Effect of solvent extraction of *Centella asiatica* on antioxidant activity and antibacterial activity against *Salmonella enterica* Enteritidis

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Abstract

Nowadays, there are increasing trends of using natural products. Interestingly herbs are considered to be one of the alternatives consumers choose to use. In Thailand, Centella asiatica can be found in local market and locally called Buabok. C. asiatica is famous in Ayurvedic medicine for the treatment of leprosy, insanity, asthma, ulcers, eczema, skin tuberculosis, wounds, stomach aches, arthritis, varicose veins and high blood pressure (Ariffin et al, 2011 and Hakono et al, 1999). Due to the benefits of C. asiatica and its easily accessible, this studied had been done to evaluate the effect of solvent extraction of C. asiatica on antibacterial activity against Salmonella enterica Enteritidis and found out that ethanol and chloroform showed highest antibacterial activity against S. Enteritidis (9.33 ± 0.5774 and 9.50 ± 0.5000 mm., respectively) while hexane extract showed lowest activity (6.67 ± 0.1443 mm.) The minimum inhibitory concentrations (MICs), using a broth dilution method, were found to be 8 mg/ml for ethanol and chloroform. While hexane extract was found to be 32 mg/ml. The minimum bactericidal concentrations (MBCs) were greater than 32 mg/ml. Similar results were found in the antioxidant activity of C. asiatica referring to the amount of phenolic content. Total phenolic contents using Folin-Ciocalteu method, found out that ethanol extract contains highest phenolic content followed by chloroform with slightly lower in phenolic content and the lowest phenolic content was hexane extract (23.8020 ± 0.5241, 22.1718 ± 0.1403 and 7.9612 ± 1.6350 µg GAE/mg, respectively). Ferric reducing antioxidant potential value of ethanolic extract was greater than those of chloroform and hexane extract (6.4008 ± 0.0393, 3.4779 ± 0.6744 and 1.7693 ± 0.1279 mmol Fe^{2+}/mg, respectively). According to the results, different extraction solvents affected the amount of total phenolic compounds and FRAP value. The difference in the antibacterial activity was found when using different extraction solvents.

Keywords**:** *Salmonella enterica* Enteritidis, *Centella asiatica*, antibacterial acitivity, antioxidant activity

Introduction

Nowadays, there are increasing trends of using natural products. Interestingly, herbs are considered to be one of the alternatives consumers choose to use. Herbs have been used for a large range of purposes including medicine, nutrition, flavorings, beverages, dyeing, repellents, fragrances, cosmetics, charms, smoking, and industrial uses. Since prehistoric times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century (Zheng and Wang, 2001).

Centella asiatica (L) urban belonging to the family Umbeliferae is a common perennial herbaceous creeper flourishing abundantly in moist areas and distributing widely in Asia (Dash *et al*., 2011). In Thailand, *C. asiatica* can be found in local market and locally called Buabok. *C. asiatica* is famous in Ayurvedic medicine for the treatment of leprosy, insanity, asthma, ulcers, eczema, skin tuberculosis, wounds, stomach aches, arthritis,

varicose veins and high blood pressure (Ariffin *et al*., 2011 and Hakono *et al*., 1999).

Salmonella enterica Enteritidis is a pathogenic bacterium which causes gastroenteritis associated with a high mortality rate in the absence of appropriate antibiotic treatment (Latasa *et al*., 2005). Investigation of outbreaks and sporadic cases of *S.* Enteritidis has indicated that the most common sources of *S.* Enteritidis infection are poultry and poultry derivatives (Kimura *et al*., 2004), particularly, in the case of outbreaks, undercooked and raw eggs (Branden, 2006 and Velge *et al*., 2005). Thus, inhibition of *S.* Enteritidis by *C. asiatica* would provide alternative use of natural antibacterial compounds and leads to apply in other products.

Due to the benefits of *C. asiatica* and its easily accessible, this study had been done to evaluate the effects of solvent extractions of *C. asiatica* on antioxidant activity and antibacterial activity against *Salmonella enterica* Enteritidis (human).

Methodology

A. Preparation of Sample

Centella asiatica was purchased from local markets in Bangkok, Thailand. The aerial part of *C. asiatica* was used. Fresh leaves were cleaned and trimmed into small pieces. Then, they were air dried in oven (Memmert, UM500) at 45^0C. The dried samples were finely ground into powder. The powders were kept at -4^0C before used.

B. Preparation of Crude Extracts

C. asiatica powder was mixed with different extraction solvents. The solvents were 95% v/v ethanol, hexane and chloroform. *C. asiatica* powder was mixed with the solvent using 1:10 ratio (g/ml) of powder per solvents. The mixtures were macerated for 48 hours at room temperature and shake at 120 rpm. To obtained crude extracts, the mixtures were filtered using whatman filter paper no.4. Then solvents were removed under vacuum by using the rotary evaporators at 45^0C (BUCHI Rotavapor R-205, Switzerland). The crude extracts were diluted using pure dimethylsulfoxide and were kept at -20^0C before use. All extraction conditions were done in three replications.

C. Preparation of the Culture

The stock culture was prepared by inoculating a loopful of *Salmonella enterica* Enteritidis into 5ml fresh Muller Hinton broth (MHB) and then this inoculated MHB broth was shake overnight on culture tube rotator SCI (Stuart Scientific) at 37°C. Then 1% v/v overnight culture was inoculated into 10 ml of fresh MHB and incubated at 37°C by shaking incubator (Daihan labtech, model LSI-3061R), until OD600 reached 0.1 using spectrophotometer (Unico, model SI 2000) for an early log phase (Pitinidhipat and Yasurin 2012).

D. Antibacterial Susceptibility Test

1. Disc Diffusion Method

Modified disc diffusion method (Pitinidhipat and Yasurin, 2012) was used to test the antibacterial activity of *C.asiatica* extracts. The stock solutions of each extract were prepared in DMSO to yield concentration of 50mg/ml. 100 µL inoculum from culture preparation part (approximately 1.5×10^8 CFU/ml) was swabbed on Mueller Hinton agar (MHA) and air-dried at room temperature. A sterile 6-mm paper disc was impregnated with test materials and the disc was placed on the agar surface. For positive control 0.05 g/ml penicillin G (Fluka BioChemika) was used. For negative control DMSO was used. The plates were left to dry, and then they were incubated at 37 °C for 24 hours under aerobic condition. All disc diffusion tests were performed in duplicated and the antibacterial activity was expressed as the mean of inhibition zone diameters (mm).

2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

The dilution test was performed to determine minimum inhibitory concentrations (MICs) using the standard procedure modified

from Witebsky *et al*. (1979). 1000-µl of MHB was added in each tube. The 1000-µl aliquot of stock solution of crude extract (128 mg/ml) was added, and subsequently two-fold serially diluted with MHB. The 10^6 CFU/ml inoculum suspension (1000 µl) was then added in each tube containing crude extract and MHB. The final concentrations of the extract were 32, 16, 8, 4 and 2 mg/ml. The experiments were done in duplicate. The tubes were incubated at 37°C for 24 h, and the turbidity was measured based on spectroscopic analysis. The lowest concentration that inhibited visible growth of the tested organisms was recorded as the MIC. For MBC, the tubes that showed no visible growth were streaked onto MHA plates. The plates were incubated at 37^0C for 24 hours. All experiments were performed in duplicate.

E. Antioxidant Activity Test

1. Determination of total phenolic compounds

Total phenolic compounds were determined using a modified version of the Folin–Ciocalteu method (Ragazzi and Veronese, 1973). Th 20 µl of the extract was added to 1.58 ml distilled water and 100 µl Folin–Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrun, Germany). The mixture was then allowed to stand for 8 minutes 30 seconds and 300 µl sodium carbonate was added to the mixture and then this mixture was incubated at room temperature and without light. The resulting blue complex was measured at 765 nm. Distilled water was used as blank. All determinations were carried out in triplicate, and the results were expressed as microgram gallic acid equivalent (GAE) /mg of sample. Gallic acid equivalent is based on constructed standard curve from different concentration of gallic acid.

2. Ferric reducing antioxidant potential assay

The ability of extracts to reduce ferric ions was measured according to the method described by Benzie and Strain (1996) with modification. The FRAP reagent was prepared using 300 mmol sodium acetate buffer at pH 3.6, 20 mmol iron chloride and 10 mmol 2,4,6-tripyridyl-*s*-triazine dissolved in 40 mmol hydrochloric acid at a ratio of 10 : 1:1 (v : v : v). The reagent was incubated at 37 ◦C for 10 min before use. The 20 µl of the extract was added to cuvette and then 1000 µl of FRAP reagent vigorously added to cuvette and kept in the dark for 30 min. The absorbance of this mixture was measured at 593 nm. Ferrous sulfate was used to create calibration curve. In this assay, the reducing capacity of the extracts tested was calculated with reference to the reaction signal given by a Fe^{2+} solution. FRAP values were expressed as mmol Fe^{2+}/mg of sample. All measurements were done in triplicate.

F. Statistical analysis

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with Duncan's multiple range tests by SAS software version 9.3. The level of significance was set at P = 0.05.

Results & Discussion

Effects of solvents on antibacterial and antioxidant activity of *C. asiatica* were studied. From the fact that *C. asiatica* can be found locally in Thailand and Thai people used *C. asiatica* as herbal drink or eat with foods. We interested in finding the best condition for antibacterial and antioxidant compounds extraction from *C. asiatica*.

For antibacterial parts, *Salmonella enterica* Enteritidis was chosen to test antibacterial activity because this strain of culture associated widely in eggs (Branden, 2006) and chickens (Kimura *et al*., 2004). Investigation of outbreaks and sporadic cases of *S.* Enteritidis has indicated that the most common sources of *S.* Enteritidis infection are poultry and poultry derivatives (Kimura *et al*., 2004), particularly, in the case of outbreaks, undercooked and raw eggs (Branden, 2006 and Velge et al, 2005).

The result in table 1 showed that chloroform extracts had the best antibacterial activity against *S.* Enteritidis with 9.50 ± 0.500 mm. clear zones. However, statistically chloroform and ethanolic extract showed no significant in inhibition zone. For MIC value, the chloroform extract had MIC at 8 mg/ml while in order to kill *S.* Enteritidis higher concentration than 32 mg/ml was needed. Similar results found in ethanolic extract. However, hexane extract showed lowest antibacterial activity against *S.* Enteritidis for both inhibition zone and MIC value.

Table 1. Antibacterial activity of *Centella asiatica* extract on *Salmonella enterica* Enteritidis

Extracts	Disc diffusion (mm)	MIC (mg/ml)	MBC (mg/ml)
Ethanol	9.33 ± 0.5774[a]	8	>32
Chloroform	9.50 ± 0.5000[a]	8	>32
Hexane	6.67 ± 0.1443[b]	32	>32

Note: Positive control using 0.05 g/ml penicillin G showed 23 mm inhibition zone diameters and using DMSO for negative control.

From the work of Dash *et al* (2011), they studied antibacterial and antifungal activity of several extracts of *C. asiatica* L. against *Proteus vulgaris, Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli*. Their research reported that the petroleum ether, ethanol and chloroform extracts of *C. asiatica* have higher antimicrobial activities than the hexane and water extract. Similar to our research, the chloroform and ethanolic extract seem to have better activity than hexane. Thus this may be effected by the polarity of solvent. Polarity index of chloroform and ethanol are 4.1 and 5.2, respectively while that of hexane is 0. From that we could assume that compounds inside *C. asiatica* that have antibacterial activity may mostly be polar compounds.

Table 2. Ferric reducing capacity and total phenolic content of *C. asiatica* extracts

Extracts	Total phenolic content (µg GAE/mg)	FRAP (mmol Fe^{2+}/mg)
Ethanol	23.8020 ± 0.5241 [a]	6.4008 ± 0.0393 [a]
Chloroform	22.1718 ± 0.1403 [a]	3.4779 ± 0.6744 [b]
Hexane	7.9612 ± 1.6350 [b]	1.7693 ± 0.1279 [c]

Not only in the aspect of our study, had that *C. asiatica* possessed the antibacterial activity but also in term of the ability to catch free radical compounds. From the works of Ikigai *et al*. (1993) and Otake and *et al*. (1991), they suggested that the antimicrobial activity of plant in form of extract is most likely due to the combined effects of adsorption of polyphenols to bacterial membranes with membrane disruption and subsequent leakage of cellular contents. Herbs and spices also rich in phenolic compounds and besides exerting antimicrobial effect they may preserve the foods by reducing lipid oxidation as they are reported to have significant antioxidant activity (Scwarz *et al*., 2001; Shahidi *et al.*, 1997; Shan *et al.*, 2009; Tanabe *et al*., 2002; Yanishlieva *et al.*, 2006). From the work of Brinkhaus (2000) found out that the chemical constituent inside *C. asiatica* oil are flavone derivatives, sesquiterpenes, triterpenic steroids, triterpenic acids and triterpenic acid sugar esters. This could contribute to total phenolic contents and ferric reducing potentials as can be seen in table 2. The ferric ion reducing activities of *C. asiatica* showed that ethanol extract possessed highest ferric reducing activity followed by chloroform and hexane extract. Similar results were found in total phenolic contents. The ethanolic extract had highest total phenolic content. Even though ethanolic extract showed slightly higher total phenolic contents than chloroform extract. However there is no significant different in term of statistic. While hexane extract had lowest total phenolic content.

Conclusion

In conclusion, the results showed that different in extraction solvents did affect the antioxidant and antibacterial activity of *C. asiatica*. Ethanol and chloroform showed highest antibacterial activity against *S.* Enteritidis (9.33 ± 0.5164 and 9.50 ± 0.5477 mm, respectively). Similar results also found in total phenolic content and FRAP value. Further investigation on chemical constituent profiles should be performed.

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