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報　文

Antimicrobial Activity of Cloves and Cinnamon Extracts against Food Borne Pathogens and Spoilage bacteria, and Inactivation of *Listeria monocytogenes* in Ground Chicken meat with their Essential oils

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Abstract

Ethanol, aqueous extracts, and essential oils of Cloves (*Syzygium aromaticum*), and Cinnamon (*Cinnamomum cassia*) were analyzed for determination of antibacterial activity against 21 food borne pathogens: *Listeria monocytogenes* (5 strains), *Staphylococcus aureus* (4 strains), *Escherichia coli* O157: H7 (6 strains), *Salmonella* Enteritidis (4 strains), *Vibrio parahaemolyticus* and *Bacillus cereus* and 5 food spoilage bacteria: *Pseudomonas aeroginosa*, *P. putida*, *Alcaligenes faecalis*, and *Aeromonas hydrophila* (2 strains). Screening of cloves and cinnamon extracts showed antibacterial activity against the test organisms. The MIC values for ethanol, aqueous extracts, and essential oil from cloves ranged from 0.5 to 5.5 mg/ml, 0.8 to 5.5 mg/ml, and 1.25 to 5 %, respectively. The MIC values for ethanol, aqueous extracts, and essential oil from cinnamon ranged from 1.0 to 3.5 mg/ml, 2.5mg/ml, and 1.25 to 5.0 %, respectively. The effect of temperature and pH on the antibacterial activity of essential oils of cloves and cinnamon against cocktails of different strains of *L. monocytogenes*, *E coli* O157: H7 and *Salmonella* Enteritidis were determined. The essential oils (EO) of cloves and cinnamon showed antibacterial activity after treatment at 100℃ for 30 min suggesting that the high temperature does not affectthe activity of these EO. The highest antibacterial activity was found at pH 5.0 for EO of cloves and cinnamon against most of the bacterial mixtures except for *L. monocytogenes*, where the highest activity was found at pH 7.0. The EO of cloves (10 %) and cinnamon (5%) were appliedin ground chicken meat inoculated with a cocktail of 5 strains of *Listeria monocytogenes*. The result showed that EO of cloves reduced all *Listeria monocytogenes* cells to an undetectable level in ground chicken meat within 1 day of exposure. However, the EO of cinnamon reduced *Listeria monocytogenes* in ground chicken meat by 2.0 log CFU /g within 1 day with only slight reductions or no further decline in cell population throughout the 15 days incubation period. Therefore, EO of clove could be useful to control *L. monocytogenes* in ground chicken meat.

Key word : Antibacterial Activity, Cinnamon extracts, Cloves extracts, Essential oil, Food Borne Pathogens and Spoilage Bacteria

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INTRODUCTION

The growing concern about food safety has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria. Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavor[1]. The antimicrobial properties of some spices and their components have been documented[2) 3) 4) 5]. Studies done previously confirm that garlic, onion,cinnamon, cloves, thyme, sage, and other spices inhibit the growth of both Gram-positive and Gram--negative food borne pathogens or spoilage bacteria, yeast, and molds[1) 6].

The antibacterial activity of spices may differ between strains within the same species. Moreover, the antimicrobial properties of spices may differ depending on the form of spices added, such as fresh, dried, or extracted forms[7] and also differ depending on the harvesting seasons[8) 9] and between geographical sources[10]. However, there is evidence that the essential oils of spices are more strongly antibacterial than is accounted for by the additive effect of their major antimicrobial components; minor components to play a significant role[6) 11].

Clove and cinnamon have been used in foods since antiquity[10]. Major antimicrobial components in clove and cinnamon have been reported to be eugenol and cinnamaldehyde, respectively[12], which have been given special attention to find their antibacterial activity against food borne pathogens. Eugenol has been reported to inhibit the growth of E. *coli* O157 : H7 and *L. monocytogenes*[13]. Cinnamaldehyde has been reported to inhibit the growth of *S. aureus*[14], *E. coli* O157 : H7, and *Salmonella* Typhimurium[15].

Listeria monocytogenes is a frequent food contaminant and is commonly recovered from raw meat, poultry, and seafood, as well as numerous varieties of processed dairy items, meat, seafoods and delicatessen products[16]. *Listeria monocytogenes* most often is found in cooked/ready-to eat foods as a post processing contaminant, since it is typically found within the manufacturing environment[17]. Although rapid growth of *L. monocytogenes* has been reported in processed meats[18], liquid eggs[19], and various seafoods including smoked salmon[20], growth of *L. monocytogenes* in pate and certain soft surface-ripened cheeses having a pH $>$ 6.5 appear to pose the greatest threat of listeriosis. The tolerance of *L. monocytogenes* to certain preservatives has resulted in an extensive effort to develop processes to control its growth in foods[21].

The objective of this study was to assess 1) the *in vitro* antibacterial activity of different extracts of cloves and cinnamon against selected food borne pathogens and spoilage bacteria, 2) the minimum inhibitory concentration (MIC) against each bacterium, 3) the effect of pH and temperature on the antibacterial activity of their EOs, and 4) application of clove and cinnamon EOs to inactivate *L. monocytogenes* in ground chicken meat.

Materials and Method

Test organisms

A total of 26 strains or species of frequently reported food borne pathogens and food spoilage bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli* O157: H7, *Salmonella* Enteritidis, *Bacillus cereus*, *Pseudomonas* spp., *Alcaligenes faecalis*, and *Aer-omonas hydrophila* were used in the study (Table 1). The stock cultures of the test organisms in 20% glycerol-containing medium in cryogenic vials were kept at $-$ 84℃ . Working cultures were kept at 4℃ on Trypto Soy Agar (TSA) slants (Nissui, Japan) and were periodically transferred to fresh slants.

Preparation of clove and cinnamon extracts

Dried cloves and cinnamon were purchased from local retail markets and transported to the Laboratory. The dried clove and cinnamon were individually ground using a grinder (Model A-210, IWATANI, Japan) into a fine powder.

Ethanol extracts:

TABLE 1. Test organisms used in this study

Code No.	Organisms	Type culture	Origin
1Lm	*Listeria monocytogenes*	ATCC 43256	Mexican style cheese
2Lm	*L. monocytogenes*	ATCC 49594	Scott A
3Lm	*L. monocytogenes*	JCM 7671	Lax Ham
4Lm	*L. monocytogenes*	JCM 7672	Roasted beef
5Lm	*L. monocytogenes*	JCM 7676	Salami sauces
6Sa	*Staphylococcus aureus*	JCM 2151	Unknown
7Sa	*S. aureus*	JCM 2179	Unknown
8Sa	*S. aureus*	JCM 2874	Wound
9Sa	*S. aureus*	IFO 13276	Human lesion
10Vp	*Vibrio parahaemolyticus*	IFO 12711	Shirasu food poisoning, Japan
11Ec	*Escherichia coli* O157 : H7	MN 28	Bovine feces
12Ec	*E. coli* O157 : H7	CR 3	Bovine feces
13Ec	*E. coli* O157 : H7	DT 66	Bovine feces
14Ec	*E. coli* O157 : H7	MY 29	Bovine feces
15Ec	*E. coli* O157 : H7	E 615	Tomato juice
16Ec	*E. coli* O157 : H7	JCM 1649	Urine
17 Sal	*Salmonella* Enteritidis	SE 1	Chicken feces
18 Sal	S. Enteritidis	SE 2	Bovine feces
19 Sal	S. Enteritidis	SE 3	Chicken feces
20 Sal	S. Enteritidis	IDC 7	Egg
21Bc	*Bacillus cereus*	IFO 3457	Unknown
22 Pa	*Pseudomonas aeroginosa*	PA 01	Unknown
23 Pp	*P. putida*	KT 2440	Unknown
24 Af	*Alcaligenes faecalis*	IFO 12669	Unknown
25 Ah	*Aeromonas hydrophila*	NFRI 8282	Unknown
26 Ah	*A. hydrophila*	NFRI 8283	Unknown

One hundred grams of cloves and cinnamon were soaked in 400 ml of ethanol (WAKO, Japan) in sterilized bottles (800 ml) with constant agitation (130 rpm) overnight at 20℃ in a temperature-controlled bioshaker (BR-40 LF, TAITEC)). The ethanol fraction was separated using sterilized cheesecloth and filtered through sterilized Whatman filter paper (No. 2).

Aqueous Extracts :

The residual materials of each sample after ethanol extraction was dried at 40℃, overnight in an oven. Then 400 ml of sterilized distilled water was added to each dried residue and agitated (130 rpm) overnight at 20℃ in the temperature-controlled bioshaker. The aqueous fraction was separated by sterilized cheesecloth and sterilized Whatman filter paper (No. 2).

All the extracts were then concentrated using a rotary vacuum evaporator (EYELA) at 40℃, and the concentrated extracts were diluted to 10 mg/ml using 10 % DMSO as solvent, sterilized by filter (0.45 μm), and kept at −20℃ until use (approximately 2 months).

Extraction of essential oils

The essential oils (EO) of clove and cinnamon were extracted using a solvent-solvent extraction method[22]. Ground cloves or cinnamon were added to hexane (Nacalai tesque Inc. Tokyo, Japan) and was kept at 20℃ in the bioshaker at 150 rpm for 24 h, and then hexane fraction was separated by squeezing through sterilized cheesecloth. The hexane from the fraction was evaporated using a vacuum evaporator (EYELA), which left mass of organics called concrete. Ethanol (99.5 %) was added to the concrete, and the material was transferred into a separation funnel, vigorously shaken, and kept for several hours for sedimentation of the ethanol insoluble part, which was mostly wax. The ethanol soluble part was poured into an evaporator flask

and concentrated by vacuum evaporator until all of the ethanol was completely evaporated, leaving the absolute essential oil.

Antimicrobial sensitivity testing :

The antimicrobial activity of all the clove and cinnamon extracts including EOs was determined according to the method of Bauer *et al*[23]. Eight mm in diameter discs (ADVANTEC; Toyo Roshi Kaisha, Ltd. Japan) were impregnated with 50 μl of different concentration of each plant extracts before being placed on the inoculated agar plates. The inocula of the test organisms were prepared by transferring a loopful of culture into 9 ml of sterilized Moeller Hinton Broth (MHB) (Difco) and incubated at 37℃ for 5 to 6 h except for *Listeria monocytogenes*, where overnight grown cells were used. The bacterial culture was compared with McFarland turbidity standard (10^8 CFU/ml) (24) and streaked evenly in 3 planes with the cotton swab at a 60^0 angle on the surface of the Mueller Hinton agar plate (5×40 cm). Excess suspension was removed from the swab by rotating it against the side of the tube before the plate was seeded. After the inocula dried, the impregnated discs were placed on the agar using forceps dipped in ethanol and flamed, and were gently pressed down to ensure contact. Plates were kept at 4℃ for 30 to 60 min for better absorption, during this time microorganisms will not grow, but absorption of the extracts will take place. Negative controls were prepared using the same solvent without the plant extract. A reference antibiotic, gentamycin, was used as a positive control. The inoculated plates containing the impregnated discs were incubated in an upright position at 37℃ overnight for 24 to 48 h. The results were expressed as the zone of inhibition around the paper disk (8 mm).

Determination of the minimum inhibitory concentration (MIC).

The minimum inhibitory concentrations (MICs) of all the extracts were determined by microdilution tech-niques in Mueller-Hinton broth according to Sanches *et al*[25]. The inocula were prepared at a density adjusted to 0.5 McFarland turbidity standard [10^8colony-forming units (CFU/ml)] and diluted 1:10 for the broth microdilution procedure. Microtiter plates were incubated at 37℃, and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of extract at which the microorganism tested did not demonstrate visible growth. Minimum bactericidal concentration (MBC) was defined as the lowest concentration yielding negative subcultures or only one colony.

Effect of pH and temperature of clove and cinnamon extracts on antimicrobial activity.

The effect of temperature and pH on the antibacterial activity of clove and cinnamon extracts was determined by the methods as described by *Lee et al*[26]. All of the extract solutions were incubated at 37, 50, 75, and 100℃, respectively, in a water bath for 30 min. Then, the extracts heated at the different temperatures were cooled down and stored at 4℃ until use. To evaluate the effect of pH, the pH of the clove and cinnamon extracts was adjusted to a range of 5.0 to 9.0 with 50mM Phosphate buffer. Then the pH-adjusted mixtures were filtered with a 0.45 μm of membrane filter, stored at 4℃, and used within 30 min.

Use of Clove and cinnamon EOs to inactivate *L. monocytogenes* in ground chicken meat.

A 1250 g-ground chicken meat sample was inoculated with a nalidixic acid resistant five-strain mixture (50 ml) of *L. monocytogenes* to obtain a final concentration of 10^6 CFU/g. Inoculated ground chicken meat samples were mixed for 1 min with a sterilized spoon. The clove and cinnamon EOs were then added at a concentration of 10 and 5 % (w/v), respectively and chicken meat samples were mixed for another 1 min. After the 1-min mixing period to achieve uniform dispersal throughout the sample, meat samples (25 g each) were packed in a Ziploc pack (Tebik Co Ltd Tokyo Japan) and stored at −18℃ for 15 days. Microbiological analysis of non-inoculated, and inoculated mixed samples was done on days 0, 1, 3, 5, 7, 10, and 15 days of post storage. Each sample was homogenized for 1 min in sterilized peptone water

(0.1 %) using a stomacher (ILU Instrument, model CE -97. Barcelona, Spain)). From this mixture, serial dilutions were prepared and surface plated (0.1 ml, in duplicate) on Trypto Soya Agar containing 50 μg/ml nalidixic acid (Wako, Japan) (TSAN) and modified Oxford medium (Oxoid) containing 50 μg/ml nalidixic acid (MOXN). Plating on media containing nalidixic acid greatly minimized interference due to colony development by naturally occurring microorganisms, thus facilitating detection of the test pathogen on the recovery media. The plates were incubated at 37℃ for 24 to 48 h before presumptive colonies were counted.

Inactivation of *L. monocytogenes* in PBS

Five-strain mixture of *L. monocytogenes* inocula was prepared in PBS with approximately 10^6 CFU/ml of cells. One hundred microlitter of EO of clove or 0.05 ml of EO of cinnamon was added in 0.8 ml or 0.85 ml of cell suspensions in PBS (pH 7.2), respectively, in centrifuge tubes. The final concentrations of the EOs of clove or cinnamon were 10 % and 5 %, respectively. The tubes were then incubated at −18℃ for 60 min and the bacterial count was done periodically at 0, 2, 5, 10, 20, 30 and 60 min of post incubation. The diluted and undiluted sample was surface plated on TSAN and MOXN agar plate and the plates were incubated at 37℃ for 48 h before presumptive colonies were counted.

Statistical analysis

The inhibition zones were calculated as means ± S. D. (n = 3). The significance among different data was evaluated by analysis of variance (ANOVA) using the Microsoft Excel program. Significant differences in the data were established by least significant difference at the 5 % level of significance. The inactivation of *L. monocytogenes* experiments were done two times with triplicate samples being analyzed at each sampling time. *Listeria monocytogenes* levels were expressed as the log CFU per gram recovered by direct plate counts.

RESULTS

Antibacterial activity of clove and cinnamon.

The antibacterial activity of clove and cinnamon extracts were tested using different bacteria and the results are listed in Table 2. It was found that the ethanol extract of clove was potentially active against *L. monocytogenes*, *S. aureus*, *V. parahaemolyticus*, pseudomanads, aeromonads, and *Alcaligenes faecalis* with zones of inhibition ranging from 13.4 to 26.3 mm. The aqueous extract of clove was active against all *S. aureus* strains and *V. parahaemolyticus* IFO 12711. However, ethanol and aqueous extracts of clove were unable to inhibit the *E. coli* O157 : H7 and *Salmonella* Enteritidis strains tested. For cinnamon, the ethanol extract was active only against *Staphylococcus aureus* strains except *S. aureus* JCM 2874 and *V. parahaemolyticus* with zones of inhibition ranging from 10.0 to 11.4 mm. The EOs from clove or cinnamon have antimicrobial properties and shown to inhibit all test organisms. The EOs of clove and cinnamon showed maximum inhibition for *A. hydrophila* NFRI 8282 (32.0 mm) and *B. cereus* IFO 3457 (46.5 mm), respectively, with zones of inhibition larger than those observed against the antibiotic, gentamycin (Table 2).

MICs of clove and cinnamon extracts

The MIC values of the ethanol and aqueous extracts of clove and cinnamon ranged from 0.5 to 5.5 mg/ml and 1.0 to 3.5 mg/ml, respectively (Table 3). The MIC of the clove ethanol extract showed the highest inhibition for *A. faecalis* IFO 12669 (0.5 mg/ml) and *A. hydrophila* NFRI 8282 (0.5 mg/ml) and the lowest inhibition for *P. aeruginosa* PA 01(5.5 mg/ml). However, the aqueous extract of clove showed the highest inhibition for *A. faecalis* IFO 12669 (0.8 mg/ml) and the lowest inhibition for *V. parahaemolyticus* IFO 12711 (5.5 mg/ml). The highest MIC value of the EOs of clove was 1.25 % against *A. hydrophila* and the lowest was 5 % against almost all the test organisms (Table 3).

The MIC of the cinnamon ethanol extract showed the highest inhibition for *V. parahaemolyticus* IFO

TABLE 2. Antibacterial activity of clove and cinnamon extracts against food borne pathogens and spoilage bacteria

Test Organisms (Code Nos.)	Plant extracts					
	Zones of inhibition[a]					
	Clove			Cinnamon		Antibiotic
	EtOH	H_2O	EO	EtOH	EO	GM
1Lm	14.1 ± 0.67	−	16.0 ± 0.50	−	27.3 ± 0.26	18.8 ± 0.64
2Lm	14.0 ± 1.05	−	16.5 ± 0.47	−	30.0 ± 0.59	22.9 ± 0.12
3Lm	26.3 ± 2.36	−	13.5 ± 1.00	−	33.0 ± 0.50	23.0 ± 0.50
4Lm	16.3 ± 0.92	−	14.1 ± 0.12	−	37.0 ± 0.68	15.5 ± 0.50
5Lm	16.1 ± 0.85	−	11.0 ± 0.25	−	38.4 ± 0.40	22.6 ± 0.12
6Sa	17.7 ± 0.85	17.9 ± 1.13	20.0 ± 0.95	10.0 ± 0.45	44.0 ± 0.50	24.5 ± 0.42
7Sa	14.7 ± 0.29	14.6 ± 0.59	18.5 ± 0.50	10.3 ± 0.58	24.0 ± 0.81	22.0 ± 0.35
8Sa	15.0 ± 0.9	14.0 ± 1.00	15.7 ± 1.66	−	31.5 ± 0.20	20.4 ± 0.12
9Sa	15.5 ± 0.5	16.0 ± 0.50	25.5 ± 0.50	11.0 ± 0.20	39.5 ± 0.59	18.1 ± 0.12
10Vp	19.4 ± 1.2	11.3 ± 0.35	21.5 ± 0.17	11.4 ± 0.15	20.5 ± 0.25	30.0 ± 0.40
11Ec	−	−	17.3 ± 0.90	−	21.0 ± 1.00	18.5 ± 0.44
12Ec	−	−	13.0 ± 0.66	−	20.0 ± 0.65	18.0 ± 0.23
13Ec	−	−	13.0 ± 0.70	−	20.0 ± 0.72	13.4 ± 0.15
14Ec	−	−	18.0 ± 0.50	−	21.5 ± 0.42	21.0 ± 0.31
15Ec	−	−	15.6 ± 0.10	−	21.5 ± 0.15	21.5 ± 0.15
16Ec	−	−	16.5 ± 0.50	−	21.5 ± 0.58	20.8 ± 0.25
17Sal	−	−	16.4 ± 0.10	−	22.2 ± 0.30	22.5 ± 0.17
18Sal	−	−	16.3 ± 0.41	−	20.3 ± 0.57	19.0 ± 0.99
19Sal	−	−	14.2 ± 0.68	−	19.8 ± 0.25	21.0 ± 0.10
20Sal	−	−	17.0 ± 0.51	−	23.1 ± 0.60	19.0 ± 0.78
21Bc	14.2 ± 0.25	−	12.5 ± 0.80	−	46.5 ± 0.50	22.0 ± 0.21
22Pa	15.4 ± 0.71	−	11.5 ± 0.52	−	12.0 ± 0.45	21.5 ± 0.12
23Pp	13.4 ± 0.21	−	22.0 ± 1.00	−	11.0 ± 0.50	21.0 ± 0.25
24Af	20.3 ± 1.0	22.5 ± 0.52	19.8 ± 0.40	−	22.2 ± 0.57	9.7 ± 0.30
25Ah	25.0 ± 0.06	12.0 ± 0.87	32.0 ± 0.47	−	31.9 ± 0.12	19.1 ± 0.17
26Ah	23.0 ± 2.16	9.7 ± 0.58	30.0 ± 0.45	−	29.5 ± 0.42	15.0 ± 0.20

Concentration of all of the plant extracts were 10.0 mg/ml and 10 % (EO)
[a]Represents mean ± S. D. mm (n = 3) ; $p < 0.05$; GM, Gentamycin (10 μg)

12711 (1.0 mg/ml) and the lowest inhibition for *S. aureus* IFO 13276 (3.5 mg/ml). The essential oil of cinnamon showed the highest lethal activity for all *L. monocytogenes* strains, *B. cereus*, and all strains of *A. hydrophila* (2.5 %), and the lowest activity was found against *P. aeruginosa* (Table 3).

Antimicrobial activity of clove and cinnamon extracts at different temperatureand various pH

The effect of temperature and pH on the antibacterial activity of ethanol and aqueous extracts of cloves against bacterial cocktails of different strains of *L. monocytogenes* and *S. aureus* were determined. The antibacterial activity of the extracts was almost unchanged below 50℃, and then the activity was slightly reduced except for aqueous extract of clove (Table 4). With the EO from cloves and cinnamon, the antibacterial activities were found unchanged at all temperatures applied suggesting that the active components of EO were not destroyed at high temperatures even with the 30 min treatment at 100℃. Moreover, the antibacterial activities of cinnamon EO were found to increase with increasing temperature. This might be due to the partial

TABLE 3. MIC of clove, cinnamon extracts against food borne pathogens and spoilage bacteria

Test organism (Code Nos.)	MIC/MBC (mg/ml)				
	Plant extracts				
	Clove			Cinnamon	
	EtOH	H_2O	EO	EtOH	EO
1Lm	2.5 (3.0)	−	2.5/5.0	−	1.25/2.5
2Lm	2.0 (2.5)	−	2.5/5.0	−	1.25/2.5
3Lm	1.0 (1.5)	−	2.5/5.0	−	1.25/2.5
4Lm	1.5 (2.0)	−	2.5/5.0	−	1.25/2.5
5Lm	2.0 (2.5)	−	2.5/5.0	−	1.25/2.5
6Sa	1.5 (2.0)	2.0 (2.5)	2.5/5.0	2.5 (3.0)	2.5/5.0
7Sa	2.5 (3.0)	2.5 (3.0)	2.5/5.0	2.0 (2.5)	2.5/5.0
8Sa	2.5 (3.0)	2.0 (2.5)	2.5/5.0	−	2.5/5.0
9Sa	2.0 (2.5)	2.5 (3.0)	2.5/5.0	3.5 (4.0)	2.5/5.0
10Vp	1.0 (1.5)	5.5 (6.0)	5.0/10.0	1.0 (1.5)	2.5/5.0
11Ec			2.5/5.0		2.5/5.0
12Ec			2.5/5.0		2.5/5.0
13Ec			2.5/5.0		2.5/5.0
14Ec			2.5/5.0		2.5/5.0
15Ec			2.5/5.0		2.5/5.0
16Ec			2.5/5.0		2.5/5.0
17Sal			2.5/5.0		2.5/5.0
18Sal			2.5/5.0		2.5/5.0
19Sal			2.5/5.0		2.5/5.0
20Sal			2.5/5.0		2.5/5.0
21Bc	4.5 (5.0)	−	2.5/5.0		1.25/2.5
22Pa	5.5 (6.0)	−	5.0/10.0	−	5.0/10.0
23Pp	5.5 (6.0)	−	5.0/10.0	−	5.0/10.0
24Af	0.5 (0.6)	0.8 (0.9)	5.0/10.0	−	1.25/2.5
25Ah	0.5 (0.6)	1.5 (2.0)	1.25/2.5	−	1.25/2.5
26Ah	0.8 (0.9)	5.0 (5.5)	1.25/2.5	−	1.25/2.5

exhaustion of solvent in EO at high temperature or destruction of interfering components present in the EO of clove and cinnamon (Table 4).

The pH 5.0 showed antibacterial activities of the ethanol extracts of cloves against the cocktail of *L. monocytogenes*, however, the activity was diminished against the cocktail of *L. monocytogenes* at pH 9.0. The ethanol and aqueous extracts of clove showed higher activity against cocktail of *S. aureus* at pH 9.0 compared to pH 5.0 and 7.0 (Tables 5).

The pH 5.0 showed the antibacterial activities of EO of clove and cinnamon against most of the cocktails except for *L. monocytogenes*, where the highest activities were found at pH 7.0.

Inactivation of *L. monocytogenes* populations in inoculated ground chicken meat.

The levels of inactivation of *L. monocytogenes* population in inoculated ground chicken meat with EOs of clove and cinnamon are shown in Fig. 1. *Listeria monocytogenes* was not detected in non-inoculated ground chicken meat. The viable count of 6.25 log CFU/g *L. monocytogenes* in the inoculated meat sample decreased slightly within 5 days and/or remained constant throughout the 15 days incubation period at −18℃. However, treatment with 5 % cinnamon EO, the *L. monocytogenes* population decreased up to 4.75 log CFU/g in 1 day and thereafter a further reduction was observed on day 3. There was a 2.5 log CFU/g

Table 4. Effect of temperature on antibacterial activities of clove and cinnamon extracts against cocktails of L. monocytogenes (5), S. aureus (4), S. Enteritidis (4), and A. hydrophila (2)

Plant extracts	Mixtures of test organisms (Code Nos.)	Zones of inhibition[a, b]					
		Temperature (℃)					
		4	25	37	50	75	100
Clove							
EtOH	*L. monocytogenes* (1Lm to 5Lm)	20.0 ± 0.15	14.0 ± 0.30	12.5 ± 0.20	11.0 ± 0.31	10.0 ± 0.25	9.0 ± 0.0
EtOH	*S. aureus* (6Sa to 9Sa)	11.0 ± 0.00	11.0 ± 0.35	11.0 ± 0.46	11.0 ± 0.35	10.0 ± 0.29	10.0 ± 0.0
H_2O	*S. aureus* (6Sa to 9Sa)	12.0 ± 0.00	12.0 ± 0.25	12.0 ± 0.10	13.0 ± 0.87	13.5 ± 0.15	14.5 ± 0.15
EO	*L. monocytogenes* (1Lm to 5Lm)	13.6 ± 0.06	13.0 ± 0.06	13.0 ± 0.15	13.4 ± 0.17	13.8 ± 0.06	14.4 ± 0.06
	S. aureus (6Sa to 9Sa)	16.0 ± 0.00	16.0 ± 0.24	16.0 ± 0.24	14.4 ± 0.36	14.6 ± 0.05	15.3 ± 0.49
	E. coli O157 : H7 (11Ec to 16EC)	15.0 ± 0.26	15.0 ± 0.06	15.0 ± 0.00	13.5 ± 0.06	14.4 ± 0.15	14.4 ± 0.06
	S. Enteritidis (17Sal to 20Sal)	15.0 ± 0.00	15.0 ± .15	15.0 ± 0.31	14.0 ± 0.20	14.0 ± 0.21	14.0 ± 0.21
	A. hydrophila (25Ah & 26Ah)	22.5 ± 0.06	22.9 ± 0.10	23.0 ± 0.06	24.0 ± 0.35	25.0 ± 0.31	25.5 ± 0.49
Cinnamon[b]							
EO	*L. monocytogenes* (1Lm to 5Lm)	24.2 ± 0.71	27.3 ± 0.14	27.3 ± 0.28	26.5 ± 0.14	30.0 ± 0.00	34.0 ± 0.14
	S. aureus (6Sa to 9Sa)	29.7 ± 0.14	29.2 ± 0.14	29.2 ± 0.00	32.0 ± 0.57	32.5 ± 1.41	33.5 ± 0.85
	E. coli O157 : H7 (11Ec to 16EC)	19.8 ± 1.56	19.5 ± 0.58	18.5 ± 0.71	21.4 ± 0.14	22.1 ± 0.14	24.3 ± 0.14
	S. Enteritidis (17Sal to 20Sal)	17.3 ± 0.14	18.0 ± 0.14	18.0 ± 0.00	19.7 ± 0.14	20.3 ± 0.14	22.3 ± 0.28
	A. hydrophila (25Ah & 26Ah)	31.0 ± 0.28	31.3 ± 0.49	30.4 ± 0.57	30.5 ± 0.00	32.2 ± 0.14	33.5 ± 0.14

Concentration of all of the plant extracts were 10.0 mg/ml and 10 % (EO),
[a]Represents mean ± S.D. mm (n = 3) ; $P < 0.05$, [b]Represents mean ± S.D. mm (n = 2)

reduction observed after 15 days of incubation at −18℃ (Fig. 1).

Treatment with 10 % clove EO, no viable counts of *L. monocytogenes* were detected on day 1 and thereafter throughout the incubation period. In this study, both nonselective TSAN and selective MOXN were used for the enumeration of *L. monocytogenes* on treated and untreated meat. Regardless of the meat conditions or treatments, higher populations of *L. monocytogenes* were recovered on TSAN than on MOXN. *L. monocytogenes* counts ranged from 0.91 to 1.25 log CFU/g higher when the inoculated meat samples were plated on TSAN compared to plating on the selective medium.

Inactivation of *L. monocytogenes* in PBS

Inactivation of *L. monocytogenes* population in PBS is shown in Fig. 2. The EO (10 %) of clove reduced the *L. monocytogenes* population to an undetectable level within 10 min at −18℃, however, the EO (5 %) of cinnamon only reduced *L. monocytogenes* by 2.0 log CFU/ml within 60 min (Figure 2). In this condition too, higher populations of *L. monocytogenes* were recovered on TSAN than on MOXN.

Table 5. Effect of pH on antibacterial activities of clove and cinnamon extracts against mixtures of L. monocytogenes (5), S. aureus (4), E. coli O157 : H7 (6), S. Enteritidis (4), and A. hydrophila (2)

Plant extracts	Mixtures of test organisms (Serotype Code Nos.)	Zones of inhibition[a]		
		pH		
		5.0	7.0	9.0
Clove				
EtOH	*L. monocytogenes* (1Lm to 5Lm)	25.0 ± 0.42	23.0 ± 0.28	0.0 ± 0.00
EtOH	*S. aureus* (6Sa to 9Sa)	12.5 ± 0.28	13.5 ± 0.14	14.1 ± 0.14
H_2O	*S. aureus* (6Sa to 9Sa)	10.0 ± 0.00	13.0 ± 0.28	17.6 ± 0.28
EO	*L. monocytogenes* (1Lm to 5Lm)	12.0 ± 0.85	13.2 ± 0.28	13.5 ± 0.28
	S. aureus (6Sa to 9Sa)	13.5 ± 0.14	13.6 ± 0.14	13.5 ± 0.71
	E. coli O157 : H7 (11Ec to 16EC)	13.2 ± 0.14	13.5 ± 0.42	14.3 ± 0.14
	S. Enteritidis (17Sal to 20Sal)	13.1 ± 0.14	13.9 ± 0.14	12.6 ± 0.07
	A. hydrophila (25Ah and 26Ah)	21.9 ± 0.85	22.9 ± 0.14	22.7 ± 0.07
Cinnamon				
EO	*L. monocytogenes* (1Lm to 5Lm)	23.7 ± 0.99	28.4 ± 0.92	26.1 ± 0.64
	S. aureus (6Sa to 9Sa)	34.0 ± 0.28	32.3 ± 0.35	30.5 ± 0.14
	E. coli O157 : H7 (11Ec to 16EC)	22.3 ± 0.78	21.7 ± 0.42	20.0 ± 1.41
	S. Enteritidis (17Sal to 20Sal)	20.8 ± 0.57	19.8 ± 0.85	18.4 ± 0.99
	A. hydrophila (25Ah and 26Ah)	33.4 ± 0.14	31.3 ± 0.57	29.6 ± 0.85

Concentration of all of the plant extracts were 10.0 mg/ml and 10 % (EO)
[a]Represents mean ± S.D. mm (n = 2); $P > 0.05$

DISCUSSION

The results of the disk diffusion test revealed that the crude ethanol extracts of clove and cinnamon showed different degrees of growth inhibition, depending upon the bacterial strains (Table 2). The ethanol extracts of clove and cinnamon showed notable antibacterial activity against Gram-positive bacteria. It is well known that most spices are more active against Gram-positive bacteria than Gram-negative bacteria[27], although ethanol extracts of both spices inhibited the growth of *V. parahaemolyticus* (Table 2).

This study showed that ethanol and water extracts of clove and the ethanol extract of cinnamon were more effective against Gram-positive bacteria than Gram-negative bacteria *in vitro*. But EOs of both spices was effective against both Gram-positive and Gram-negative bacteria, which is similar to other reports describing the use of EOs components[27,28,29].

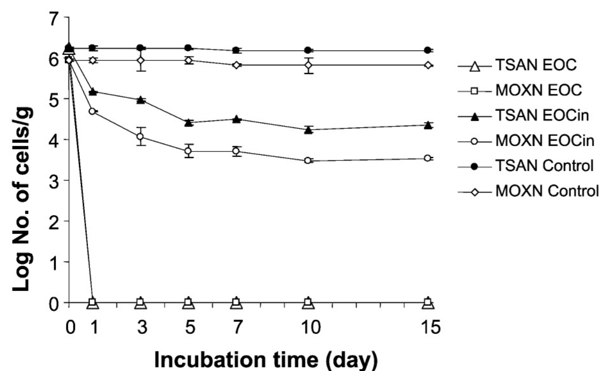

Fig. 1. Survival of Listeria monocytogenes in ground chicken meat exposed to EOs of clove (10%) and cinnamon (5 %) and stored at −18℃. Counts are means ± S.D. (n=3). Bars indicate error of standard deviation ($p<0.05$). EOC, indicates essential oil of cloves ; EOCin, indicates essential oil of cinnamon.

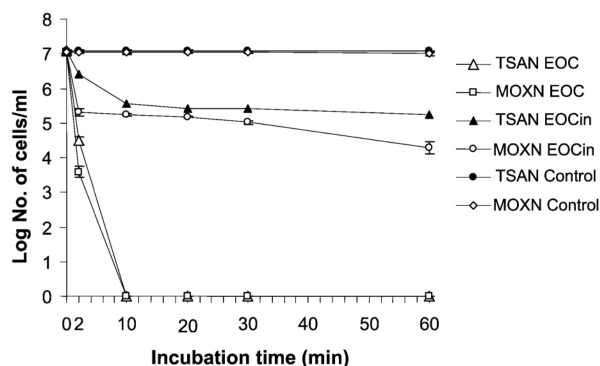

Fig. 2. Survival of Listeria monocytogenes in PBS at −18℃ exposed to EOs of clove (10%) and cinnamon (5 % and stored at −18 ℃). Counts are means ± S.D. (n=2). Bars indicate error of standard deviation ($p<0.05$). EOC, indicates essential oil of cloves ; EOCin, indicates essential oil of cinnamon.

The essential oils from clove and cinnamon showed the strongest antibacterial activity against all food borne pathogens and spoilage bacteria tested. Clove bud oil contains a high eugenol (70-90 %) content[30], which is an antimicrobial compound having wide spectra of antimicrobial effects against enterobacteria[7,31,32]. Similar findings have been reported by other investigators[33,34]. The results of the current study using the EO of clove correlated with the findings of other investigators[27,31]. Cressy *et al*[21] who showed the antibacterial activity of clove oil against *L. monocytogenes*, *Campylobacter jejuni*, S. Enteritidis, *E. coli* and *S. aureus*. Only a few studies have been conducted to determine the antimicrobial activity of herbs against *L. monocytogenes* in actual food products[35,36]. Results presented by Smith-Palmer *et al*[27] showed that the EO of clove was among the most capable for controlling *L. monocytogenes*.

Smith-Palmer *et al*[37] reported the reduction of *L. monocytogenes* from 6.0 log CFU/g to less than 1.0 log CFU/g in low and high fat cheese with low concentration of clove essential oil. In another study, higher concentration (10-fold) was used to inactivate the pathogen in pork sausages, 50-fold in soup and 25 to 100-fold in soft cheese to produce the similar efficacy compared to *in vitro* studies[38]. The presence of protein or fat in foods could protect food from the effect of essential oils. Therefore, higher concentrations of EOs are needed to effectively control the microorganism in food compare to *in vitro* studies.

In this experiment, 10 % of clove EO and 5 % of cinnamon EO was used, which is four times higher than that of their MIC values (Table 3).

CONCLUSION

Ethanol and aqueous extracts and the EOs from clove and cinnamon exhibited antibacterial activity against food borne pathogens *in vitro*. Gram-positive organisms were more sensitive to EOs of clove and cinnamon than Gram-negative organisms. EOs from clove and cinnamon exhibited bactericidal and bacteriostatic activity against *L. monocytogenes*, respectively, in ground chicken meat. Therefore, EOs of clove and cinnamon can be useful to control *L. monocytogenes* in ground chicken meat. However, there are some limitations in using spices like clove or cinnamon, such as 1) the antibacterial activity is decreased when spices are added to food materials containing protein, carbohydrate, and fat, and 2) the strong flavor. The flavor of the food products may not be acceptable by some consumer groups if large amounts of spices are added to the products to inhibit the food borne pathogens. Therefore, the use of spices along with preservatives

such as acid, salt, sugar and with processing and storage conditions can help in controlling microorganismsin food products.

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シナモンとクローブ抽出物の食中毒菌・腐敗菌に対する抗菌活性およびこれらハーブ由来の製油を用いた鶏挽肉中の接種 Listeria monocytogenes の不活化

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要　約

クローブ（*Syzygium aromaticum*）とシナモン（*Cinnamomum cassia*）のエタノール抽出画分，水溶性画分および製油画分の食中毒菌 21 株［*Listeria monocytogenes*（5 株），*Staphylococcus aureus*（4 株），*Escherichia coli* O157: H7（6 株），*Salmonella* Enter-itidis（4 株），Vibrio parahaemolyticus および *Bacillus cereus*］および腐敗菌 5 株［*Pseudomonas aeroginosa, P. putida, Alcaligenes faecalis*，および *Aeromonashy drophila*（2 株）］に対する抗菌活性を調べた．クローブからのエタノール抽出画分，水溶性画分および製油画分のこれら細菌に対する最小増殖阻止濃度（MIC）はそれぞれ 0.5 ～ 5.5mg/ml, 0.8 ～ 5.5mg/ml および 1.25 ～ 5％の範囲であった．シナモンからのエタノール抽出画分，水溶性画分および製油画分のこれら細菌に対する最小増殖阻止濃度（MIC）は 1.0 ～ 3.5mg/ml, 2.5mg/ml および 1.25 ～ 5％の範囲であった．クローブとシナモンからの製油画分の *L. monocytogenes*，*E. coli* O157: H7 および *Salmonella* Enteritidis の菌株カクテルに対する抗菌活性への温度と pH の影響を調べた．100℃，30 分間処理後も両ハーブの製油画分（EO）は抗菌活性を示したことから，高温によりこれら EO 活性は影響されないことが示唆された．両ハーブの EO は，*E. coli* O157:H7 と *Sal-monella* Enteritidis の菌株ミクスチャに対しては pH5.0 で，一方 *L. monocytogenes* の菌株カクテルに対しては pH7.0 で最大の抗菌活性を示した．*L. monocytogenes* 5 菌株のカクテルを接種した鶏挽肉へのクローブ（10％）およびシナモン（5％）の添加効果を検討した．その結果，クローブの EO は添加 1 日後に挽肉中の接種 *L. monocytogenes* 菌数を検出限界以下まで減少させた．一方，シナモンの EO は挽肉中の接種 *L. monocytogenes* 菌数を添加 1 日後に 2.0log CFU/g 程度減少させその後の菌数減少は保存期間（15 日）を通じてほとんどなかった．従って，鶏挽肉中の *L. monocytogenes* 制御には，クローブの EO が優れており利用できる可能性が明らかとなった．