 µg/mL) against all the tested microorganisms except for

Table 4 Antimicrobial activities of 4-methoxysalicylaldehyde and its derivatives against foodborne bacteria

Compound	Conc. (mg/disc)	Bacterial species					
		Gram-positive bacteria			Gram-negative bacteria		
		<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
4-Methoxysalicylaldehyde	2.0	+++ ^a	+++	++	+++	++	+++
	1.0	++	++	+	++	++	++
	0.5	+	+	–	++	+	+
4-Hydroxysalicylaldehyde	2.0	++	+++	+++	+++	+	+++
	1.0	+	++	++	++	+	++
	0.5	–	++	+	++	–	++
	0.25	–	+	–	+	–	+
	0.125	–	+	–	–	–	–
Salicylaldehyde	2.0	++	+	–	+	+	–
	1.0	+	–	–	+	+	–
3-Methoxysalicylaldehyde	2.0	–	–	–	–	–	–
5-Methoxysalicylaldehyde	2.0	+	++	+	+++	++	+++
	1.0	–	+	+	++	+	++
	0.5	–	–	–	+	–	+
3-Methylsalicylaldehyde	2.0	–	–	–	–	–	–
5-Methylsalicylaldehyde	2.0	–	–	–	–	–	–

Cultured on Mueller–Hinton agar at 37 °C for 24 h in an incubator

^aDiameter of inhibition zone > 30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and < 10 mm, –

Table 5 Minimum inhibition concentrations (MIC) of 4-methoxysalicylaldehyde and its derivatives against foodborne bacteria

Compound	MIC ($\mu\text{g/mL}$) ^a					
	Gram-positive bacteria			Gram-negative bacteria		
	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. typhimurium</i>	<i>S. flexneri</i>	<i>S. sonnei</i>
4-Methoxysalicylaldehyde	41.7	51.9	67.3	30.1	59.8	40.2
4-Hydroxysalicylaldehyde	61.5	44.3	47.2	41.1	59.1	52.5
Salicylaldehyde	62.8	81.5	100 <	78.8	74.8	100 <
3-Methoxysalicylaldehyde	100 <	100 <	100 <	100 <	100 <	100 <
5-Methoxysalicylaldehyde	92.1	70.0	80.7	48.5	58.2	41.3
3-Methylsalicylaldehyde	100 <	100 <	100 <	100 <	100 <	100 <
5-Methylsalicylaldehyde	100 <	100 <	100 <	100 <	100 <	100 <

Cultured on Mueller–Hinton broth at 37 °C for 24 h in an incubator

^aMIC values < 100 $\mu\text{g/mL}$

S. intermedius and *S. sonnei*. However, 3-methoxysalicylaldehyde, 3-methylsalicylaldehyde, and 5-methylsalicylaldehyde did not show inhibitory effects against six foodborne bacteria. Several studies suggested that structural features of a compound determined the differences in their antimicrobial effects [2, 4, 25, 26].

The utilization of natural products may enhance food safety and reduce microbial contamination in many foods. In conclusion, our study showed the possibility of pathogen reduction in foods using natural food preservatives containing 4-methoxysalicylaldehyde and its derivatives. Furthermore, this study elucidated the structural properties of compounds derived from essential oil of *P. sepium* manifesting antibacterial activity.

Acknowledgments This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (2016R1A2A2A05918651).

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