Research Article

Simulated Gastrointestinal System Study of *Centella asiatica* Extract-loaded Gelatin Nanoparticles on Antioxidant and Antimicrobial Activities

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Abstract
The aim of this study is to improve antibacterial activity and antioxidant activity of *Centella asiatica* in simulated gastrointestinal system. Gelatin one-step and two-step desolvation nanoparticle methods were used to prepare *C. asiatica*-loaded gelatin nanoparticles (CGNP) on three different ratio of 95% ethanolic *C. asiatica* crude extracts:Gelatin (1:2, 1:3, and 1:4). One-step CGNP (OSCGNP) was tested on antibacterial activity by using well agar diffusion method with different concentrations (100, 200, and 300 µg/ml) against seven foodborne pathogens and antioxidant activity by using DPPH method. The inhibition zones of OSCGNP showed highly significant effective at concentration of 300 µg/ml in oesophagus-stomach section against *E. coli* ATCC25822 and *B. subtilis* respectively. In addition, *S. aureus*, *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone were strongly inhibited by OSCGNP at concentration of 100 µg/ml. The highest inhibition zone of OSCGNP was 1.00±0.17 cm at pH 2.0 using gelatin one-step desolvation method. The highest antioxidant activity was 22.70±4.69 µg GAE/ml per 10 mg of OSCGNP with ratio of 1:2 occurred in stomach at pH 2.0. Moreover, antioxidant activity of CGNPs (One-step and two-step gelatin nanoparticles) were dropped when they reached duodenum section. The results indicated that CGNPs gave lower antioxidant activity than crude extract.

Keywords: *Centella asiatica*, Simulated gastrointestinal system, Nanoparticle, Antibacterial activity, Antioxidant activity

1 Introduction

Centella asiatica has been introduced into the group of medicinal plants since the ancient period. The region of *C. asiatica* is located in the tropical and subtropical areas mostly in South East Asia and spread through Western part of the earth. It generally consists of various chemical compositions which have ability to broadly cure many symptoms and diseases related to mentality and injury. Active compounds are mainly compartmentalized in the roots and leaves. It also inhibits the carcinogenesis in the intestines of rats [1]–[4].

There are several research works investigated on the efficiency of Extracted *C. asiatica* including antioxidant activity and antimicrobial activity.

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The comparison of antioxidant activity in Extracted *C. asiatica* from 3 different solvents; water, ethanol and petroleum ether, has shown that *C. asiatica* extracted by ethanol gave the highest antioxidant activity [5]. *C. asiatica* is also able to inhibit the creation of oxidation [6]. Undoubtedly, *C. asiatica* is extracted by ethanol showed higher Ferric Reducing Antioxidant Power (FRAP) value than the one that is extracted by chloroform and hexane. Extracted *C. asiatica* also has ability to inhibit the growth of *B. cereus* and *L. monocytogenes* at normal, osmotic stress, and high acidic conditions, the growth of pathogenic bacteria in intestines and the growth of both Gram-positive and Gram-negative bacteria [7]–[10].

As stated by several researchers, those experiments on the beneficial effects of *C. asiatica* were tested with rats which have a possibility to enhance the human's health. However, a research study reported that the wound healing requires one kg of *C. asiatica* to receive one mg of the active form of asiaticoside through oral administration. Therefore, it is impossible to consume one kg of *C. asiatica* at a time in order to get only one mg of active form. The acidic condition and enzyme secretion along the gastrointestinal tract and the permeability of membrane in intestines that could inhibit or reject medicinal components in *C. asiatica* to access into human's bodies causes extremely low absorption [11].

The world of technology is changing rapidly so there are many available techniques which can be applied to the existing drugs or herbs in order to increase the efficiency of drugs. Nanoparticles technique has been selected to apply in various types of medicinal components and it is considered as one of the most effective technique to help in the enhancement of drug delivery system and bioavailability. As shown in the various study, nanoparticles technique is applicable to enhance the effectiveness of herbal drug properties to inhibit foodborne pathogens twice larger inhibition zone than crude and improve drug delivery system via oral administration [12]–[15].

There are various types of materials are being used in the development field of nanoparticles to improve the drug delivery system. Gelatin is a biomaterial that has been approved to use for drug delivery [16]. Gelatin is produced from animal collagen by partial acid hydrolysis or partial alkaline hydrolysis. It is economical, readily available, biodegradable, and harmless. Moreover, it consists of functional groups which can accessibly bind with various chemical substances.

Gelatin has shown high potential to apply in biomedical and pharmaceutical fields so gelatin nanoparticles were chosen to prepare for developing the drug delivery. They act as drug carrier and protector to help the drug released at the specific time and guard from being damaged [13]. There are two famous methods for producing gelatin nanoparticles which are one-step gelatin desolvation method and two-step gelatin desolvation method. Two-step gelatin desolvation method is beneficial in making less aggregation and stable gelatin nanoparticles, whereas one-step gelatin desolvation method appears crumbly large particles and less stable [13].

Therefore, the aim of this present study is to improve antibacterial activity and antioxidant activity of *C. asiatica* in *In-vitro* gastrointestinal tract by the application of one-step and two-step desolvation gelatin nanoparticles.

2 Materials and Methods

2.1 *Preparation of C. asiatica crude extract*

The mixture of *C. asiatica* with 95% ethanol in ratio of 1:10 (g/ml) was soaked at 30°C , 120 rpm, for 48 hours. Mixture was filtered through Whatmann filter paper no. 4 after 48 hours. The crude extract was evaporated at 45°C by rotary evaporators (BUCHI Rotavapor R-205, Switzerland) and stored at –20°C prior to use in preparation of *C. asiatica*-gelatin nanoparticles (CGNP) [7].

2.2 *Preparation of gelatin one-step desolvation C. asiatica nanoparticles*

Gelatin was prepared under constant heat and pH at 40 ± 1°C, pH 3 (adjusting by 0.1 M HCl) by dissolving 600 mg of gelatin in 30 ml sterile distilled water. The gelatin nanoparticles were formed after adding *C. asiatica* crude extract at the ratio of 1:2, 1:3 and 1:4 and adding 30 ml acetone dropwise. 100 µl 8% v/v glutaraldehyde solution was added to stabilize of CGNP and the solution was stirred gently for 2 hours. CGNP solution was centrifuged at speed of 4,000 rpm for 5 min. CGNP residue was purified by

centrifuging in sterile distilled water for three times. After purification, 3% w/w mannitol was added to CGNP particles. Then, the particles were freeze-dried to obtain the free-flowed powder of *C. asiatica* extract-loaded GNP [13].

2.3 *In-Vitro simulated gastrointestinal system*

Simulated digestive system of *C. asiatica*-gelatin nanoparticles was modified from Verruck *et al*., 2015 [17] by dissolving 1 mg/ml of *C. asiatica*-gelatin nanoparticles and extract crude in mastication step after pH adjusted to 6.9 with addition of 1 mol/l $NaHCO_3$ and *C. asiatica*-gelatin nanoparticles were treated with 100 U/ml of α-amylase and 1 mmol/l $CaCl_2$ as saliva solution at the rate of 0.6 ml/min for 2 min, 200 rpm [18]. The treated sample was continuously added with 1 mol/l HCl until pH reached 2.0 to create the oesophagus-stomach condition in addition 0.05 ml/g of sample pepsin solution was added and the sample was stirred at 130 rpm for 90 min. The sample was added with 0.25 ml/g of sample pancreatin-bovine bile salts solution and the condition of sample was changed to pH 5 by adding of 1 mol/l $NaHCO_3$. Sample was stirred at 45 rpm for 20 min as the duodenum section. Lastly, pH of sample increased to 6.5 by adding 1 mol/l $NaHCO_3$ and stirred at 45 rpm for 90 min. The processing sequence of simulated gastrointestinal system was presented in table 1. The mixture in all sections were incubated at 37 ± 1°C and centrifuged at 7,000 rpm for 10 min. The pellet was collected for further analysis.

Table 1: The processing sequence of simulated gastrointestinal system (adapted from Verruck *et al*. [17])

Step	Simulated Conditions	Shaking (rpm)	Final pH	Time (min)
Mouth	α-amylase +$CaCl_2$	200	6.9	2
Oesophagus-Stomach	Pepsin+HCl	130	5.5	10
			4.6	10
			3.8	10
			2.8	20
			2.3	20
			2.0	20
Duodenum	Pancreatin +bile salts +$NaHCO_3$	45	5.0	20
Ileum	$NaHCO_3$	45	6.5	90

2.4 *DPPH antioxidant assay*

Brand-William *et al*. reported α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method to measure antioxidant activities [19].

This method is modified to determine the antioxidant activities of *C. asiatica* crude extract and *C. asiatica* gelatin nanoparticles. The mixture of 100 µl *C. asiatica* crude extract or *C. asiatica* gelatin nanoparticles and 3.9 ml of 50 µM methanol DPPH solution is shaken thoroughly and kept in the dark room for 30 min. The mixture is measured by UV-vis spectrophotometer at 517 nm. The unit of µg/ml of Gallic Acid Equivalent (GAE) per 10 mg sample is used to express results.

2.5 *Antibacterial activity*

100 µl of bacterial cultures (approx. 1.5×10^8 CFU/ml) were swabbed on Mueller Hinton Agar (MHA) plates. 100 µl of *C. asiatica* crude extract and *C. asiatica* gelatin-loaded nanoparticles were added. This method was referred to the modified agar well diffusion method adapted from Rattanakom [7]. Penicillin G and DMSO were used as positive and negative controls, respectively. After 24 hours of incubation, the inhibition zone were measured to determine how effective *C. asiatica* crude extract and *C. asiatica* gelatin-loaded nanoparticles could inhibit certain microorganisms which were *Escherichia coli* ATCC25822, Bacillus cereus, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone.

2.6 *Statistical analysis*

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with Duncan's multiple range tests ($P < 0.05$) by SAS software version 9.3.

3 Results and Discussions

3.1 *Antibacterial activity*

Hydrophilicity and drug regulated release are beneficial properties from the application of nanoparticles with

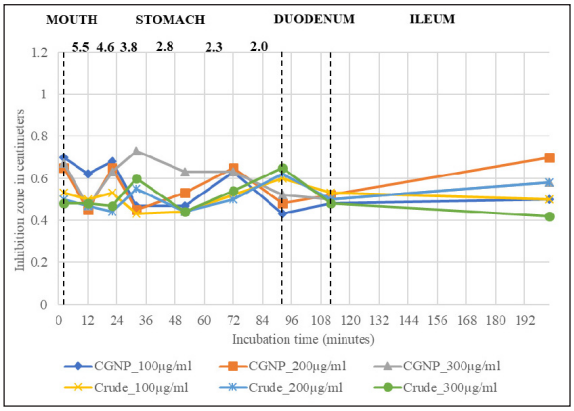

Figure 1: The inhibition zone of one-step CGNP and crude against *E. coli* in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

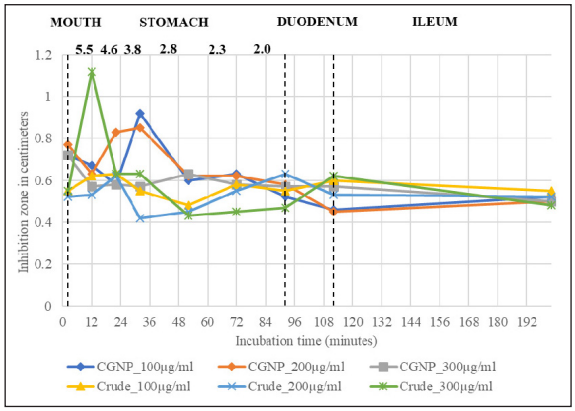

Figure 2: The inhibition zone of one-step CGNP and crude against *B. cereus* in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

bioactive hydrophobic compounds could enhance their effectiveness for the drug throughout *In-vitro* digestive system [20], [21]. CGNP from ethanolic *C. asiatica* crude extract and gelatin with the ratio concentration of 1:4 was generated by using one-step gelatin desolvation methods [13]. One-step CGNP (OSCGNP) and free crude were treated with enzymes and pH adjustment based on simulated digestive system. The antibacterial activity on OSCGNP and crude extract were determined by well agar diffusion method [7]. OSCGNP and crude extract of 100, 200, and 300 µg/ml were chosen to test against seven foodborne pathogens (*Escherichia coli* ATCC25822, Bacillus cereus, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone). The results showed that antibacterial activity of ethanolic *C. asiatica* extract-loaded gelatin desolvation nanoparticles showed significantly increasing trend comparing with ethanolic crude extract ($P<0.05$). as shown in Figure 1–7 The OSCGNP showed the highest effective at the concentration of 300 µg/ml in oesophagus-stomach section against *E. coli* ATCC25822 in Figure 1 and *B. subtilis* in Figure 3 as indicated by the inhibition zone of 0.73±0.05 and 0.92±0.08 cm, respectively. In addition, *S. aureus* in Figure 4, *S. enterica* Enteritidis (human) in Figure 6, and *S. enterica* 4, 5, 12: i: - (human) US clone in Figure 7 were strongly inhibited by OSCGNP at concentration of 100 µg/ml with the clear zone of

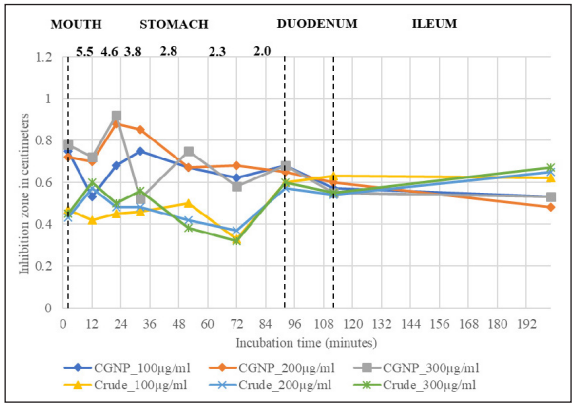

Figure 3: The inhibition zone of one-step CGNP and crude against *B. subtilis* in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

0.87±0.29, 1.00±0.17, and 0.92±0.12 cm, respectively. The highest inhibition zone of OSCGNP was 1.00±0.17 cm against *S. enterica* Enteritidis (human) located in stomach section at pH 2.0 at concentration of 100 µg/ml. While OSCGNP showed moderate antibacterial activity against *B. cereus* as shown in Figure 2. The OSCGNP also show good antibacterial activity against *S. enterica* Typhimurium U302 (DT104b) at Duodenum as shown in Figure 5. *C. asiatica* extracted crude could poorly inhibit foodborne pathogens because of its bioactive hydrophilic compounds as shown in previous study. *C. asiatica* impossibly adheres

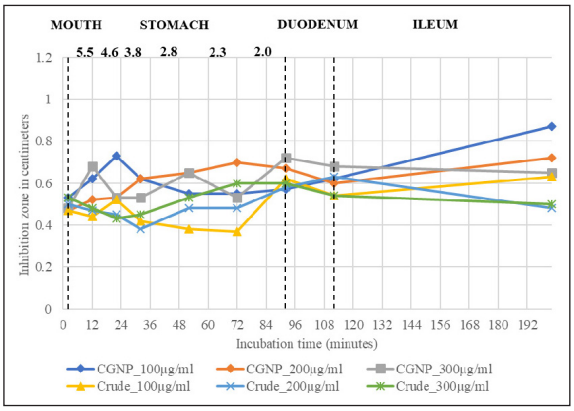

Figure 4: The inhibition zone of one-step CGNP and crude against *S. aureus* in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

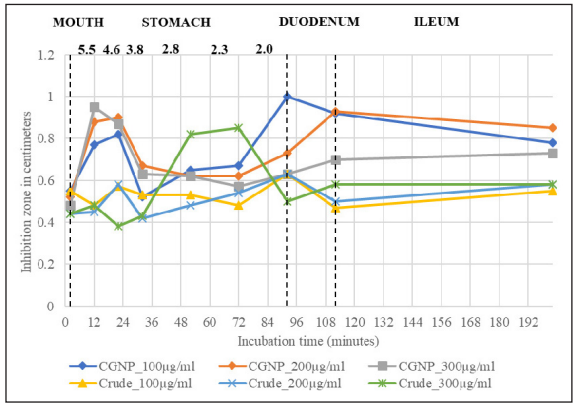

Figure 6: The inhibition zone of one-step CGNP and crude against *S. enterica* Enteritidis (human) in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

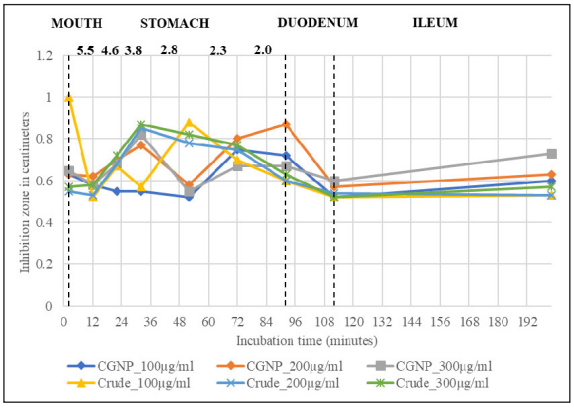

Figure 5: The inhibition zone of one-step CGNP and crude against *S. enterica* Typhimurium U302 (DT104b) in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

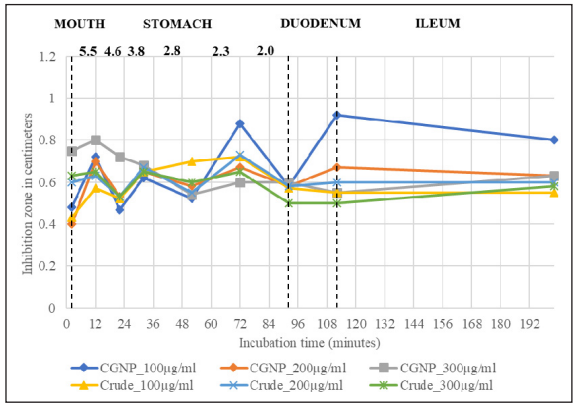

Figure 7: The inhibition zone of one-step CGNP and crude against *S. enterica* 4,5,12:i:- (human) US clone in SGS condition. The upper left indicates simulated gastrointestinal system sections and pH values.

and penetrates pathogenic bacteria's cells due to the property of cell membrane of pathogenic bacteria is hydrophobic which causes water-soluble compounds. Moreover, gelatin which was chosen to produce nanoparticles is an economical, readily, and non-toxic material can accessibly entrap bioactive compounds and positively enhance those compounds to embed to bacterial cells. The gelatin desolvation nanoparticles has been proven that it can slowly release active compounds [13]. From research claim above, bacterial cells are effectively interfered by the drug loaded in gelatin nanoparticles. From the result, OSCGNP could affected to both gram-negative (*E. coli* ATCC25822, *S. enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4, 5, 12: i: - (human) US clone) and gram-positive bacteria (*B. cereus*, *B. subtilis*, and *S. aureus*). The factor that can help to explain the interference of OSCGNP to foodborne pathogenic cells is the structure of gram-negative and gram-positive bacteria which are different in the way that gram-positive has an inner membrane covered with thick layer of peptidoglycan comparing to gram-negative bacteria is constructed from inner membrane, thin layer made of peptidoglycan, and outer membrane

[22], [23]. Therefore, location in gastrointestinal tract and concentration were independent to antibacterial activity of OSCGNP against all pathogenic bacteria and gelatin desolvation nanoparticles can be applied to improve antibacterial activity of ethanolic extracted *C. asiatica* crude against foodborne pathogens.

3.2 *Antioxidant activity*

Active compounds are commonly found in herbal products in large quantity [24]. These active compounds can help to inactivate free radical generating either from food consumption or stress [25]. Scavenging method is widely chosen to measure antioxidant activity in which free radical compounds are caught by active compound [26]. *C. asiatica* contains various types of active compounds which can function as antioxidant activity. Different active compounds probably have different functional properties. Inactivation of free radicals, reactive oxygen species scavenging, and chain-breaking activity are some examples of functions of active compounds in *C. asiatica* [6], [27], [28]. DPPH radical scavenging were used for evaluating antioxidant activity of CGNPs (One-step and two-step gelatin desolvation nanoparticles), and ethanolic crude extracts [19]. The results were expressed as the Gallic Acid Equivalent (GAE) in the unit of µg GAE/ml per 10 mg of CGNPs and crude extract and interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.3. As shown in Figure 8, ethanolic crude extract showed significantly higher activity than both CGNPs ($P < 0.05$). Due to the change in structure of active compounds to inactive compounds and binding with protein can lead to the loss of activity after generating of CGNPs in DPPH radical scavenging. Furthermore, active compounds are tightly encapsulated into gelatin desolvation nanoparticles in which active compounds are regulated to release at the specific area. However, types of gelatin desolvation methods did not show significant difference in antioxidant activity ($P<0.05$). The result of antioxidant activity was agreed with a research work where the chloroform crude extract showed higher DPPH radical scavenging than *C. asiatica* loaded in BSA nanoparticles [12]. Surprisingly, DPPH radical scavenging presented relatively high in all types and ratio of CGNPs comparing with crude extract in late stomach section. However, there is

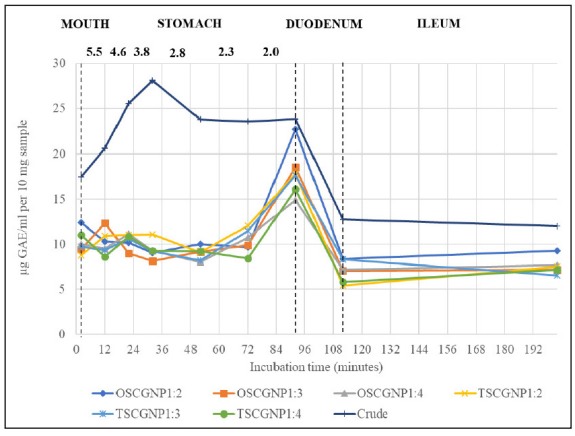

Figure 8: DPPH radical scavenging expressed in µg GAE/ml per 10 mg of sample exposed to simulated gastrointestinal conditions throughout incubation time in minute. The upper left indicates simulated gastrointestinal system sections and pH values.

no significant difference in statistic for antioxidant activity in late stomach section between CGNPs and crude extract. The highest antioxidant activity was 22.70±4.69 µg GAE/ml per 10 mg of OSCGNP with ratio of 1:2. On the other hand, antioxidant activity of CGNPs from both methods were dropped when they reached duodenum section at pH 5.0. The explanation on the release of active compounds in stomach at pH 2.0 and rapidly dropped of antioxidant values in duodenum section at pH 5.0 is that the gelatin is naturally unfolded or denatured when the pH lower than 4.0 and it can be renatured at pH 5.0 or above [29], [30]. Therefore, gelatin desolvation nanoparticles could help to delay the release of active compounds at certain area in gastrointestinal system.

4 Conclusions

The application of gelatin desolvation nanoparticles can improve herbal properties especially antibacterial activity whereas it helps in active compounds release at targeted area. Ethanolic *C. asiatica* extract-loaded one-step gelatin desolvation nanoparticles had a highly effect on antibacterial activity comparing with crude extract. The inhibition zones of OSCGNP showed the highest effective at concentration of 300 µg/ml in oesophagus-stomach section against *E. coli* ATCC25822 and *B. subtilis* were 0.73±0.05

and 0.92±0.08 cm, respectively. In addition, *S. aureus*, *S. enterica* Enteritidis (human), and *S. enterica* 4, 5, 12: i: - (human) US clone were strongly inhibited by OSCGNP at concentration of 100 µg/ml with the clear zone of 0.87±0.29, 1.00±0.17, and 0.92±0.12 cm, respectively. The highest inhibition zone of OSCGNP was 1.00±0.17 cm against *S. enterica* Enteritidis (human) located in stomach section at pH 2.0 using at concentration of 100 µg/ml. Ethanolic crude extract showed significantly higher antioxidant activity than CGNPs except in late stomach section ($P < 0.05$). The highest antioxidant activity was 22.70±4.69 µg GAE/ml per 10 mg of OSCGNP with ratio of 1:2 occurred in stomach at pH 2.0. On the other hand, antioxidant activity of CGNPs were dropped when they reached duodenum section at pH 5.0. The results indicated that OSCGNP showed the promising to increase antibacterial activity and release active compounds at targeted area in gastrointestinal system however CGNPs gave lower antioxidant activity than crude extract.

Acknowledgments

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