Effect of Extraction and Shaking Time on Antibacterial Activity of Dry *Centella asiatica* againts Food Pathogenic Microorganisms

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Abstract

The outbreaks caused by pathogenic bacteria still being a major human health and food industry problem. Since time immemorial, people used plant material as medicine and nowadays, it widely used as tea preparation. Centella asiatica is a potential herbs related to its antibacterial activity. Therefore, this study aimed to investigate the antibacterial activity of dry C. asiatica crude 95% ethanolic extract under different extraction time (1, 3, 5, and 7 days) and shaking action (120 rpm) against three human pathogenic bacteria; Salmonella enterica typhimurium U302 (DT104b), S. enterica enteritidis (human), S. enteric 4, 5, 12:i:- (human) US clone, and Bacillus cereus by using agar disc diffusion method. The results showed that the all extracts showed antibacterial activity against B. cereus with inhibition zone range of 0.214 ± 0.053 to 0.557 ± 0.559 mm but has not significantly effect on Salmonella sp. The highest antibacterial activity of dry C. asiatica achieved on the three days extraction time. The shaking action could increase antibacterial activity. The MIC (minimum inhibitory concentration) was between 40-80 µl/ml and MBC (minimum bactericidal concentration) was 80-160 µl/ml. This showed that C. asiatica dry extract could inhibit gram-positive bacteria rather than gram-negative and shaking effect gives promising factor to increase effectiveness of plant ethanol extraction.

Keywords: *Centella asiatica, antibacterial activity, Salmonella sp, Bacillus cereus, ethanol extraction*

Introduction

Foodborne diseases caused by pathogenic bacteria are still a major threat to public health. *B. cereus* is responsible for the majority of foodborne illness attributed to *Bacillus.* During 1993-1997, *B. cereus* was linked to 14 outbreaks and caused 691 reported cases of foodborne illness in the United States (Keith *et al*., 2004). *B. cereus* is a Gram-positive, mobile, facultative and aerobic sporeformer. The optimum growth temperature occuring between 28-35^{0}C and has pH limit 4.9-9.3 (Gilbert *et al*., 1981). In fact, 19 of 22 strains of B. cereus is produce enterotoxin which is known as diarrheagenic toxin, diarrheal agent, fluid accumulation factor, vascular permeability factor, dermonecrotic toxin and intestinonecrotic toxin (Spira and Goepfert, 1972).

The other bacteria that commonly cause foodborne illness is *Salmonella spp* which is gram-negative with the optimal temperature for growth is 35-43^{0}C (Jay *et al.*, 2003; ICMSF, 1996). Over 99% of human *Salmonella sp* infection are caused by *S. enterica* subsp. *Enteric* (Crum, 2008). It produce a heat labile enterotoxin which causing diarrhea and some of them also produce cytotoxin which may cause general enteric symptoms and inflammation (Jay *et al.,* 2003). *Salmonella spp* infection (salmonellosis) incidence in southeastern Turkey range from 210 to 320 cases per 100.000 populations (Ministry of health, Republic of Turkey, 1994). The highest incidence for *S. typimurium* was observed in

2008 with 315 cases per 100.000 among children below one year. In that year, significantly more children age <6 months were admitted to hospital compared to infants 6-12 months (Jensen *et al.*, 2012).

Nowdays, medical plants are used by 80% of the world population (Hashiim *et.al.*, 2010) as tea preparation. An example of the herbal tea plant is *Centella asiatica* (L.) Urban, an stoloniferous perennial herb belonging to the plant *family Apiaceae (Umbilliferae*) that is distributed in many parts of Asia such as India, Sri Lanka, Indonesia, Malaysia, and Vietnam (Gandhi and Giri, 2012). *C. asiatica* is claimed to posses a wide range of pharmacological effects, one of them is antibacterial purposes. *C. asiatica* reported to be useful in the treatment of inflammations, diarrhea, asthma, tuberculosis and various skin lesions (Ullah *et.al.*, 2009). A major component that are responsible for those properties are triterpene derivates including asiatic acid and asiaticoside, phenolic compounds with flavonoids and volatile oil (Zainol *et.al.,* 2003). In order to gain maximum antibacterial compound, extraction process have to be optimized. Therefore, this research attempts to observe the effect of extraction and shaking time on antibacterial activity of dry *Centella asiatica* by using disc diffusion method against gram-negative and gram-positive bacteria.

Materials and Methods

A. Materials

Materials used in this research are *Centella asiatica*, bacteria culture of *Salmonella enterica typhimurium U302* (DT104b), *Salmonella enterica enteritidis* (human), *Salmonella enteric* 4, 5, 12:i:- (human) US cloneand *Bacillus cereus,* MHB (Muller Hinton Broth), 95% ETOH, agar, DMSO, and 0.95% NaCl

B. Plant sample preparation

C. asiatica were obtained from local fresh market in Bangkok, Thailand. For making dry crude extract, herb was cut into small pieces and air dried in oven (Memmert, UM500) at 40^0C until the weight is constant. Dried herb was stored in refrigerator at 6^0C over night then blended in food blender to reduce the size.

C. Extraction Condition

Extraction process was using 1:5 v/v of 95% ethanol. Firstly, 3 g and 6 g of herb powder extracted with 30 ml of 95% ETOH and soaked for 1, 3, 5 and 7 days at room temperature. Sample that kept for 24 hour were given two different treatments which is with and without incubation shaker at 30^0C, 120 rpm. The crude extract was separated with filter paper to collected the liquid part then was concentrated in rotary evaporator at 40^0C until become very concentrate slurry then dissolved with DMSO to make concentration 0.05 mg/l. Crude extract was kept in freezer at -20^0C until use.

D. Inoculum Preparation

Stock culture of *Salmonella enterica typhimurium U302* (DT104b), *Salmonella enterica enteritidis* (human), *Salmonella enteric* 4, 5, 12:i:- (human) US clone, *Escherichia coli,* and *Bacillus cereus* were used to test antibacterial activity of three herbs crude extract in this research. 1-looped of *S. enterica typhimurium U302* (DT104b), *S. enterica enteritidis* (human), *S.enteric* 4, 5, 12:i:- (human) US clone and *B. cereus* were inoculated into MHA (Muller Hinton Agar) then incubated at 37^0C for 24 hours (Jouan incubator, model EB280). Single colony from each culture was transferred into fresh MHB then incubate using rotator over night. After that, 1% of cultures were moved to sterile MHB flask and incubated at 37^0C, 150 rpm by culture tube rotator SCI (Stuart Scientific) until OD_{600} reach 0,1 (SPECTRONIC, model GENESYS 5) which is their early log phase. The OD (optical density) was measured with spectrophotometer.

E. Antimicrobial Assay

The antimicrobial activities of the test samples were carried out by disc diffusion method (Bauer *et.al.*, (1966), NCCLS (2000)). In this method, MHB were used as culture media and the discs in diameter 0.6 mm were

placed aseptically. 100µl of culture was swabbed on the agar. Sterile paper disc contained 20 µl of crude dry extract were placed in appropriate position on the plate. All plates were incubated at 37^0C for 24 hours.

Clear zone result was measured by millimeter scale as antibacterial activity then data were collected. All experiment was performed in duplicate. Sterile DMSO (10µl/disc) was used as negative control and Penicillin-G DMSO (10µl/disc) was used as positive control for comparison of the antibacterial activity.

F. MIC and MBC Determination

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) methods were modified from BSAC Disc Diffusion Method for Antibacterial Susceptibility Testing version 8 by The British Society for Antimicrobial Chemotherapy. For MIC test, Each culture was inoculated in the broth then incubated at 37^0C for 24 hours until reach 0.1 OD_{600}. Then 10% v/v of 0.1 OD_{600} culture was transferred to fresh broth to make working culture solution. Crude extract were added to the 1 ml working culture solution in different amount as following 160, 80, 40, 20, and 10 µl. The MIC test negative result tubes were chosen for MBC Test then incubated at 37^0C for 24 hours.

Results and Discussion

Ethanol extraction

In this research, we use 95% ethanol as the solvent. A general principle in solvent extraction is 'like dissolve like", which means that solvents only extracts those phytochemicals which have similar polarity with the solvents (Zhang *et al.,* 2007). *C. asiatica* could be extracted with either ethanol or water. However, extraction using ethanol had a greater yield as compared to water extraction. Taemchuay *et al* (2009) reported that the ethanol had an inhibitory effect against *Staphylococcus aureus* with MIC_{50} value of 8 mg/ml and the MBC value of 16 mg/ml. The water extracts has MIC value of 32-256 mg/ml and these concentrations could not kill *S. aureus*. This may be caused due to ethanol can extract more components than water. Based on Chew *et al.* (2011), the highest total phenolic compound was achieved at 60% ethanol concentration if compared to 0%, 20%, 40%, 80% and 100% ethanol. Hence, they proposed that most of the phenolic compounds in *C. asiatica* had a moderately polar characteristic.

Ethanol extract of *C. asiatica* contains some bioactive compound that has inhibitory effect on some microorganisms among others alkaloids, saponins, tannin, phlobatannins, flavonoids, anthraquinones and cardiac glycosides (Udoh *et al.,* 2012). Another research reported that the major component responsible for antibacterial properties are triterpene derivates including asiatic acid and asiaticoside, phenolic compounds with flavonoids and volatile oil (Zainol *et.al.,* 2003).

If we observe visually, it can be seen that dry *C. asiatica* extract have dominant green color. This indicates the presence of chlorophyll as a green pigment. *C. asiatica* powder which is dried in 40^0C contains 35.38 SPAD value of chlorophyll in average. This value was measured using the Minolta Chlorophyll meter; Model SPAD 500 (Rozalian *et al.*, 2008). Chlorophyll and its derivatives is used as a food additive and alternative medicine which has positive effects on inflammation, oxidation and wound healing (Inanc, 2011). Mowbray (1957) reported that Chlorophylin, Chlorophyll derivatives, have bacteriostatic action againts gram-positive organisms. Chlorophylin is a surface-active agents and may increase the sensitivity of microorganisms to antibiotics under the conditions of an agar diffusion assay.

Disc Diffusion Analysis

Disc diffusion test is used to determine the antimicrobial activity of *C. asiatica*. It is performed in duplicate and repeated three times to gain the representative result. As reference, we use penicillin which is known had antibacterial properties as positive control and DMSO used as the negative control which has no inhibition zone (Fig. 2).

Fig. 1 showed comparison between penicillin and *C. asiatica* against human pathogenic microorganisms. Inhibition zone were measured from outer diameter zone with diameter of disc was 0.6 mm. Although,

C.asiatica has no antibacterial activity as strong as penicillin, but with this comparison proved that *C. asiatica* dry extract still have ability to inhibit the growth of *B. cereus* but

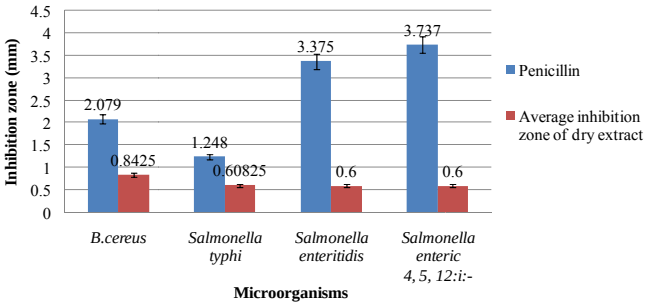

Fig 11. Comparison of inhibition zone between penicillin and *C. asiatica* dry extract by using disc diffusion test

not significantly gives effect on *S. typhi* nor *S. eteritidis* and S. *enteric 4,5,12:i:-*. The result presented in Table 1 confirm that the dry extract of *C. asiatica* which is extracted by ethanol 1:5 v/v has the ability to inhibit the growth of *B. cereus* but not for *salmonella spp*. The dry extract achieved a highest inhibitory activity of *B. cereus* when it is extracted over 24 hour with shaking.

Table 1. Inhibition zone of *C. asiaticacrude* extract on *Salmonella spp* and *B. cereus*

	Diameter of clear zone (mm)			
Extraction time (day)	*Bacillus cereus*	*Salmonella typhimurium*	*Salmonella enteritidis*	*Salmonella enteric* 4, 5, 12:i:-
1	0.21 ± 0.05	0.033 ± 0.05	-	-
1 + S	0.58 ± 0.56	-	-	-
3	0.28 ± 0.08	-	-	-
5	0.26 ±0.11	-	-	-
7	0.22 ±0.12	-	-	-

*1+ S : Incubate with shaker incubator for 24 hr
** - : no clear zone

Dry extract of *C. asiatica* can inhibit the growth of *B. cereus* with the clear zone diameter range for 0.214 to 0.557 mm, but neither for *S. enterica typhimurium*, *S. enterica enteritidis* or *S.enteric* 4, 5, 12:i:- (human) US clone. This is may be due to the difference between those bacteria related to their gram-types. According to Silhavy (2010), the major difference between gram-positive (*B.cereus*) and gram-negative (*Salmonella sp*) is the thickness of peptidoglycan surrounding the plasma membrane. Gram-negative peptidoglycan is only a few nanometers thick, representing one to a few layers, gram positive peptidoglycan is 30-100 nm thick and contains many layers. In other words, gram-positive bacteria have thicker peptidoglycan than gram-negative. However, it is not the factor why extract of *C.asiatica* cannot inhibit the growth of *salmonella sp*.

Gram negative bacteria are more resistant than gram-positive (Silhavy, 2010). There are three principal layers in the gram-negative envelope; the outer membrane (OM), the peptidoglycan cell wall and the cytoplasmic or inner membrane (Silhavy, 2010). The OM is a distinguishing feature of gram-negative bacteria; gram-positive bacteria lack this organelle (Kamio and Nikaido, 1976). The only known function of the OM is to serve as a protective barrier and this is why this organelle is essential. Gram-negative bacteria have a protective outer membrane that prevents the entry of a variety of larger antibiotics included plant extract, such as the glycopeptides vancomycin (Pages *et al.*, 2008).

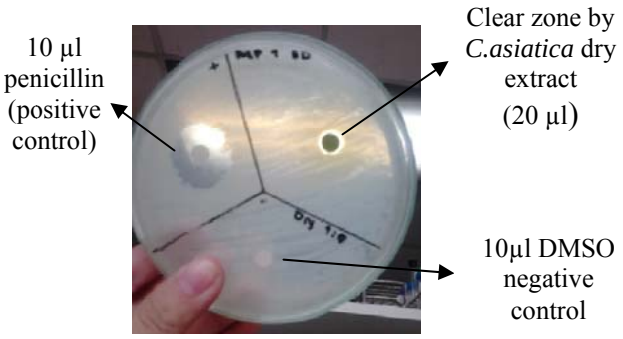

Fig 2. Result of disc diffusion method of *C. asiatica* dry extract with extraction time of three days against *B. cereus*

This result is similar to Zaidan *et al* (2005) reported that *Centella asiatica* have potential antibacterial activities to gram negative *S. aureus* (5 mm) but did not showed the inhibition zone to gram negative *E. coli* and *Klebsiella pneumoniae* by using disc diffusion. Another research reported triterpene substance, isolated by dry column chromatography did not show the antibacterial activity to *Salmonella typimurium* and *Escherichia coli* by diffusion method Besovic *et al*. (2009) which is both of them are gram-negative bacteria. Thus, we

proposed that triterpene derivatives contained in *C. asiatica* has the ability to penetrate the membrane of gram-positive but not strong enough to break the protective membrane of gram-negative.

Extraction Time Evaluation

Extraction time is crucial in determining the yield of bioactive compound that can be extract and also in minimizing cost of the extraction process for industry. In general, dry extract of *c. asiatica* increased as the increasing of extraction time and achieved the highest inhibition action at extraction time of three days for *B. cereus* as pictured in Fig 3. After this point the inhibition zone were decreased. It was believed that prolonged extraction time would lead to exposure of more oxygen and thus increase the changes for occurrence of oxidation on phenolic compound (Naczk and Shahidi, 2004; Chirinos *et al.*, 2007) which is play the important role as antibacterial compound in *C. asiatica.* Moreno *et al.*, (2006), reported that an antimicrobial action of phenolic compounds was related to inactivation of cellular enzymes, which depended on the rate of penetration of the

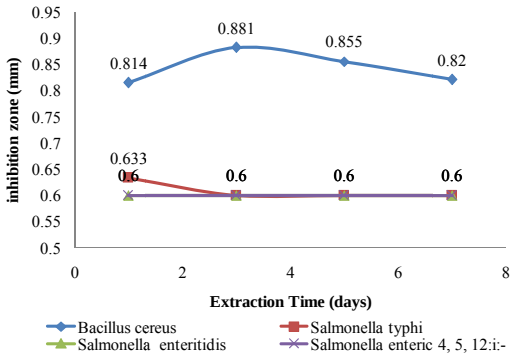

Fig 3. Inhibition zone (mm) versus extraction time

substance into the cell or caused by membrane permeability changes. Generally, prolonged the extraction time will increase the extraction yield. But, as it have already explained before, at the same time it will prolonged exposure of solvent assistance, affects analyte nature, stability and contribute the extraction of impurities (Luque Dea Castro and Garcia-Ayuso, 1998 on Sampath, 2013).

Effect of shaking in ethanol extraction

From Figure 4, we can see a significant difference of inhibition zone diameter between the samples that was incubate with shaker and the sample that was not, over 24 hours. This proved that shaking treatment could optimize the extraction process. According to Kain *et al.* (2009) when molecules move from high concentration to low concentration through semi permeable membrane that needs appropriate driving force. This is means shaking action used as the driving force to achieve the optimum movements of bioactive compounds in *C. asiatica* to solvent. Sampath (2013) said that the basic mechanisms is that the increasing driving force could increase the mass transfer rate and facilitate the concentration gradients between inside and outside plant cells, which consequently prompted diffusion rate of solute particles and made more bioactive compounds enter to the solution (solvent). Thus, this study strongly suggested that the shaking effect is the promising factor for the extraction of maximum yield of bioactive compounds from *C. asiatica* or other plant material.

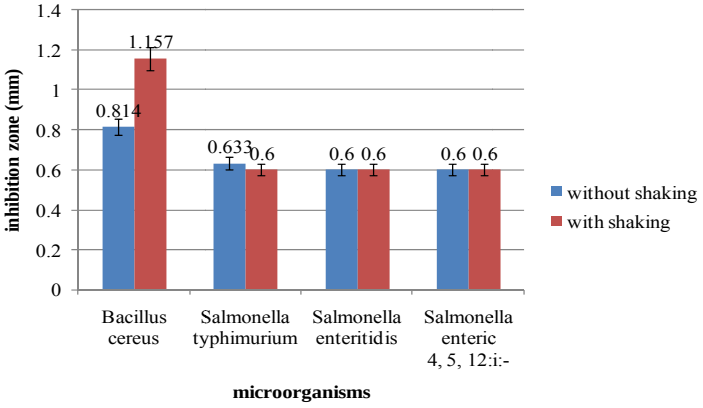

Fig 4. Comparison of inhibition zone (mm) between extraction for 24 hour without and with shaker by disc diffusion method

Determination of MIC and MBC value of *C. asitica* dry extract

Since, *C. asiatica* has the effect to inhibit the growth of *B.cereus* according to disc diffusion result, then the gram-positive bacteria was choosen to MIC and MBC test. Plan MHB media added with extract used as negative control showed no inhibitory effect.

Table 2. MIC and MBC of C. asiatica 95% crude extract on *B. cereus*

Extraction time (day)	MIC (µl/ml)	MBC (µl/ml)
1	80	80
1 + S	80	80
3	80	160
5	80	160
7	40	160

1+ S : Incubate with shaker incubator for 24 hr

The MIC is defined as the lowest concentration of drug/compound that will inhibit the visible growth of an organism *in vitro* after overnight incubation (Barros *et al.*, 2007). The lowest concentration that can inhibit the growth of bacteria were noted and considered as the MIC value for each of the bacteria strain. This study indicated the presence of antibacterial activity of *C. asiatica* dry powder ethanol extract. Concentration of 40-80 µl/ml has ability to inhibit the growth of *B. cereus* which extraction over seven days give strongest inhibition action with MIC 40 µl/ml, but this concentration could not kill *B. cereus* (Table 2).

The MBC values of dry extract of *C. asiatica* were determined by touching the loop from each of MIC tubes and streaking it on a MHA and were incubated at 37^0C for 24 hours. The extraction and shaking time give no effect on MBC value. The minimum bactericidal concentration of dry *C. asiatica* to kill *B. cereus* is 80 µl/ml on extraction time up to two days and 160 µl/ml on three up to seven days extraction (Fig 5).

Fig 5. MBC evaluation of *C. asiatica* which is extracted for three days
(80 µl and 160 µl)

Conclusion

Centella asiatica (L) Urban dry extract show antibacterial properties against *Bacillus cereus* (gram positive bacteria) effectively but has no antibacterial effect against *Salmonella typhimurium U302*, *S. enteritidis* (human) and *Salmonella enteric* 4, 5, 12:i:- (human) US clone (gram negative bacteria).The optimum extraction is three days with shaking AND Concentration of 40-80 µl/ml has ability to inhibit the growth of *B. cereus.* The minimum bactericidal concentration of dry *C. asiatica* to kill *B. cereus* is 80 µl/ml and 160 µl/mg

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