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Potential Roles of Essential Oil and Extracts of *Piper chaba* Hunter to Inhibit *Listeria monocytogenes*

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Abstract: The *in vitro* anti-listerial potential of essential oil and various organic extracts of *Piper chaba* Hunter (Piperaceae) were evaluated. The chemical composition of the essential oil was analyzed by the GC-MS. Fifty four compounds representing 95.4% of the total oil were identified, of which α-humulene (16.4%), caryophyllene oxide (12.2%), veridiflorol (8.1%), globulol (7.4%), β-selinene (7.1%), spathulenol (6.2%), trans-nerolidol (5.1%), linalool (4.5%), 3-pentanol (3.5%), tricyclene (2.2%) and p-cymene (1.6%) were the major compounds. The oil and organic extracts revealed a great potential anti-listerial effect against all five strains of *Listeria monocytogenes* ATCC 19111, 19116, 19118, 19166 and 15313. Also the essential oil had a strong inhibitory effect on the viable cell count of the tested *Listeria* spp. Our findings demonstrate that the essential oil and extracts derived from the leaf of *P. chaba* might be a potential source of natural preservatives used in food industries.

Keywords: *Piper chaba* Hunter; essential oil; α-humulene; anti-listerial activity; GC-MS.

1. Introduction

Food safety is a fundamental concern of both consumers and the food industry, especially as the number of reported cases of food-associated infections continues to increase and is rapidly changing [1]. *Listeria monocytogenes* is an important food-borne pathogen due to the severity of infection with a high mortality rate. This Gram-positive bacterium causes gastroenteritis and more importantly listeriosis, a serious disease with high mortality in immune compromised people, unborn children, and neonates. It is regarded as one of the most important food-borne pathogens of the recent

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years, and numerous food-borne outbreaks and sporadic cases of listeriosis have been reported, mostly in Canada, North America and Europe [2,3]. Most reports associate listeriosis with the consumption of contaminated ready-to-eat foods such as dairy products, processed or cured meat and poultry, salads, seafood and uncooked eggs [4]. Therefore, still a need for new methods of reducing or eliminating *L. monocytogenes* to ensure public health.

A range of synthetic antimicrobial agents have been used to inhibit the growth of *L. monocytogenes* in foods, although concerns about the safety of these chemicals have increased consumer demand for naturally processed food. Hence, there has been recent interest in testing natural products, including plant-derived compounds, for anti-listerial properties as these may be used as natural preservatives in foods [5]. Thus plant essential oils and extracts are promising natural antimicrobial agents with potential applications in food industries for controlling of food-borne pathogenic microbes.

The genus Piper (Piperaceae) comprises of 500 species, which are distributed in tropical and subtropical regions. They are mostly shrubs and rarely herbs or trees [6]. The plant *Piper chaba* Hunter is a branched, rambling shrub which is cultivated in Bangladesh, India and Malay [7]. In Bangladesh a number of *Piper* species are noted for their ethnomedical properties, of which the reputed stimulant, carminative, diuretic and diaphoretic agents of *P. nigrum* are probably the best known. Traditionally, the leaves and stems of *P. chaba* are used as spices in foods and also used as a substitute for *P. longum*. Like other plants of *Piper* genus, the plant enjoys vast folklore uses, as traditional medicine. The root of *P. chaba* is alexiteric; useful in asthma, bronchitis, and consumption. The fruit has stimulant and carminative properties, and is used in haemorrhoidal affections [7]. Stem is used to reduce post-delivery pain in mothers and also useful in rheumatic pains and diarrhoea [8]. However, there is no report available in the literature on the analyses of essential oil from leaves of *P. chaba* and its anti-listerial property. Hence, efforts have been made to investigate the role of essential oil and various extracts of *P. chaba* as an anti-listerial potential.

In this study, we examined the chemical composition of the essential oil from leaves of *P. chaba* by GC-MS; and tested the efficacy of the oil and leaf extracts of *P. chaba* for controlling the growth of *Listeria* spp. with emphasis for the possible future use of the oil and extracts as alternative antibacterial agents.

2. Materials and Methods

2.1. Plant Material

The leaves of *P. chaba* were collected from Jessore area of Bangladesh in June 2007 and identified by a senior taxonomist Dr. M. Oliur Rahman, Bangladesh National Herbarium, Dhaka, Bangladesh, where a voucher specimen (DACB 32565) has been deposited.

2.2. Isolation of the essential oil

The air-dried leaves (250 g) of *P. chaba* were subjected to hydrodistillation using a Clevenger type apparatus and extracted with 2000 mL of water for 3 h (until no more essential oil was obtained). The oil was dried over anhydrous sodium sulphate and preserved in a sealed vial at 4^{o}C until further analysis.

2.3. Preparation of various organic extracts

The air-dried leaves (50 g) of *P. chaba* Hunter was extracted with hexane, chloroform, ethyl acetate and 80% methanol, separately in a 1000 mL Erlenmeyer flask at room temperature for seven days. The process was repeated three times to ensure complete extraction. The solvents from the combined extracts were then filtered by Whatman filter paper no.1, and the filtrates were evaporated

by vacuum rotary evaporator (EYELA N1000, SB-1000; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The extraction process yielded in hexane (7.5 g), chloroform (6.6 g), ethyl acetate (5.4 g) and methanol (6.3 g) extracts. Solvents (analytical grade) for extraction were obtained from commercial sources.

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of the essential oil was performed using a Shimadzu GC-MS (GC-17A) equipped with a ZB-1 MS fused silica capillary column (30 m × 0.25 i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 mL/min. Injector and mass transfer line temperature were set at 220 and 290°C, respectively. The oven temperature was programmed from 50 to 150°C at 3°C/min, then held isothermal for 10 min and finally raised to 250°C at 10°C/min. Diluted samples (1/100, v/v, in methanol) of 1 μL was manually injected in the splitless mode. The relative percentage of the oil constituents was expressed as percentage by peak area normalization.

The identity of the components of the essential oil was assigned by comparison of their retention indices (RI), relative to a series n-alkane indices on the ZB-1 capillary column and GC-MS spectra from the Wiley 6.0 MS data and literature data [9]. The relative amounts (RA) of individual components of the essential oil were expressed as percentages of the peak area relative to the total peak area.

2.5. Microorganisms

Five strains of *Listeria monocytogenes* ATCC 19111, ATCC 19116, ATCC 19118, ATCC 19166 and ATCC 15313 were used in this study. The strains were obtained from the Korea Food and Drug Administration (KFDA), Daegu, Republic of Korea. Active cultures for experimental use were prepared by transferring a loopful of cells from stock cultures to flasks and inoculated in BHI (Brain Heart Infusion, Difco) broth medium at 37°C for 24 h. Cultures of each *Listeria* strain were maintained on BHI agar medium at 4°C.

2.6. Anti-listerial activity assay

The dried leaf extracts were dissolved in the same solvent used for their extraction to a final concentration of 40 mg/mL and sterilized by filtration through 0.22 μm sterilizing Millipore express filter (Millex-GP, Bedford, OH). *Listeria* strains were suspended in sterile water and diluted to 10^6~10^8 CFU/mL. The anti-listerial test was then carried out by agar disc diffusion method [10] using 100 μL of standardized inoculum suspension containing 10^6~10^8 CFU/mL of *Listeria* strains. The essential oil was diluted 1:5 (v/v) with methanol and aliquots of 5, 10 and 15 μL were spotted onto the filter paper discs, respectively; while10 μL of 40 mg/mL of each organic extract (400 μg/disc) was applied on the filter paper discs (6 mm diameter) and placed on the inoculated BHI agar. Negative controls were prepared using the same solvents employed to dissolve the samples. Standard reference antibiotic, streptomycin (20 μg/disc, from Sigma-Aldrich Co., USA), was used as positive control for the tested *Listeria* strains. The plates were then sealed with parafilm and incubated at 37°C for 24 h. Anti-listerial activity was evaluated by measuring the diameter of the zones of inhibition against the tested pathogens. Each assay in this experiment was replicated three times.

2.7. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the essential oil and organic extracts was tested by two-fold serial dilution method [11]. The test samples of oil and various extracts were first dissolved in methanol, and incorporated into BHI broth medium to obtain a concentration of 2000 μg/mL and serially diluted to achieve 1000, 500, 250 125, 62.5 and 31.25 μg/mL, respectively. The

final concentration of methanol in the culture medium was maintained at 0.1% (v/v). A 10 mL standardized suspension of each tested organism (10^6~10^8 CFU/mL) was transferred to each tube. The control tubes contained only listerial suspension, were incubated at 37°C for 24 h. The lowest concentration of the test samples at which the tested organism did not demonstrate visible growth was determined as MIC.

2.8. Effect of essential oil on viable counts of Listeria spp.

Active cultures for viable count assay were prepared in BHI broth medium [12]. For each strain, 1 mL of active stock solution was transferred to 4 mL of BHI broth. All treated cultures were kept under microaerobic conditions at 37°C for 2 h. The cultures were then centrifuged at 10,000 rpm for 10 min. The pellets were retained and resuspended with 1 mL of phosphate-buffered saline. For viable counts, each of the tubes containing resuspended bacterial suspension (10^6~10^8 CFU/mL) of *L. monocytogenes* ATCC 19111, ATCC 19116 and ATCC 15313 was inoculated with the minimum inhibitory concentration of the essential oil in 10 mL BHI broth, and kept at 37ºC. Samples for viable cell counts were taken out at 0, 20, 40, 60, 80 and 100 min time intervals. The viable plate counts were monitored as followed: After incubation, 1 mL of the resuspended culture was diluted into 9 mL buffer peptone water, there by diluting it 10-fold. 0.1 mL sample of each treatment was diluted and spread on the surface of BHI agar. The colonies were counted after 24 h of incubation at 37ºC. The controls were inoculated without essential oil for each listerial strain with the same experimental condition as mentioned above.

3. Results and Discussion

The hydrodistillation of the air-dried leaves of *P. chaba* gave the dark yellowish oil with a yield of 0.31% (w/w). GC-MS analyses of the oil led to the identification of 54 different compounds, representing 95.4% of the total oil. The identified compounds are listed in Table 1. The oil contains a complex mixture consisting of mainly oxygenated mono- and sesquiterpenes, and mono- and sesquiterpene hydrocarbons. The major compounds detected were α-humulene (16.4%), caryophyllene oxide (12.2%), veridiflorol (8.1%), globulol (7.4%), β-selinene (7.1%), spathulenol (6.2%), trans-nerolidol (5.1%), linalool (4.5%), 3-pentanol (3.5%), tricyclene (2.2%) and p-cymene (1.6%). Also, piperitone (1.2%), cis,trans-farnesol (1.1%), α-selinene (1.1%), citronellyl acetate (1.0%), β-pinene (0.9%), 1,8-cineole (0.8%), α-pinene (0.5%) and camphene (0.3%) were found to be the minor components of *P. chaba* leaf oil in the present study.

Table 1. Chemical composition of the essential oil from leaves of *Piper chaba* Hunter

No	RT[a]	Components	% RA[b]	Identification[c]
1	780	Hexanal	0.4	RI, MS
2	858	Hexanol	0.3	RI, MS
3	920	Tricyclene	2.2	RI, MS
4	932	α-Pinene	0.5	RI, MS
5	940	Camphene	0.3	RI, MS
6	963	β-Pinene	0.9	RI, MS
7	966	3-Octanol	1.1	RI, MS
8	1000	δ-3-Carene	0.4	RI, MS
9	1001	1, 4-Cineole	0.6	RI, MS
10	1003	p-Cymene	1.6	RI, MS
11	1005	1,8-Cineole	0.8	RI, MS
12	1025	3-Octen-2-one	0.2	RI, MS
13	1075	Linalool	4.5	RI, MS
14	1082	Terpinolene	0.4	RI, MS
15	1102	3-Pentanol	3.5	RI, MS
16	1136	Phenyl ethyl alcohol	0.5	RI, MS
17	1159	α-Terpineol (p-Menth-1-en-8-ol)	0.3	RI, MS
18	1163	Citronellyl acetate	1.0	RI, MS
19	1248	Piperitone	1.2	RI, MS
20	1252	Linalyl acetate	1.1	RI, MS
21	1278	Isobornyl acetate	0.3	RI, MS
22	1320	Dihydrocarvyl acetate	0.2	RI, MS
23	1342	α-Terpineol acetate	0.4	RI, MS
24	1368	Geranyl acetate	0.4	RI, MS
25	1375	α-Copaene	0.6	RI, MS
26	1381	β-Bourbonene	0.3	RI, MS
27	1390	β-Elemene	0.7	RI, MS
28	1404	α-Cedrene	0.2	RI, MS
29	1428	β-Copaene	0.2	RI, MS
30	1438	Aromadendrene	0.3	RI, MS
31	1447	α-Humulene	16.4	RI, MS
32	1455	trans-β-Farnesene	0.4	RI, MS
33	1470	β-Chamigrene	0.2	RI, MS

Table 1 *Continued.*.

34	1486	β-Selinene	7.1	RI, MS
35	1490	Germacrene D	0.9	RI, MS
36	1493	α-Selinene	1.1	RI, MS
37	1494	Ledene	0.3	RI, MS
38	1513	β-Bisabolene	0.4	RI, MS
39	1518	Myristicin	0.2	RI, MS
40	1532	Elemol	0.8	RI, MS
41	1548	Germacrene B	0.2	RI, MS
42	1549	trans-Nerolidol	5.1	RI, MS
43	1551	Spathulenol	6.2	RI, MS
44	1554	Elemicin	0.2	RI, MS
45	1561	Caryophyllene oxide	12.2	RI, MS
46	1566	Globulol	7.4	RI, MS
47	1569	Veridiflorol	8.1	RI, MS
48	1574	Epiglobulol	0.5	RI, MS
49	1580	Ledol	0.2	RI, MS
50	1602	Humulene oxide	0.3	RI, MS
51	1660	Junipher camphor	0.2	RI, MS
52	1697	*cis,trans*-Farnesol	1.1	RI, MS
53	1761	Aristolone	0.2	RI, MS
54	1768	Tetradecanoic acid	0.3	RI, MS
		Total	**95.4**	

[a]Retention indices relative to n-alkanes C8 – C20 on ZB-1 capillary column.; [b]Relative area (peak area relative to the total peak area); [c]Identification: MS, comparison of mass spectra with MS libraries; RI, comparison of retention index with bibliography.

Recurring outbreaks of food-borne illness caused by *Listeria monocytogenes* have sustained the demand for preservation systems that limit the proliferation of this psychrotrophic pathogen in refrigerated foods. A novel way to reduce the proliferation of microorganisms is the use of essential oils and plant extracts. The oils are secondary metabolites of plants, which because of their antibacterial, antifungal, antioxidant and anticarcinogenic properties, can be used as natural additives in many foods [13,14]. The *in vitro* anti-listerial activities of essential oil and various organic extracts of *P. chaba* leaf against the employed *Listeria* were qualitatively assessed by the presence or absence of inhibition zones. At the concentrations of 5, 10 and 15 µL/disc of 1:5 (v/v) dilution of oil with methanol, the oil exhibited a potent inhibitory effect against all five *Listeria* strains with their respective diameter of inhibition zones of 12.0~16.2, 17.2~21.0 and 22.1~26.2 mm, respectively, as shown in Table 2. Organic extracts of *P. chaba* also revealed a great potential of anti-listerial effect against all strains of *Listeria* tested, at the concentration of 400 µg/disc (Table 3). Methanol and ethyl acetate extract showed the strongest anti-listerial effect against all strains of *L. monocytogenes* (ATCC 19111, ATCC 15313, ATCC 19116, ATCC 19118 and ATCC 19166) in the diameter of inhibition

zones ranging from 17.1~30.2 and 18.2~27.2 mm, respectively. On the other hand, chloroform and hexane extracts showed remarkable anti-listerial effect with inhibition zones in the range of 19.1~22.2 and 11.0~18.0 mm, respectively. In this study, in most of the cases, the oil and organic extracts of methanol, ethyl acetate and chloroform exhibited higher anti-listerial activity compared to streptomycin. The blind control did not inhibit the growth of the *Listeria* strains tested.

Table 2. Anti-listerial activity of the essential oil of *Piper chaba* Hunter

Bacteria	Essential oil				Antibiotic
	DD[a]	DD[b]	DD[c]	MIC	DD[d]
L. monocytogenes ATCC 19111	13.1 ± 1.1	19.3 ± 0.5	25.0 ± 0.4	62.5	nd
L. monocytogenes ATCC 19116	16.2 ± 0.5	21.0 ± 0.5	26.2 ± 0.6	62.5	15.2 ± 0.6
L. monocytogenes ATCC 19118	12.0 ± 0.6	17.2 ± 0.7	22.2 ± 0.5	125	15.0 ± 0.5
L. monocytogenes ATCC 19166	16.0 ± 0.7	19.2 ± 0.5	22.1 ± 1.1	125	14.0 ± 0.5
L. monocytogenes ATCC 15313	16.2 ± 1.2	20.0 ± 0.7	25.0 ± 0.5	62.5	nd

Values are given as mean ± S.D. (n =3). nd, not detected;DD: Diameter of inhibition zone (mm) around the discs (6 mm); [a]Essential oil 5 µL/disc; [b]10 µL/disc; [c]15 µL/disc of 1:5 (v/v) dilution of oil with MeOH; [d]Streptomycin (20 µg/disc). MIC: Minimum inhibitory concentration (µg/mL).

Table 3. Anti-listerial activity of the various extracts of *Piper chaba* Hunter

Bacteria	Extracts[a]				Antibiotic[b]
	HE	CE	EE	ME	
L. monocytogenes ATCC 19111	18.0 ± 0.6	22.2 ± 0.7	27.2 ± 1.1	30.2 ± 1.4	nd
L. monocytogenes ATCC 19116	14.1 ± 0.5	19.1 ± 1.2	22.0 ± 1.3	22.1 ± 1.2	15.2 ± 0.6
L. monocytogenes ATCC 19118	11.0 ± 0.6	16.2 ± 0.7	18.2 ± 0.5	17.2 ± 0.5	15.0 ± 0.5
L. monocytogenes ATCC 19166	15.2 ± 0.7	19.1 ± 1.1	22.2 ± 1.2	17.1 ± 0.6	14.0 ± 0.7
L. monocytogenes ATCC 15313	18.0 ± 1.1	21.1 ± 0.6	26.1 ± 1.1	22.2 ± 1.2	nd

Values are given as mean ± S.D. (n =3). nd, not detected;[a]Diameter of inhibition zone (mm) around the discs (6 mm) impregnated with 10 µL extracts (400 µg/disc). HE: Hexane; CE: $CHCl_3$; EE: EtOAc; and ME: MeOH extracts; [b] Streptomycin (20 µg/disc).

As shown in Table 2, the MIC values for the oil were found more effective against *L. monocytogenes* ATCC 19111, ATCC 19116 and ATCC 15313 (62.5 µg/mL for each) than for *L. monocytogenes* ATCC 19118 and ATCC 19166 (125 µg/mL for each). On the other hand, MIC values of the organic extracts of chloroform, ethyl acetate and methanol against the tested *Listeria* spp. were found in the range of 62.5~250 µg/mL (Table 4). Hexane extract also showed good inhibitory effect against all the tested *Listeria* with the MIC values of 125~500 µg/mL.

Based on the susceptibility, further study was carried out to evaluate the effect of essential oil on the viable count of *L. monocytogenes* ATCC 19111, ATCC 19116 and ATCC 15313 (Fig. 1). All the tested strains of *Listeria* were found sensitive to the essential oil. The essential oil exerted its maximum bactericidal activity as evident by the significant reduction in microbial counts at 40 and 60 min exposure and complete inhibition of cell viable counts at 80-100 min exposure. Similar to our findings, cilantro essential oil also exerted bactericidal activity against *Listeria* spp. [15].

Table 4. MIC of the various extracts of *Piper chaba* Hunter against *Listeria* spp.

Bacteria	MIC (μg/mL)			
	HE	CE	EE	ME
L. monocytogenes ATCC 19111	125	125	62.5	62.5
L. monocytogenes ATCC 19116	250	250	125	125
L. monocytogenes ATCC 19118	500	250	250	250
L. monocytogenes ATCC 19166	250	250	125	125
L. monocytogenes ATCC 15313	125	125	62.5	125

MIC: Minimum inhibitory concentration; HE: Hexane; CE: $CHCl_3$; EE: EtOAc; and ME: MeOH extracts.

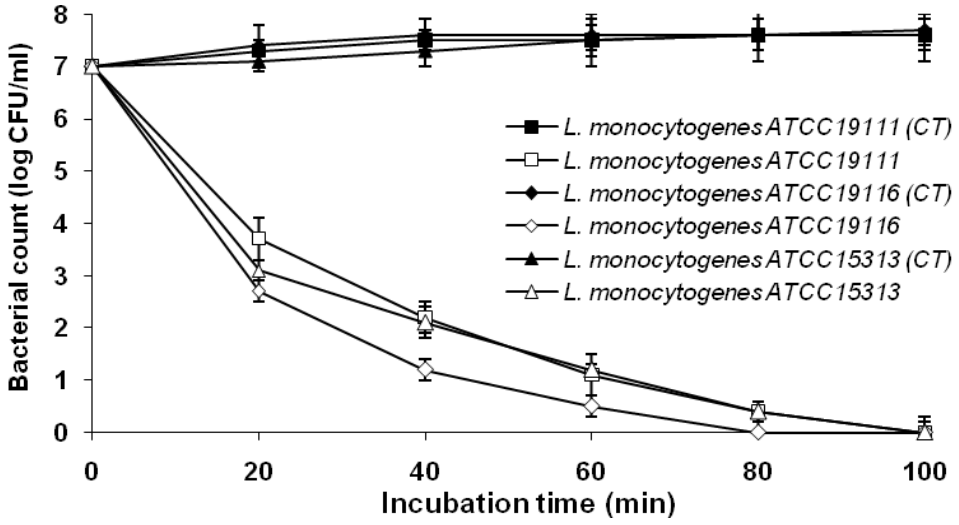

Figure 1. Effect of leaf essential oil from *P. chaba* Hunter on viability of the tested *Listeria*. at minimum inhibitory concentration; CT: control without treatment.

In our opinion, major components of *P. chaba* leaf oil, α-humulene, caryophyllene oxide, veridiflorol, globulol, β-selinene, spathulenol, trans-nerolidol, linalool, 3-pentanol and p-cymene have key roles for their anti-listerial activities [16-18]. On the other hand, the components in lower amount such as α-selinene, β-pinene, 1,8-cineole, α-pinene and camphene also contributed to anti-listerial activity of the oil [19,20]. It is also possible that the minor components might be involved in some type of synergism with the other active compounds [21]. Also, the anti-listerial activity of the various extracts could be attributed to the presence of some bioactive compounds (e.g., piperamine 2,4-decadienoic acid piperidide, pellitorine, lignan, chabamide and some alkamides such as piperine, sylvatine, piplartine and β-sitosterol) in *P. chaba* and these finding are in agreement with the previous work reports [22,23].

In conclusion, the results of this study showed that essential oil and extracts of *P. chaba* have potential anti-listerial activities. We hope the natural compounds derived from essential oil and extracts of *P. chaba* might be suitable for using in food industries as preservatives to control food-borne pathogens.

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