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Antibacterial Activity of *Chrysanthemum indicum, Centella asiatica* and *Andrographis paniculata* on *Bacillus cereus* and *Listeria monocytogenes* under Low pH Stress

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Abstract

Classic food preservation methods do not guarantee to inhibit *Bacillus cereus* and *Listeria monocytogenes* growth. The application of natural antibacterial agent is an alternative way to control their growth. Asia herbs, *Chrysanthemum indicum*, *Centella asiatic*a, and *Andrographis paniculata* were used in this experiment to test their antibacterial activity under low pH stress. Their antibacterial activities of 95% ethanolic crude extracts were tested on *B. cereus and L. monocytogenes* 10403S under different low pH stress by agar disc diffusion method. The best antibacterial effect on both bacteria was found at low pH stress condition. Lowering pH also acts as one inhibitory effect. The result of in vitro antibacterial effect as inhibition zone at pH 7.0, 6.5, 6.0, 5.5, showed that the inhibition zone diameters of *C. indicum* extracts were 7.62±1.18. 7.87±2.35, 6.25±3.06 and 9.50±2.14 cm, while the inhibition zone diameters of *C. asiatica* extracts were 8.75±1.03, 8.75±2.66, 7.75±2.37 and 9.12±1.96 cm and *A. paniculata* extracts were 9.75±1.75, 5.87±3.52, 8.5±1.23 and 9.33±1.63 against *L. monocytogenes* 10403S, respectively. Under the same condition, the inhibition zone diameters of *C. indicum* extracts at 2.12±0.64, 1.37±0.92, 0.93±0.78 and 6.00±3.25 cm, the inhibition zone diameters of *C. asiatica* extracts at 0.62±0.44, 2.25±0.46, 1.75±0.28 and 6.50±1.60 cm and *A. paniculata* extracts at 0.87±0.79, 1.25±0.60, 2.00±1.65 and 6.00±1.31, respectively against *B. cereus.* All 95% ethanolic crude extracts showed more inhibition effect on *L. monocytogenes* 10403S than *B.cereus.* However, the promising active antibacterial compounds in all three herbs are needed to be identified. The MBCs of *A. paniculata*, *C. asiatica* and *C. indicum* showed 4, 16 and >32 µl/ml against *B. cereus* while *A. paniculata* and *C. asiatica* showed 16 and >32 µl/ml against *L. monocytogenes* 10403S.

Keywords: Antibacterial, *Chrysanthemum indicum*, *Centella asiatica*, *Andrographis paniculata*, *Bacillus cereus*, *Listeria monocytogenes* 10403S, Low pH stress, Herb

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1. Introduction

Bacillus cereus is a gram-positive aerobic or facultative anaerobic spore-forming rod. It is a cause of food poisoning, which is frequently associated with the consumption of rice-based dishes. *B. cereus* can be frequently isolated from soil and some food. Spores of *B. cereus* are more resistant to heat and chemical treatments than vegetative pathogens such as *Salmonella*, *Escherichia coli* and *Campylobacter*. When *B. cereus* grows in food, it can cause two different types of food borne illness in humans; vomiting very shortly after eating contaminated food or diarrhea after a longer incubation [1].

Listeria monocytogenes is a gram-positive rod-shaped bacterium. It is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria. Listeriosis has been recognized as an important public health problem in the United States. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems. Listeriosis is a serious disease for humans; the overt form of the disease has mortality greater than 25 percent. The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial infections. Microscopically, *Listeria* species appear as small, gram-positive rods, which are sometimes arranged in short chains. In direct smears they may be cocci, so they can be mistaken for streptococci [2].

Bacillus cereus and *L. monocytogenes* are two pathogenic bacteria that can cause many dangerous illness and even death if they are too much consumed. Both of them have strong resistance against many preservatives. They are marked resistant to classic methods of food preservatives such as low temperature, and high salt (osmotic stress) conditions. These food borne pathogens require certain processing to inhibit the growth. This process might cause undesirable flavor or texture to food product. Introducing of natural antibiotics to food product might be another possible process for food preservation to keep the desirable flavor and texture. Natural substances or bioactive compound might be effective to kill the food borne pathogenic bacteria without decreasing the quality of the food.

Essential oils are highly concentrated substances extracted from various parts of aromatic plants and trees. The aromatic substances from parts, such as petals, leaves, roots, barks, fruits, peels, roots or the whole plant are usually captured by distillation or solvent extraction method. The chemistry components of pure essential oil are very complex. Most consist of hundreds of components, such as terpenes, alcohols, aldehydes and esters. Essential oil has any benefits which are analgesic, anticoagulant, antidepressant, antiseptic, astringent, cicatrisant, cytophylatic and insecticide [3, 4].

Extract of *Chrysanthemum indicum*, *Centella asiatica*, and *Andrographis paniculata* are chosen as the antimicrobials in this study against *Bacilus cereus* and *Listeria monocytogenes* 10403S because of many criteria. They are easy to find (widely available), cheap and safe for human consumption, and contain many effective bioactive compounds. Therefore the extracts of *C. indicum, C. asiatica*, and *A. paniculata* are chosen to study their antibacterial activity against *B. cereus* and *L. monocytogenes* 10403S under different low pH stress conditions.

2. Materials and Methods

2.1 Plant sample preparation and extraction

Chrysanthemum indicum's flower (chrysanthemum, mums), *Centella asiatica*'s leave and stem (pennywort, gotu kola), and *Andrographis paniculata*'s leave and stem (kariyat, creat, chuanxinlian) were obtained from local fresh market in Bangkok, Thailand. Herbs were cut into small pieces and air dried in oven (Memmert, UM500) at 45 °C for overnight. Then, dried herbs were blended in food blender to reduce the size. Herb powder was stored in refrigerator at 6 °C until use. The 20 g herb powder was weighed on top-loaded balance (1 decimal, ZEPPER model

ES-300), then 180 ml 95% ethanol (supported from Rung-Sap Co.,Ltd., Thailand) was added and soaked for 48 hours at room temperature and stirred every 12 hours. After 48 hours, liquid part was separated by filtering through thin cloth. Then the crude extract was centrifuged (Chermle model Z230A) at 5000 rpm for 5 min. Supernatant was collected and concentrated in water bath (Schutzart DIN40050 – IP20) at 45 °C until it became very concentrate slurry. This crude extract was kept in freezer at -20 °C until use. The crude extract was diluted to 100 mg/ml by 95% ethanol. Diluted crude extract was steriled by 0.2 µm CE filter paper (Minisart®) and kept in freezer at –20°C.

2.2 Antibacterial assay and growth condition

Bacillus cereus and *L. monocytogenes* 10403S obtained from the Faculty of Biotechnology Stock Culture, Assumption University, were used. One loop of *B. cereus* was inoculated into Nutrient Broth (NB) and one loop of *L. monocytogenes* was innoculated into Brain Heart Infusion (BHI) broth incubated at 37 °C 24 hours (Jouan incubator, model EB280, USA). Incubated broth (1% v/v) was transfer to fresh NB and BHI broth and incubated at 37 °C in Culture Tube Rotator (Stuart Scientific, UK), until reaching 0.1 OD_{600} (SPECTRONIC, model GENESYS 5, UK) which is an early log phase. BSAC disc diffusion method for antimicrobial susceptibility testing version 8 by the British Society for Antimicrobial Chemotherapy was used for antibacterial activity assay. Culture (100 µl) was swabbed on the media agar. Sterile paper disc contained 15 µl of 100 mg/ml of each crude extract, 100 mg/ml Penicillin-G, and 95% ethanol as the positive control, were placed on NA and BHI agar at pH 7.0, 6.5, 6.0, and 5.5. All plates were incubated at 37 °C for 48 hours. Positive antibacterial activity was measured, using inhibition zone diameter as criterion. The data were collected; mean and standard deviation of data were calculated. All experiments were performed in duplicate and repeated four times

2.3 MIC and MBC determination MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)

The methods for determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) used in this study were modified from BSAC disc diffusion method for antimicrobial susceptibility Testing version 8 by The British Society for Antimicrobial Chemotherapy. For MIC test, crude extracts were added to the 1ml of fresh broth in different concentration as follows: 32, 16, 8, 4, 2, 1 ml, 0.5 and 0.25 µl/ml. Each of bacterial culture with 0.1 OD_{600} (100 µl) was inoculated and then incubated at 37 °C 18 hour. The MIC test negative result tubes were chosen for MBC test, then incubated at 37 °C 18 hours. All experiments were performed in duplicate and repeated three times

3. Results and Discussion

3.1 Antibacterial activity

Listeria monocytogenes is a major concern to manufacturers worldwide due to the high mortality rate of listeriosis in susceptible populations and to the resistance of the pathogen to a number of food preservation practices. In particular, the ability of the organism to grow at refrigeration temperatures and on dry surfaces and its ability to tolerate acidic conditions make it well adapted to food environments which normally restrict bacterial growth [5]. But this bacterial growth is limited in the low pH and *L. monocytogenes* failed to grow at or below pH 5.4 [6].

Bacillus cereus is a food poisoning bacterium in general description. The presence of large numbers of *B. cereus* cells, greater than 10^6 cell/g in foods is indicative of active growth and proliferation of the organism and is consistent with a potential hazard to health. Low pH is again likely to be one of the stresses encountered by this pathogen in food. *Bacillus cereus* undergoes acid adaptation at pH 6.3, resulting in enhanced protection when subsequently exposed to pH 4.6.

The resistance of both bacteria in low pH is different. *Bacillus cereus* has a higher resistance in low pH than *L. monocytogenes*, and the survival rate of bacteria cultures can be observed in different pH conditions. However, the herb extracts are more effective on *L. monocytogenes* than *B. cereus* [7].

From Tables 1 and 2, the results showed that all three crude extract of herbs had antibacterial activity against the growth of both *B. cereus* and *L. monocytogenes* under normal and low pH stress condition. The lower pH condition showed the better antibacterial activity on both *B. cereus* and *L. monocytogenes* in all three herbs.

Table 1 shows that at pH 5.5, the antibacterial activity of all 95% ethanolic crude extract gave the highest inhibition against the growth of *B. cereus*. A significant increase of antibacterial activity was observed when pH was lower (from pH 6 to pH 5.5) in all crude extracts.

The results from Tables 1 and 2 showed that the clear zone of all crude ethanolic extracts had more antibacterial activity effect on *L. monocytogenes* than *B. cereus*. Both bacteria possess a myriad of acid resistance systems that can help them to overcome the challenge posed by different acidic environments. In general both bacteria can survive in the low pH longer than other bacteria. Using these three crude ethanolic extracts showed the better antibacterial activity against both of them than many traditional preservatives although the antibacterial resistance of all crude ethanolic extracts between *L. monocytogenes* and *B. cereus* was different.

pH plays a role in determining the ability of bacteria to grow or thrive in particular environments. The pH affects the ionization and therefore the binding and interaction of a myriad of molecular processes. pH also affects the solubility of many substances that bacteria need. There is also no certain pH level for maximum growth for bacteria in general. This phenomena is possible to increase the microbial activity of crude extract. Natural antibiotics provide a great benefit over other antibiotics in term of their working mechanism that mechanism of natural antibiotic are usually not specific binding sites [8]. This mechanism also made bacterial cell more difficult to produce resistance for natural antibiotic due to their random binding site [8]. However, previous research mentioned that salt condition might decrease bacterial cell ABR (scale of antibiotic resistance), then lead to lower activity of antibiotic on *E. coli* and *S. aureus* [9, 10].

GC-MS study by the former independent laboratory showed that 73 active compounds were identified from *C. indicum*'s essential oil extracts and its essential oil extracts had stronger antibacterial activity than its individual antibacterial active compounds; monoterpene hydrocarbons α-pinene, oxygenated monoterpenes camphor, 1,8-cineole, terpinon-4-ol, borneol and sesquiterpene hydrocarbon β-caryophyllene on 15 oral bacterial strains; *Streptococcus* sp., *Prevotella intermedia* ATCC 25611, and *Porphylomonas gingivalis* ATCC 33277 (MICs , 0.1 to 1.6 mg/ml ; MBCs , 0.2 to 3.2 mg/ml) [3].

Another previous study showed that *C. asiatica* had antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Shigella boydii* [9, 11]. This result also confirms the result from this experiment that *C. asiatica* crude extract had antibacterial activity.

Andrographis paniculata's active compounds have an antibacterial activity [4]. The major active compounds of *A. paniculata* are lactone group; andrographolide, deoxy-andrographolide, neoandrographolide, dehydroandrographolide [4]. Diterpenoids, flavanoids and polyphenols are also major active compounds in *A. paniculata* [12].

3.2 The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs)

The minimum inhibitory concentrations (MICs) of *A. paniculata, C. asiatica* and *C. indicum* against *B. cereus* were 4, 16, and 16 µl /ml, respectively. The MICs of *A. paniculata* and *C. asiatica* against *L. monocytogenes* 10403S were 16 and 8 µl /ml, respectively. The Minimum Bactericidal Concentrations (MCBs) of *A. paniculata*, *C. asiatica* and *C. indicum* against *B. cereus* were 4, 16, and >32 µl /ml, respectively. The MCBs of *A. paniculata* and *C. asiatica* against *L. monocytogenes* 10403S were 16 µl /ml, and >32 µl /ml, respectively. The results from Table 3

showed that among three herbs, crude extract of *A. paniculata* gave the highest antibacterial activity (the lowest MIC, 4 µl/ml) against *B. cereus* while crude extract of *C. asiatica* had the highest antibacterial against *L. Monocytogenes* 10403S (the lowest MIC, 8 µl/ml).

Table 1 Antibacterial activity of three crude 95% ethanolic extracts on *B. cereus* under different low pH stress conditions.

pH	Inhibition zone diameter (cm)				
	C. indicum	***C. asiatica***	***A. paniculata***	**95% Ethanol**	**Penicillin-G**
7	2.12±0.64	0.62±0.44	0.87±0.79	0.67±0.86	9.04±4.87
6.5	1.37±0.92	2.25±0.46	1.25±0.60	1.04±0.70	11.75±1.14
6	0.94±0.78	1.75±0.27	2.00±1.65	0.46±0.40	9.50±3.90
5.5	6.00±3.22	6.50±1.60	6.00±1.31	2.87±4.34	14.42±9.03

Note: clear zones were measured from edge of paper disc to the end of clear zone in cm unit. 95% ethanol and 100 mg/ml penicllin-G were used as positive control.

Table 2 Antibacterial activity of three crude 95% ethanolic extracts on *L. monocytogenes* 10403S under different low pH stress conditions.

pH	Inhibition zone diameter (cm)				
	C. indicum	***C. asiatica***	***A. paniculata***	**95% Ethanol**	**Penicillin-G**
7	7.62±1.19	8.75±1.03	9.75±1.75	4.83±2.37	18.75±6.15
6.5	7.88±2.36	8.75±2.66	5.88±3.52	6.08±2.23	17.67±5.19
6	6.25±3.06	7.75±2.37	8.50±1.22	7.27±1.10	16.82±5.30
5.5	9.50±2.14	9.12±1.96	9.33±1.63	8.64±3.20	19.45±6.09

Note: clear zones were measured from edge of paper disc to the end of clear zone in cm unit. 95% ethanol and 100 mg/ml penicllin-G were used as positive control.

Table 3 MICs and MBCs of crude extracts derived from herb samples against *B. cereus* and *L. monocytogenes* 10403S

B. cereus	**Plant**	**MIC (µl/ml)**	**MBC(µl/ml)**
	C. indicum	16	> 32
	C. asiatica	16	16
	A. paniculata	4	4
L. monocytogenes	*C. asiatica*	8	> 32
	A. paniculata	16	16

4. Conclusions

Chrysanthemum indicum, *C. asiatica*, and *A. paniculata* showed to produce promising antibacterial substances against *B. cereus* and *L. monocytogenes*. The lower pH stress condition gave the better antibacterial effect on both *L. monocytogenes* and *B. cereus* in all herb extracts. All of the extracts showed more inhibition effect on *L. monocytogenes* than *B. cereus*. However, the antibacterial substances, its individual antibacterial activity, combination of antibacterial activity, and antibacterial mechanism under low pH stress in each crude extract have to be further investigated for application in food safety and medicine purpose.

References

[1] Drobnieski, F.A., **1993**. *Bacillus cereus* and related species. *Clinical Microbiology Reviews*, 6, 4, 324-338.

[2] Todar, K., **2011**. T*odar's Online Textbook of Bacteriology Listeria monocytogenes*. [on line] Available at:< http://textbookofbacteriology.net/Listeria.html>

[3] Jung, E.K., **2009**. Chemical composition and antimicrobial activity of the essential oil of *Chrysanthemum indicum* against oral bacteria. *Journal of Bacteriology and Virology*, 39, 61-69.

[4] Singhamutra, S., **1992**. *Properties of 200 herbs.* Bangkok: SO Printing House.

[5] Barker, C. and Park, S.F. **2001**. Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. *Applied and Environmental Microbiology*, 67, 1594-1600.

[6] Ryser, E.T. and Marth, E., **1998**. Growth of *Listeria monocytogenes* at different pH values in uncultured whey or whey cultured with *Penicillium camemberti*. *Canadian journal of microbiology*, 34, 730-734.

[7] Cotter, P.D. and Hill, C., **2003**. Surviving the Acid Test: Responses of Gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews*, 67, 429-453.

[8] Vigil, A .L. M., Palou, E. and Alzamora S.M., **2005**. Naturally Occurring Compounds - Plant Sources. **In** D. P. Michael, J. N. Sofos and A.L Branen, eds. 2005. *Antimicrobials in Food*. Taylor & Francis Group, LLC., pp.429-452.

[9] Pitinidhipat, N. and Yasurin, P., **2012**. Antibacterial Activity of *Chrysanthemum indicum, Centella asiatica* and *Andrographis paniculata* against *Bacillus cereus* and *Listeria monocytogenes* under Osmotic Stress. *AU Journal of Technology*, 15,4, 239-245.

[10] McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S. and McDowell, D.A. **2007**. Environmental Stress and Antibiotic Resistance in Food Related Pathogens. *Applied and Environmental Microbiology*, 73, 211-217.

[11] Panthi, M.P. and Chaudhary, R.P., **2006**. Antibacterial Activity of some selected folklore medicinal plants from west Nepal. *Scientific World*, 4, 16-21.

[12] Chao, W.W. and Lin, B.F., **2010**. Isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). *Chinese Medicine*, 5, 17.