Note

Antimicrobial Activity of the Carnivorous Plant *Dionaea muscipula* Against Food-Related Pathogenic and Putrefactive Bacteria

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Solvent extracts from the carnivorous plant *Dionaea muscipula* (Venus flytrap) were prepared using eight different organic solvents, and examined for antibacterial activity against food-related pathogenic and putrefactive bacteria. All solvent extracts showed higher antibacterial activity against gram positive bacteria than against gram negative bacteria. The TLC-bioautography analysis of the extracts revealed that a yellow spot was detected at *R_f* value of 0.85, which showed strong antibacterial activity. The UV, MS, and NMR analyses revealed that the antibacterial compound was plumbagin.

Key words : Antimicrobial activity / *Dionaea muscipula* / Food-related borne pathogenic bacteria / Plumbagin / Carnivorous plant.

Recently, the bacteriostatic and bactericidal effects of functional ingredients in plants against microorganisms have been examined, particularly in plants such as wasabi (Inouye et al., 1983), green tea (Toda et al., 1989), moso bamboo (Nishina and Uchibori., 1991), Job's Tears (Ishiguro et al., 1993), hops (Larson et al., 1996), persimmon leaves (Yamada et al., 1999), grapefruit peels (Negi and Jayaprakasha, 2001), grape seed (Jayaprakasha, et al., 2003), pomegranate peel (Negi and Jayaprakasha, 2003), Japanese basil (Vardar-Ünlü et al., 2003), flowers (Alzoreky and Nakahara., 2003), and cranberries (Wu et al., 2008). Some of them are used as food additives to increase the shelf life of food products.

Carnivorous plants are angiosperm spermatophytes (seed plants) and have acquired insectivorous behaviors. As many of these carnivorous plants grow in oligotrophic marshlands that are nitrogen deficient, these plants have developed the ability to trap insects

and extract the nitrogen they require for growth and propagation (Juniper et al., 1989). Interestingly, despite coming into close contact with insects, carnivorous plants rarely become infected by the pathogenic bacteria transmitted by many insects, suggesting the presence of some sort of defense mechanism. Few studies have been conducted on antibacterial activity of carnivorous plants, although the inhibitory effects of carnivorous plant extracts on intestinal bacteria have been reported (Park et al., 2005).

Dionaea muscipula (Venus flytrap) is a well-known carnivorous plant that catches and digests insects and spiders, and is also popular as a cultivated plant. Based on the possibility that carnivorous plants may employ special antibacterial defense mechanisms, we examined the antibacterial activity of *D. muscipula* extracts against food-related pathogenic and putrefactive bacteria in this study.

Dionaea muscipula was cultivated from parent plants. Small pieces of plant tissue were taken and transferred to a Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The

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tissue samples were then cultured for approximately two months at room temperature. After removing dirt and impurities from the selected *D. muscipula* specimens, the plants were weighed, frozen in liquid nitrogen and ground using a mortar and pestle. Five grams of the ground tissue were added to 50 ml of ethanol, methanol, acetone, methyl ethyl ketone, ethyl acetate, 2-propanol, chloroform and hexane, and extraction was performed by agitation for 24 h at 4°C. Each extract was filtered, and then concentrated to 5 ml under vacuum.

The bacterial strains used in this study were the gram positive bacteria, *Bacillus cereus* IAM 1110, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* CSJ 1923 and *Enterococcus faecalis* ATCC 29212 (n=5), and the gram negative bacteria, *Escherichia coli* CSJ 1922, *Escherichia coli* ATCC 35150 (O157:H7), *Pseudomonas aeruginosa* CSJ 1853, *Salmonella* Enteritidis IFO 3313, *Salmonella* Typhimurium IID 971, *Serratia marcescens* ATCC 13880 and *Yersinia enterocolitica* JCM 1677 (n=7). All strains were incubated for 24 h at 37°C, except *M. luteus* and *Y. enterocolitica* which were incubated at 30°C.

First, we attempted to examine the antibacterial activity of the extracts. Assessments of the activity were conducted using a National Committee for Clinical Laboratory Standards disk diffusion method (NCCLS., 1990). Bacterial strains were subcultured in tryptic soy broth (TSB; Becton Dickinson Co. Franklin Lakes, NJ, USA) at 30°C or 37°C for 24 h and then smeared on Mueller–Hinton Agar (MHA; Becton Dickinson Co. Franklin Lakes, NJ, USA). Colonies were then collected and used to prepare bacterial solutions with a McFarland turbidity of 0.5. These were then smeared on MHA plates with a sterile cotton swab before a paper disk (diameter = 6 mm, Toyo Roshi Kaisha, Ltd. Tokyo, Japan) inoculated with 30 µl of the extract was placed on the bacterial cultures. The MHA plates were then incubated at 30°C or 37°C for 18 h and the inhibition zone diameter was measured.

Table 1 summarizes the antibacterial activity of solvent extracts from *D. muscipula*. The antibacterial activity of each extract was assessed against the gram positive bacterium, *S. aureus*, and the gram negative bacterium, *E. coli*. All of the solvent extracts showed higher antibacterial activity against gram positive bacteria than against gram negative bacteria. Among them, the extracts with ethanol, acetone and chloroform showed large inhibition zones of ≥ 27 mm against *S. aureus*. Although the sizes of the inhibition zone against *E. coli* were smaller than those against *S. aureus*, methanol, ethanol, methyl ethyl ketone and 2-propanol extracts showed higher antibacterial activity than those of other extracts.

The components in the extracts were analyzed by using high performance thin layer chromatography (HPTLC). Silica gel 60 F₂₅₄ with a 0.25 mm thickness (10 × 10 cm, Merck Ltd., Darmstadt, Germany) was used for HPTLC analysis. Five to nine spots were observed in different solvent extracts, and some of them were detected at the same *Rf* values, although the amounts (spot size) were different depending on the solvent used (Fig. 1). These results implied that the common compounds observed in all solvent extracts, specifically the compounds detected at the same *Rf* values, may show antibacterial activity.

From the results of HPTLC analysis, the methanol and ethanol extracts contained several compounds, and therefore the antibacterial activity of these extracts was examined against various food-related bacteria (Table 2). When tested against five species of gram positive bacteria and seven species of gram negative bacteria, antibacterial activity was observed against all 12 test strains. Large inhibition zones measuring 29.0 ± 1.8 to 31.1 ± 1.7 mm in diameter were observed around the gram positive bacteria (*B. subtilis*, *M. luteus* and *S. aureus*), indicating strong antibacterial activity. On the other hand, the inhibition zones around the gram negative bacteria were smaller (≤ 11 mm in diameter), indicating that these extracts were less effective against the test bacteria. Although no differences in the bactericidal efficacy between ethanol and methanol extracts were observed, the ethanol extract showed slightly larger inhibition zones.

The TLC-bioautography which combined microbial detection with TLC separation technique was used to assess the antibacterial activity of the separated spots. As shown in Fig. 2, the clear zone on the agar plate showed a *Rf* value of 0.85, which appeared at the same position as the yellow spot observed in the HPTLC analysis, suggesting the presence of an antibacterial compound in the yellow spot.

TABLE 1. Antibacterial activity of *Dionaea muscipula* extracts against *S. aureus* and *E. coli*

Each solvent	Inhibition zone diameter (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
Methanol	++	++
Ethanol	+++	++
2-propanol	++	++
Acetone	++	+
Ethyl acetate	+++	+
Ethylmethylketone	++	++
Chloroform	+++	+
Hexane	++	+

Each extracts (30 µl) was used in paper disk (Inhibition zone, including diameter of the disk, 6.0 mm)

+: ≤ 10 mm, ++: $> 10 \sim \leq 27$ mm, +++: > 27 mm

FIG. 1. HPTLC of different solvent extracts from *Dionaea muscipula*

1, methanol; 2, ethanol; 3, 2-propanol; 4, acetone; 5, ethyl acetate; 6, ethyl methyl ketone; 7, chloroform; 8, hexane. The sample powder (5 g) was incubated with different solvents (50 ml) at 4 °C for 24 h. After filtration and evaporation, the evaporate was dissolved in each solvent (5 ml). An aliquot (10 μ l) of the extract was withdrawn for HPTLC and spotted. The HPTLC was used with diethylether – hexane (1:1) as a developing solvent. The images were captured on a plate under UV light at 254 nm.

FIG. 2. TLC-bioautography of ethanol extract from *Dionaea muscipula*

The samples were directly deposited on the TLC plates (8 $\times$ 23 cm, Eiken Chemical Co. Ltd., Tokyo, Japan), and then the TLC plates were developed with diethyl ether – hexane (1:1, v/v) as a developing solvent. Each TLC plate was gently placed face down for 15 min on a square Petri dish that contained MHA inoculated with *B. subtilis* ATCC 6633. After separating the TLC plate from the agar, the Petri dishes were incubated for 20 h at 37°C. The antibacterial zone diameter was measured.

TABLE 2. Antibacterial activity of solvent extracts from *Dionaea muscipula* extracts against food-related pathogenic and putrefactive bacteria.

Bacterial strains		Inhibition zone diameter (mm)	
		Methanol	Ethanol
Gram positive bacteria	<i>B. cereus</i>	20.3 $\pm$ 1.5	20.9 $\pm$ 1.7
	<i>B. subtilis</i>	29.0 $\pm$ 1.8	30.1 $\pm$ 1.0
	<i>M. luteus</i>	29.1 $\pm$ 0.9	30.5 $\pm$ 1.9
	<i>S. aureus</i>	31.1 $\pm$ 1.7	30.9 $\pm$ 1.2
	<i>S. faecalis</i>	18.8 $\pm$ 0.5	18.9 $\pm$ 0.5
Gram negative bacteria	<i>E. coli</i>	10.9 $\pm$ 0.9	10.8 $\pm$ 0.7
	<i>E. coli</i> O157:H7	9.8 $\pm$ 0.8	10.2 $\pm$ 0.7
	<i>P. aeruginosa</i>	8.6 $\pm$ 0.9	8.2 $\pm$ 0.7
	<i>S. Enteritidis</i>	9.7 $\pm$ 1.3	10.2 $\pm$ 0.4
	<i>S. Typhimurium</i>	8.9 $\pm$ 0.4	9.5 $\pm$ 0.2
	<i>S. marcescens</i>	9.3 $\pm$ 0.9	9.4 $\pm$ 0.9

Each extracts (30 μ l) was used in paper disk (Inhibition zone, including diameter of the disk, 6.0 mm)

To identify the antibacterial compound, preparative TLC and HPLC purifications and spectroscopic analyses were performed. The methanol extract (400 ml) was prepared using 40 g of *D. muscipula* lyophilized powder as described above. After being concentrated to 40 ml, the solutions were applied onto the preparative TLC plates (Silica gel 60, 20 $\times$ 20 cm, thickness 0.7 mm, Merck Ltd.) using diethyl ether – hexane (1:1, v/v) as a mobile phase. The band detected at *R_f* value of 0.85, showing antibacterial activity in TLC-bioautography assay, was scraped out and extracted

using acetone. After evaporation to dryness (175 mg), the fraction was dissolved in 10 ml of 80% acetone. The solution was subjected to preparative HPLC (column: Shiseido Capcell Pak C18, 250 $\times$ 10 mm, eluent: 80% acetone, flow rate: 2 ml/min, monitoring wavelength: 420 nm, column oven: 40°C) to give a crystalline compound (60 mg). The isolated compound was subjected to spectroscopic analyses. The FAB-MS spectrum (JEOL JMS-SX 102A mass spectrometer, positive mode) showed molecular ion related peaks at *m/z* 190 [*M*+2*H*]⁺, suggesting a reduced type of

naphthoquinone (Matsuura and Yamaguchi, 1996). From the results of the UV, MS, and NMR analyses, the isolated compound was finally identified as plumbagin (Fig. 3). The chromatographic and spectroscopic analyses of authentic compound also confirmed the structure. The spectroscopic data are as follows: UV-vis (benzene); 423 nm ($E^{1\%}_{1\text{cm}}=38.5$), FAB-MS (+); m/z 190 $[M+2H]^+$ (100), $^1\text{H-NMR}$ (chloroform- d); δ 2.19 (3H, d, $J=2.0$ Hz, $-\text{CH}_3$), 6.80 (1H, d, $J=2.0$ Hz, H-3), 7.25 (1H, dd, $J=2.0, 7.5$ Hz, H-6), 7.59 (1H, m, H-7), 7.62 (1H, m, H-8), 12.0 (1H, s, $-\text{OH}$), $^{13}\text{C-NMR}$; δ 16.5 ($-\text{CH}_3$), 115.1 (C-4a), 119.3 (C-8), 124.1 (C-6), 132.0 (C-8a), 135.4 (C-3), 136.1 (C-7), 149.6 (C-2), 161.1 (C-5), 184.8 (C-1), 190.3 (C-4).

When the carnivorous plant *D. muscipula* digests insects, the possibility exists that it could become infected by any pathogenic bacteria present in those insects. Instead, the plant appears to absorb the nutrients it requires from the insects that it ingests without becoming infected. Plumbagin may play a role in protecting *D. muscipula* against bacterial pathogens. The results of this study demonstrated the antibacterial activity of plumbagin against food-borne pathogens and other bacteria and suggested that *D. muscipula* was equipped with a mechanism for preventing infection. Previous reports related to plumbagin in *D. muscipula* have shown that this compound is involved in protecting the plant from herbivores (Tokunaga et al., 2004). In addition, plumbagin has also been shown to inhibit insect molting (Kubo et al., 1983). Plumbagin is also found in other plant taxa, including members of the Plumbaginaceae, such as *Plumbago auriculata*. It is also used in Africa as a traditional medicine for headaches, vomiting, skin diseases and other problems (Elgorashi et al., 2003) and has been reported in Japanese *Diospyros* spp. (Ito et al., 1995). Demma et al. (2009) reported that plumbagin induced DNA damage at high concentrations, but showed antigenotoxic effects at low concentrations, and concluded that it was an agent with dual actions. Future studies on this compound, such as quantification studies or studies on its stability and

toxicity, are required to examine its potential application as an antimicrobial agent or repellent.

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FIG. 3. Chemical structure of plumbagin

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