IMPROVED BIOAVAILABILITY AND DRUG DELIVERY SYSTEM ON CONVENTIONAL CENTELLA ASIATICA EXTRACT USING GELATIN NANOPARTICLES

BY
KITTITHATYONGSRIRASAWAD
ID: 591-9711

A Thesis Submit in Partial Satisfaction of The Requirement for The Degree of Master of Science in Food Biotechnology
Department of Food Biotechnology
Assumption University
Academic Year 2016
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Assumption University
The manuscript has been read and in satisfaction of the thesis requirements for the Master of Science in Food Biotechnology

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Mr. Kittithat Yongsirasawad
September, 2017
ABSTRACT

Centella asiatica has been used as medicinal plant to treat various types of symptoms and diseases as well as to improve memory recognition. C. asiatica crude extracts showed excellent potential In-vitro but poor In-vivo bioavailability and drug delivery system resulting from its poor lipid solubility and undesired molecular size. The most economical and readily material used for generating the nanoparticles is gelatin. It can act as the carrier and primary protection for crude extracts to be able to increase bioavailability and drug delivery system. Therefore, this study was aimed to develop C. asiatica crude extract-loaded Gelatin Nanoparticles (CGNP) to improve bioavailability and drug delivery system. CGNPs were prepared by using 2 methods, gelatin one-step desolvation and gelatin two-step desolvation methods, on three different ratio of 95% ethanolic C. asiatica crude extracts: Gelatin (1:2, 1:3, and 1:4 w/w). Entrapment and loading efficiencies are parameters used to measure the ability for the bioactive compounds to be trapped into the carrier system and the quantity of bioactive compounds loaded into carrier, respectively. As the result, entrapment efficiencies in all concentration of CGNPs showed no significant difference. Whereas, loading efficiencies were varied depending on the concentration of C. asiatica crude extract that has been used. The highest loading efficiencies were 18.39±2.08 % and 16.47±4.89 % from one-step gelatin desolvation nanoparticle and two-step gelatin desolvation nanoparticle, respectively. The solubility showed that CGNPs were barely dissolved in water comparing to C. asiatica crude extract at 31.10 to 45.40 µg/ml whereas the solubility of crude extract was 216.53±32.46 µg/ml. Therefore, gelatin desolvation nanoparticles could help to deliver hydrophilic bioactive compounds to attach and penetrate cell membranes of human and pathogenic bacteria in which, their cell membranes allow only hydrophobic compounds to access. C. asiatica extract-loaded gelatin nanoparticles were investigated for stability in phosphate buffer solution pH 7.4 with an hour interval for 6 hours. One-step and two-step desolvation gelatin nanoparticles were very stable over 6 hours of study. The well agar diffusion method was used for evaluating antibacterial activity of CGNPs with different concentrations (100, 200, and 300 µg/ml) 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 the highest inhibition zone of CGNP was 1.65±0.57 cm against S. aureus using gelatin one-step desolvation
method on ratio 1:4, 200 µg/ml. There were no significant difference of CGNP’s antibacterial activity using different preparation method and ratio. The antibacterial activity of CGNP gave almost 3 times higher than 95% ethanolic *C. asiatica* crude extracts. It was found out that antibacterial activity of CGNP was not concentration-dependent against all tested foodborne pathogens. 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 OSCGNP was dropped when they reached duodenum section.

The results indicated that OSCGNP gave lower antioxidant activity than crude extract. The kinetic release of *C. asiatica* crude extract-loaded gelatin nanoparticles promised to regulate the release rate of bioactive compounds in phosphate buffer at pH 7.4 at constant rate for up to 12 hours for both one-step and two-step gelatin nanoparticles. However, the acidic condition in gastric juice at pH 2.0 could denature the protein structure of gelatin which caused the structure to unfold and bioactive compounds were released at higher rate comparing to the release rate of CGNPs in PBS pH 7.4. *C. asiatica* crude ethanolic extracts showed significantly greater ferric reducing antioxidant power with 1.33±0.31 mmol Fe²⁺/mg dried weight than both one-step and two-step CGNPs at all ratios (p<0.05). CGNPs prepared with one-step desolvation method at ratio 1:2 showed highest FRAP 0.97±0.10 mmol Fe²⁺/mg dried weight among CGNPs. Moreover, *C. asiatica* crude ethanolic extracts also showed higher radical scavenging than both one-step and two-step CGNPs in DPPH radical scavenging activity (p<0.05). CGNPs prepared with two-step desolvation method at ratio 1:2 showed highest DPPH radical scavenging activity with 1.22±0.16 µg GAE/mg sample among CGNPs. In addition, there are no significantly difference between CGNPs preparation methods, gelatin one-step and two-step desolvation methods (p<0.05). Therefore, CGNPs prepared by using one-step desolvation method at ratio 1:4 is the most effective in an economical solution to produce CGNPs because it consumed the least operating time and
materials. Moreover, antioxidant activity for both DPPH and FRAP of 1:4 one-step gelatin desolvation nanoparticle showed no significant difference comparing with others and antibacterial activity showed the highest inhibition zone at 1.65±0.57 cm against *S. aureus*.
# TABLE OF CONTENTS

Acknowledgement ........................................................................................................................... i  
Abstract ........................................................................................................................................... ii  
List of Tables ................................................................................................................................. vii  
List of Tables (continue) ................................................................................................................ viii  
List of Figures ................................................................................................................................ ix  
List of Figures (continue) ................................................................................................................ x  
Introduction ..................................................................................................................................... 1  
  
Literature review ............................................................................................................................. 4  
  
  Centella asiatica origin ............................................................................................................................. 4  
  Biological properties and applications of C. asiatica .............................................................................. 10  
  Antibacterial activity .......................................................................................................................... 10  
  Antioxidant activity ............................................................................................................................ 11  
  Anti-inflammatory ................................................................................................................................ 11  
  Wound healing .................................................................................................................................... 12  
  Anti-cancer activity ............................................................................................................................ 12  
  Cognitive enhancement and stress depression .................................................................................... 13  
  
Physical properties and applications of C. asiatica ........................................................................... 13  
  Stability ............................................................................................................................................. 13  
  
Limitation of using C. asiatica in Bioaccessibility ............................................................................... 14  
  Technologies to improve drug delivery system .................................................................................. 14  
  
Gelatin ................................................................................................................................................ 15  
  Gelatin Nanoparticles ........................................................................................................................... 16  
  Desolvation method .............................................................................................................................. 16  
  Entrapment and loading efficiencies ................................................................................................... 17  
  In-Vitro kinetic release ....................................................................................................................... 17  
  
Objectives ...................................................................................................................................... 19  
  
Materials and Methods .................................................................................................................. 20  
  Preparation of C. asiatica crude extract .............................................................................................. 20  
  Preparation of Gelatin one-step desolvation C. asiatica nanoparticles ........................................... 20  
  Preparation of Gelatin two-step desolvation C. asiatica nanoparticles ........................................... 20  
  Encapsulation efficiency and loading capacity .................................................................................... 21  
  Solubility and stability of C. asiatica-gelatin nanoparticles ................................................................ 21
Release kinetic in Vitro ................................................................. 22
Antimicrobial activity................................................................. 22
Antioxidant efficiency ............................................................... 22
   DPPH radical scavenging activity ........................................... 22
   Ferric reducing (FRAP) antioxidant power ............................ 23
In Vitro in simulated gastrointestinal conditions ....................... 23
Statistical analysis .................................................................... 23
Results and discussion ............................................................. 25
   Entrapment efficiency and loading capacity ......................... 25
Solubility and stability .............................................................. 27
In-Vitro gastrointestinal system .................................................. 29
   Antibacterial activity ........................................................... 29
   Antioxidant activity ............................................................ 34
Release kinetic .......................................................................... 36
Antibacterial activity ............................................................... 42
Antioxidant activity ................................................................. 44
Conclusion ................................................................................... 46
References ................................................................................... 47
APPENDIX A .................................................................................. 59
APPENDIX B .................................................................................. 68
LIST OF TABLES

Table 1 Bioactive compounds of Centella asiatica including classes and its biological properties

Table 2 Processing conditions used in each step of simulated gastrointestinal conditions (Adapted from Verruck et al., 2015)

Table 3 Entrapment efficiency, loading efficiency, and solubility of CGNPs and C. asiatica crude extract

Table 4 The inhibition zone of CGNPs and crude extract against 7 different microorganisms in the unit of centimeter

Table 5 Ferric reducing antioxidant potential and DPPH radical scavenging of CGNPs and C. asiatica crude extract

Table 6 Average absorbance values of C. asiatica crude extracts from 3 replications for plotting standard curve at λmax

Table 7 Absorbance value of One-step Gelatin desolvation nanoparticles at λmax

Table 8 Absorbance value of Two-step Gelatin desolvation nanoparticles at λmax

Table 9 Microgram of C. asiatica crude extract in One-step and Two-step gelatin nanoparticles

100 µl of 2 mg/ml.

Table 10 Conversion of microgram to gram of C. asiatica crude extract in 1 mg One-step and Two-step gelatin nanoparticles

Table 11 The percentage of entrapment efficiency of One-step and Two-step gelatin nanoparticles

Table 12 The percentage of loading capacity of One-step and Two-step gelatin nanoparticles

Table 13 The mean±SD of entrapment efficiency and loading capacity

Table 14 Absorbance value of one-step gelatin desolvation nanoparticles from three replications at λmax

Table 15 Absorbance value of two-step gelatin desolvation nanoparticles and crude extract from three replications at λmax

Table 16 Average of absorbance value of one-step and two-step gelatin desolvation nanoparticles and crude extract at λmax

Table 17 Solubility of one-step and two-step gelatin nanoparticles and crude extract in unit of µg/ml
List of Tables (Continue)

Table 18 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for first replication ................................................................. 65
Table 19 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for second replication ................................................................. 66
Table 20 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for third replication ................................................................. 66
Table 21 % release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for first replication ........................................................................................................ 66
Table 22 % release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for second replication ........................................................................................................ 67
Table 23 % release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH 2.0 for third replication ........................................................................................................ 67
LIST OF FIGURES

Figure 1 Basic structure of gelatin (brought from Elzogby, 2013) ............................................. 15
Figure 2 The entrapment efficiency of C. asiatica-loaded one-step and two-step gelatin desolvation nanoparticles .................................................................................................................. 25
Figure 3 The loading efficiency of C. asiatica-loaded one-step and two-step gelatin desolvation nanoparticles ................................................................................................................................. 26
Figure 4 Percentage of solubility of C. asiatica crude extract and C. asiatica-loaded one-step and two-step gelatin desolvation nanoparticles ................................................................................... 27
Figure 5 Stability of C. asiatica extract-loaded gelatin nanoparticles in PBS (pH 7.4) at 37 °C. 28
Figure 6 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 ....................... 30
Figure 7 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 ......................... 30
Figure 8 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 ..................... 31
Figure 9 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 ...................... 31
Figure 10 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 ................................................................. 32
Figure 11 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 ................................................................. 32
Figure 12 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 value ................................................................................................................................. 33
Figure 13 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 ................................................................. 36
LIST OF FIGURES (CONTINUE)

Figure 14 Release rate of C. asiatica from One-step and Two-step CGNPs in vitro in PBS (in artificial gastric juice at pH 2.0 and artificial intestinal juice at pH 7.4) at 37°C over period of 12 hours. .............................................................................................................................................. 37

Figure 15 Inhibition zone in millimeter of E. coli from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G ............................................................ 39

Figure 16 Inhibition zone in millimeter of S. enterica Thyphimurium from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G ... 39

Figure 17 Inhibition zone in millimeter of S. enterica Enteritidis from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G ... 40

Figure 18 Inhibition zone in millimeter of S. enterica 4:5:2i:- US clone from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G ... 40

Figure 19 Inhibition zone in millimeter of S. aureus from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G................................. 41

Figure 20 Inhibition zone in millimeter of B. subtilis from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G................................. 41

Figure 21 Inhibition zone in millimeter of B. cereus from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G................................. 42

Figure 22 Standard curve of absolute amount of C. asiatica crude extract against absorbance value at 416 nm.............................................................................................................................................. 60

Figure 23 Standard curve of absolute amount of C. asiatica crude extract against absorbance value at 412 nm.............................................................................................................................................. 60

Figure 24 Standard curve of absolute amount of C. asiatica crude extract against absorbance value at 413 nm.............................................................................................................................................. 61
INTRODUCTION

*Centella asiatica* is originated from tropical or sub-tropical areas in South East Asia and spread through the Western side of the world. It is categorized in the group of plant that has ability to produce medicinal substances from second metabolite inside the plant itself. Ministry of Public Health in Thai traditional and Alternative Medicine Department has stated that *C. asiatica* is one of five Thailand champion herbal products. (MOPH, 2013) Recently, Thai government has set up the first issue of National Master Plan with 4 strategies to lead the herbal market into the world market according to international standard qualification. In this plan, *C. asiatica* is selected to be one of top six herbs that have tremendous potential to produce and develop for exporting raw materials, substituting modern medicine, food supplements, and cosmetic products including *Curcuma longa* (Turmeric), *Zingiber cassumunar Roxb* (Plai), *Kaempferia parviflora* (Black galingal), and *Pueraria mirifica* (Kwao Krua Kao). (MOPH, 2016) *C. asiatica* generally consists of various chemical compositions which have ability to broadly cure many symptoms and diseases related to mentality and injury. (Das, 2011) Monoterpenes, sesquiterpene, and triterpenoids are the major bioactive compounds existing in *C. asiatica* extracts. (Rattanakom & Yasurin, 2015) Moreover, *C. asiatica* contains high quantity of phenolic contents. (Yasurin, et al., 2016) Many researchers have approved *C. asiatica* in the way that it has possibility to heal the wounds, enhance cognitive abilities and reduce an inflammatory activity. (Kartnig, 1988; Goh, et al., 1995; George, et al., 2009; Idrus, et al., 2012) *C. asiatica* also functions as the neuroprotective which could lead to the cognitive impairments in rats and it inhibits the carcinogenesis in the intestines of rats. (Gupta, et al., 2003; Soumyanath, et al., 2012; Bunpo, et al., 2004) Nevertheless, there are several research works investigated on the efficiency of extracted *C. asiatica* including antioxidant activity and antimicrobial activity. 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. (Hamid, et al., 2002) *C. asiatica* is also able to inhibit the creation of oxidation. (Hatano, et al., 1989) 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. *C. asiatica* crude extract has ability to inhibit the growth of pathogenic bacteria such as *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 were reported. (Pitinidhipat & Yasurin, 2012; Utami, et al., 2012; Mamtha, et al., 2004; Rattanakom & Yasurin, 2015) Specifically, triterpene can help to break down cell wall of microorganism as a result of weakening tissue membranes. (Mamtha, et al., 2004) The bioactive compounds in *C. asiatica* play an important role in health beneficial improvement and prevention as mentioned above. These bioactive compounds are mainly compartmentalized in the roots and leaves.

Even though bioactive compounds in triterpene group in *C. asiatica* have ability to inhibit the growth of pathogenic bacteria, they are likely destroyed by complex enzymes and gastric juice. That means only 50 \% or even less can pass through gastrointestinal tract. It has a limitation on the delivery of bioactive compounds from the crude extract of *C. asiatica* when tested under *In Vivo* and acidic condition. (Mamtha, et al., 2004; Kriengsinyos, et al., 2006) 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 could inhibit or reject medicinal components in *C. asiatica* to access into human’s bodies causes extremely low absorption. (Shukla, et al., 1999)

The technology that has a possibility to solve those problems related to bioavailability and drug delivery system there are many available techniques such as capsules, solid dispersion, dry emulsion, pellets and tablets, microsphere, nanoparticles, suppositories, and implants which can be applied to the existing drugs or herbs in order to increase the efficiency of drugs. (Khadka, et al., 2014) 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. (Kesornbuakao & Yasurin, 2016; Azimi, et al., 2014; Yu, et al., 2014; Xie, et al., 2011) The definition of nanoparticles is the solid particles with a size in the range of 1–1000 nm. (Mohanraj & Chen, 2007) The examples of nanoparticles are PGLA-nanoparticles, BSA-nanoparticles, and
Gelatin-nanoparticles which have been revealed in several research works. 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. (Coester, et al., 2000) Gelatin is defined as the hydrophilic proteins derived from collagen extracted from bones, ligaments, skin, and tendons of animal. It is the most economical, readily material, biodegradable, and harmless among other sources of material used for generating the nanoparticles. 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. (Azimi, et al., 2014) There is a method for producing gelatin nanoparticles which are one-step gelatin desolvation method. This method is beneficial in economical production and less complicated method. Moreover, it consists of functional groups which can accessibly bind with various chemical substances. (Azimi, et al., 2014; Xie, et al., 2011; Yu, et al., 2014) One research work has investigated on antibiotic delivery by using gelatin nanoparticles as carrier for the drug. The result showed that the small dose of antibiotics could treat the bacterial infection with controllable releasing drug. (Li, et al., 2014) Therefore, the aim of this present study is to improve the bioavailability and drug delivery system of C. asiatica by the application of one-step and two-step desolvation gelatin nanoparticles.
LITERATURE REVIEW

**Centella asiatica origin**

The origin of *Centella asiatica* is located in the tropical and subtropical regions of South-East Asian countries and spread through the Western side of the world. Traditionally, the consumption of *C. asiatica* is in form of fresh vegetable or herbal drink. It helps the children who are shortage of nutrients as its rich dietary nutrients. (Prasad, et al., 2016) Apart from nutrition enrichment, *C. asiatica* is mostly used to treat symptoms and diseases as well as to improve the intellectual ability for many decades. In India, *C. asiatica* is used in the well-known historical medicine as Ayurvedic medicine to treat patients infected by bacteria.

*C. asiatica* is one of the five medicinal herbs that has been announced by department for development of Thai traditional and alternative medicine under the control of Ministry of Public Health in Thailand to be Thailand Champion Herbal Products. The demand of purchasing *C. asiatica* is increasing annually. (MOPH, 2013) In 2016, government has set up 4 new strategies in Nation Master Plan to promote the Thai herbal products to meet with domestic market and international market demands, improve the quality of herbal products to meet international standard and use herbal products as an alternative way to treat diseases. *Curcuma longa* (Turmeric), *Zingiber cassumunar Roxb* (Plai), *Kaempferia parviflora* (Black galingal), *Pueraria mirifica* (Kwao Krua Kao), and *Centella asiatica* (Bao Bog) were ranked to be the top five herbs to boost economic of Thailand. (MOPH, 2016)

Since bioactive compounds in *C. asiatica* are beneficial for curing symptoms or diseases and improving health benefits in anti-inflammation, blood pressure regulation, wound healing, diuretic, memory recognition, gastrointestinal system, skin, etc. *C. asiatica* contains high quantity of phenolic contents including apigenin, catechin, flavonoids, kaempherol, naringin, quercetin, rutin, and volatile oils. These bioactive compounds are also known as phytochemical components produced in second metabolite and are categorized into three major bioactive compounds of *C. asiatica* are terpenoids and phenolic compounds. (Yasurin, et al., 2016)
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<th>Classes</th>
<th>Bioactive compounds</th>
<th>Biological properties</th>
<th>References</th>
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<tr>
<td>Terpenoids</td>
<td>Asiatic acid</td>
<td>• Anti-inflammatory&lt;br&gt;• Induces gene expression changes&lt;br&gt;• Promotes the cultivation of Glial cells&lt;br&gt;• Helps to improve memory recognitions&lt;br&gt;• Promotes wound healing&lt;br&gt;• Inhibits apoptotic protein&lt;br&gt;• Inactivates the function of acetylcholinesterase&lt;br&gt;• Stimulates granule production&lt;br&gt;• Promotes cuticle cornification&lt;br&gt;• Induces antinociceptive activity</td>
<td>Nasir, et al., 2011;&lt;br&gt;Huang, et al., 2011;&lt;br&gt;Song, et al., 2012;&lt;br&gt;Zhang, et al., 2012;&lt;br&gt;Zhou, et al., 2011</td>
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<td>Asiaticoside</td>
<td>• Antioxidant&lt;br&gt;• Anti-inflammatory&lt;br&gt;• Induces gene expression changes&lt;br&gt;• Promotes wound healing&lt;br&gt;• Diminishes scar formation&lt;br&gt;• Preserves neuronal functions&lt;br&gt;• Develops collagen biosynthesis</td>
<td>Tang, et al., 2011;&lt;br&gt;Lee, et al., 2012;&lt;br&gt;Zhou, et al., 2011; Nowwarote, et al., 2013; Paolino, et al., 2012; Xu, et al., 2012</td>
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<td>Madecassoside</td>
<td>• Induces gene expression changes&lt;br&gt;• Inhibits oxidative injury to endothelial cells</td>
<td>Zhou, et al., 2011; Bian, et al., 2012</td>
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<td>Brahminoside</td>
<td>• Red Blood Cells generation&lt;br&gt;• Regulates blood sugar&lt;br&gt;• Induces serum cholesterol&lt;br&gt;• Reduces urea level in blood</td>
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<td>• Antibacterial activity</td>
<td>Jamil, et al., 2007;</td>
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<td>• Antineoplastic</td>
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<td>• Antitumor against melanoma and neuroblastoma cells</td>
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<td>Zhou, et al., 2011</td>
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<td>Phenols</td>
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<td>• Reduces platelet aggregation</td>
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<td>• 3',5'-cAMP-phosphodiesterase inhibitor</td>
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<td>• Inhibits the generation of fatty acid</td>
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<td>• Aldose reductase inhibitor (eye lens)</td>
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<td>• Protein C kinase inhibitor</td>
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<td>• Reduces blood capillary brittleness</td>
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<td>Kaempferol</td>
<td>Apigenin</td>
<td>• Antioxidant</td>
<td>Chong &amp; Aziz, 2013; Zhou, et al., 2011</td>
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<td>• Aldose reductase inhibitor</td>
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<td>• Nodulation signal for metabiosis of pea and Rhizobium leguminosarum</td>
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<td>Quercitrin</td>
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<td>• Antibacterial activity</td>
<td>Bhandari, et al., 2007; Zhou, et al., 2011</td>
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<td>• Hepatoprotective</td>
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<td>• Antioxidant</td>
<td>• Prevents viral infections</td>
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<td>• Aldose reductase inhibitor</td>
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<td>• Insect antifeedant (Bombyx mor)</td>
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<td>• Insect phagostimulant (Gastrophysa atrioxyaea)</td>
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<td>Luteolin</td>
<td>• Antibacterial activity</td>
<td>• Anti-inflamatory</td>
<td>Sulaiman, 2015; Bhandari, et al., 2007; Zhou, et al., 2011</td>
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<td>• Promote immune system</td>
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<td>• Aldose reductase inhibitor</td>
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<td>• Antibacterial activity</td>
<td>• Enhances arterial tension</td>
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<td>• Lowers intravenous tension</td>
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<td>• Enhances blood capillary permeability</td>
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<td>• Dihydrocoenzyme I (NADH) oxidase inhibitor</td>
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<td>• Iodine-induced thyronine deiodinase inhibitor</td>
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<td>Naringin</td>
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<td>• Antibacterial activity</td>
<td>Zheng &amp; Qin, 2007; Zhou, et al.,</td>
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<td>• Anti-inflammatory</td>
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<td>Chlorogenic acid</td>
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### Biological properties and applications of *C. asiatica*

**Antibacterial activity**

Antibacterial activity of *C. asiatica* crude extracts was revealed in various research works with different extraction solvents. Extracted *C. asiatica* (95% ethanol) crude has ability to inhibit the growth of *B. cereus* and *L. monocytogenes* at normal, osmotic stress, and high acidic conditions. (Pitinidhipat & Yasurin, 2012) *C. asiatica* that was extracted by 95% ethanol also showed strongly inhibition of *B. cereus* and *L. monocytogenes* under the control condition of low pH stress. (Utami, et al., 2012) Crude extracts of *C. asiatica* from chloroform, ethanol, and hexane showed an effect on foodborne pathogens including *Bacillus cereus*, *B. subtilis*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone. (Rattanakom & Yasurin, 2015) *Staphylococcus aureus* and its methicillin-resistant strain were effectively inhibited by hot methanolic crude extracts of *C. asiatica*. (Zaidan, et al., 2005) In addition, methanolic crude extract showed antibacterial activity against *S. aureus*, *Escherichia coli*, *Streptococcus faecalis* and *S. pyogenes*. A bioactive compound known as asiaticoside has been encapsulated in form of liposome particles showed high antibacterial efficiency when tested against *Mycobacterium leprae* and *M. tuberculosis*. (Fugh-Berman, 2003) *C. asiatica* was extracted by several types of extraction solvents showed high possibility to inhibit the growth of pathogenic bacteria in intestines and the growth of both Gram-positive and Gram-negative bacteria. (Pitinidhipat & Yasurin, 2012; Utami, et al., 2012; Mamtha, et al., 2004; Rattanakom & Yasurin, 2015)
Antioxidant activity

Good sources of antioxidant come from vegetable and fruits, such as grape seed extract, rosemary extracts, sage extracts, and vitamin C, these examples showed similar antioxidant activity to *C. asiatica*. (Jaswir, et al., 2004; Ashawat, et al., 2007; Hashim, et al., 2011) 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 however, *C. asiatica* extracted by petroleum ether has no effect on antioxidant activity. (Hamid, et al., 2002) *C. asiatica* is also able to inhibit the creation of oxidation and it has a possibility to reduce amyloid-β deposition in the brain as the result, it helps to reduce the Alzheimer’s disease. (Hatano, et al., 1989; Chen, et al., 2016) Undoubtedly, *C. asiatica* is extracted by ethanol showed higher FRAP value than the one that is extracted by chloroform and hexane. (Rattanakom & Yasurin, 2015) The reduction of hydroperoxide that inhibits free radicals and chelates metal ions would hypothetically explain the reaction of antioxidant activity of *C. asiatica*. (Zainol, et al., 2003) Functional properties can be used to differentiate the antioxidant activity of *C. asiatica* such as scavenging of the reactive oxygen species, inhibition of free radical generation, inhibition of chain-breaking activity, and metal chelation. (Hatano, et al., 1989; Laranjinha, et al., 1995; Acker, et al., 1998)

Anti-inflammatory

The apparatus of host defense to restore tissue structure back to its normal condition related to neurostimulation after it was intervened by those pathogenic bacteria is known as inflammation. (Lawrence, et al., 2002; George, et al., 2009) *C. asiatica* is a famous historical herb used to reduce or prevent inflammation. Asiatic acid and madecassic acid in *C. asiatica* have ability to inhibit the enzymes (iNOS, cyclooxygenase-2 (COX-2)), interleukins (IL-6, IL-1β), cytokine tumor necrosis factor (TNF-α) expression through the down-regulation of NF-Kb activation in lipopolysaccharide (LPS) induced RAW 264.7 murine macrophage cells. (Yun, et al., 2008; Won, et al., 2010)
Wound healing

In pre-clinical stage, many researchers have discovered that *C. asiatica* exhibited high potential to ameliorate the wound naturally. *C. asiatica* has been added into cream and gel to test on the rats as the result the wound is recovering quickly due to the cellular multiplication and collagen integration. (Sunikumar, et al., 1998) Aqueous *C. asiatica* extract with low concentration promoted epithelium wound healing in rabbit corneal epithelial cells. (Ruszymah, et al., 2012) The recovery of wound by bioactive compounds, especially asiaticoside, existed in *C. asiatica* has been reported about its functions to promote the process of wound healing by thickening skin in infected area (Poizot & Dumez, 1978), multiplying collagen formation, inducing tensile strength, developing capillary permeability, and suppressing the inflammatory. (Rosen, et al., 1967; Incandela, et al., 2001)

For clinical studies, there are approvals on the usage of *C. asiatica* in crude and capsules that had been tested under in vivo and in vitro conditions showed significant effect to heal the wound and had no negative effect on 200 diabetic wound patients, respectively. (Azis, et al., 2017; Paocharoen, 2010) One research work has discovered that an active compound, known as asiaticoside, can help to heal the wound on the human skin as well as stimulate the generation of dermal fibroblasts. (Lee, et al., 2012)

Anti-cancer activity

*C. asiatica* extracted by methanol had ability to stimulate Apoptosis on human breast cancer cells, specifically in MCF-7 cells and inhibit the spreading of adenocarcinoma (MK-1) in human stomach and carcinoma (HeLa) in uterus. (Babyykuty, et al., 2009; Yoshida, et al., 2005) In addition, water-extracted *C. asiatica* also induced apoptosis in colonic crypts and influenced the prevention of tumor from cancer cells in colon of male rats F344. (Bunpo, et al., 2004) Asiatic acid induced apoptosis in human melanoma SK-MEL-2 cells which responded for skin cancer and SW480 human colon cancer cells. (Park, et al., 2005; Tang, et al., 2009)
Cognitive enhancement and stress depression

The memory improvement has been discovered when the Asiatic acid was tested in Spraque-Dawley rats with concentration of 30 mg/kg as the result, the memory and the ability to learn were developed significantly. (Nasir, et al., 2011) The whole plant usage showed a benefit to increase the memory. (Vaidyaratnam, 1994) The *C. asiatica* extract could help to prevent and improve the children who experienced with intellectual disability related to the mental problem. (Apparao, et al., 1973) *C. asiatica* plays an important role in neuroprotective in which the neuroblastoma cell line expressing amyloid beta 1-42 and embryonic cortical primary cell culture in rats were improved by the phosphorylation of cyclic AMP response element binding protein. (Xu, et al., 2008) The *C. asiatica* showed an excellent profile on depressing the stress from works, challenges, or unexpected situations. Triterpenes have a possibility to eliminate the stress by actions of improving imbalance amino acid levels and delaying immobility time in two groups of mice population involving enforced swimming group. (Chen, et al., 2003)

Physical properties and applications of *C. asiatica*

Stability

When bioactive compounds are extracted out from the original parts of plants, they are exposed to external environment which causes them to lose their medicinal properties. The stability of bioactive compounds is the most important factor that needs to be considered in term of the application in food, cosmetic, and pharmaceutical products. There are many factors can affect the stability of bioactive compounds by either intrinsic or extrinsic factors. Intrinsic factors are water activity, pH, redox potential, available oxygen, natural biochemistry; Extrinsic factors are temperature, relative humidity, UV exposure, and consumer handling. (IFTS, 1993) The stability is already used as guidelines for the quality, safety, and efficacy of herbal products by World Health Organization (WHO), European Medicine Agency, and International Conference on Harmonization (ICH). (WHO, 2009; EMA, 2011; ICH, 2003) Kaur reported that the stabilities of asiatic acid and kaempferol in ethanolic *C. asiatica* crude extract were monthly reduced by 5.09 and 5.24 %, respectively. (Kaur, et al., 2016) The temperature over 37 °C has strongly affected to the bioactive compounds including asiaticoside and madecassoside as well as the alkaline
condition at pH 8.2 and exposed to light by decomposing of those compounds when pentacyclic triterpene enriched C. asiatica extract has been used to study for stability in various conditions involving temperature, pH, and light exposure. Surprisingly, temperature at 4 and 25 and pH at 5.8 and 7.0 would not significantly interfere with stability of asiaticoside and madecassoside over 4 months. (Puttarak, et al., 2016)

**Limitation of using C. asiatica in Bioaccessibility**

*C. asiatica* is usually used to treat various types of diseases. It contained several bioactive compounds that help to heal the wound, inhibit the creation of cancer, inhibit the growth of pathogenic bacteria, and so on. On the other hand, the bioactive compounds such as asiaticoside and asiatica acid can transfer only 50% or even less through the epithelial cells on human intestine. That means it has a limit on the absorption of bioactive compounds from the crude extract of *C. asiatica* when tested under *In Vivo*. The experiment on *In Vitro* gave significantly high efficiency on the drug absorption and drug delivery system. (Kriengsinyos, et al., 2006) Another research work studied on oral bioavailability of one of major bioactive compounds, known as Asiatic acid, in gastrointestinal tract of rats, the result showed only 16.25% with low solubility and very fast metabolism. (Yuan, et al., 2015)

**Technologies to improve drug delivery system**

The improvement of bioavailability and drug delivery system can be done by various technologies such as capsules, solid dispersion, dry emulsion, pellets and tablets, microsphere, nanoparticles, suppositories, and implants. These technologies can help to increase the drug efficiency. (Khadka, et al., 2014)

Nanoparticles technology is the process of transforming objects into Nano-sized particles are considered to have size smaller than 100 nm. At this size, it is suitable for delivering the drug including the high absorption of the drug into the cells. The application of nanotechnology currently has been used with the components that are extracted from plants in order to increase the stability and efficiency of drug delivery system, and bioavailability. The materials for generating nanoparticles can be from natural or synthetic polymers, lipids, and metals. The examples of
nanotechnology are PGLA-nanoparticles, BSA-nanoparticles, and Gelatin-nanoparticles which have been revealed in several research works. (Azimi, et al., 2014; Xie, et al., 2011; Yu, et al., 2014) The application of nanotechnology to the plant extracts had been widely used due to sustainable release of active compounds, low dose requirement, activity improvement, and side effect reduction. (Ghosh, et al., 2013; Rajendran, et al., 2013)

Nanoparticles can be made in many forms such as solid lipid nanoparticles (SLNs), liquid crystal (LC) system, precursors systems for liquid crystal (PSLCs), liposomes, microemulsions, and polymeric nanoparticles. These different formulations have discovered to improve the drug delivery by inducing the effectiveness of active constituents, protecting against thermal- or photodegradation, reducing side effects, and regulating the release of active constituents. (Mainardes, et al., 2006; Grill, et al., 2010; Venugopal, et al., 2009)

However, only a few research works on C. asiatica nanoparticles were studied. C. asiatica extract loaded in nanoparticles were prepared by ionic gelation principle and also found out the ratio of chitosan to alginate did strongly affect to the size of C. asiatica extract particles. (Okonogi, et al., 2008) Kwon reported that C. asiatica-loaded gelatin nanoparticles showed an ability to inhibit hyaluronidase expression over 60% at a concentration of 0.5 mg/ml, which was higher than the levels produced by the C. asiatica crude extracts. (Kwon, et al., 2012)

**Gelatin**

![Figure 1 Basic structure of gelatin (brought from Elzogby, 2013)](image-url)
Gelatin is defined as a tasteless, odorless substance which is generated through the process of partial acidic or alkaline hydrolysis derived from collagen. The sources of collagen are from animal bones, skin, or white connective tissue. FDA has approved to use gelatin as food ingredient and a material in pharmaceutical industries. (Elzoghby, et al., 2012; Kommareddy, et al., 2005) In addition, gelatin is biodegradable, readily available, economic, non-toxic, favorable cell adhesion, and accessible for active compounds to bind with. (Wang, et al., 2012)

Gelatin structure is constructed from the combination of various amino acids in linear pattern ended with amine group and carboxylic group on another side. Gelatin contains 98-99 % wt. of protein in dry form which composes of positively charged, negatively charged, and hydrophobic groups knowing as an ampholytic polymer with molecular weight up to 250,000 g/mol. The following statement is revealing positions of different amino acids on gelatin structure based on polarity. Lysine and arginine are responsible for positively charged region. Glutamic acid and aspartic acid are responsible for negatively charged region. For hydrophobic chain, it consists of leucine, isoleucine, methionine, and valine. The proportion of cation, anion, and hydrophobic groups are in the ratio of 1:1:1. (Kommareddy, et al., 2005; Mohanty, et al., 2005)

**Gelatin Nanoparticles**

**Desolavation method**

The dehydration of aqueous solution in dissolved gelatin is known as the desolavation technique. The solvents that are normally used to desolavate are acetone and alcohol. This technique will transform the structure of gelatin from stretched to coil structure which allows the functional groups on active compounds or other chemical constituents to interact and bind with functional groups on gelatin structure as well as to form as the nanoparticles as the result of coiling formation. In fact, the aqueous molecules as water are removed by desolavating agents and the substitution of active compounds will occur due to polarity of the functional groups on both gelatin and active compounds are compatible with each other. (Elzoghby, 2013) One-step and Two-step gelatin desolavation methods have been widely used with drug and biological active compounds from various types of herbs as the advantageous aspect in controllable release of compounds, great drug

**Entrapment and loading efficiencies**

The entrapment efficiency is defined as the percentage of sample that has been entrapped into the carrier comparing with the total quantity of initial sample. Saxena who generated cycloheximide-loaded gelatin nanoparticle by two-step desolvation method discovered the entrapment efficiencies were in the range between 26.2 to 41.0 %. (Saxena, et al., 2005) Similarly, 42 % entrapment efficiency was reported for doxorubicin loaded in gelatin nanoparticle with coacervation method. (Leo, et al., 1997) The ratio of weight of entrapped sample to the weight of total carrier system is showing loading capacity. The loading capacity is varied depending on the methods for producing the particles. Gelatin nanoparticles produced by water-in-oil emulsion method that entrapped methotrexate showed 5.6-15.6 % loading capacity and entrapped chloroquine phosphate revealed 15-19 % loading capacity. (Cascone & Lazzeri, 2002; Bajpai & Choubey, 2006) The loading capacity of cycloheximide in two-step gelatin desolvation method were 7.1-18.5 %. (Saxena, et al., 2005) However, the generation of gelatin nanoparticle with the coacervation method showed better loading capacity of 30-50 %. (Leo, et al., 1999; Vandervoort & Ludwig, 2004; Kaul & Amiji, 2002) The loading capacity of sample is depending on the concentration of sample that is presented into the system that means higher concentration of initial sample possibly increases loading capacity of sample as revealed in various research works. (Dora, et al., 2010) The relationship between loading capacity and concentration of crude extract could be explained by the interaction of hydrophobic effect and hydrogen bonding under synergistic. That means only specific bioactive compounds that are available in crude extract and compatible with functional groups on gelatin structure can be attached to the matrix of gelatin. (Bennick, 2002; Yi, et al., 2006)

**In-Vitro kinetic release**

Gelatin nanoparticles have been widely applied in medicinal and herbal products with the promising to protect the drug or active compounds from unexpected conditions including intrinsic and extrinsic environments and delay the release of medicinal substances. There are several factors
affecting release rate for the drug including pH, temperature, polarity of active compounds, and the concentration of cross-linking agents. Permeation and absorption of water into hydrogel matrix, swelling of hydrogel gelatin nanoparticles, and diffusion of active compounds through swollen structure of gelatin nanoparticles are probably involved in the release mechanism from gelatin nanoparticles. (Azimi, et al., 2014) Akhter has reported that gelatin nanoparticles could help to reduce the rate for bovine serum albumin to release. (Akhter, et al., 2012) The curcumin entrapped in poly(3-hydroxybutyrate) nanoparticles trapped in gelatin nanoparticles showed the reduction in release rate over 50%. (Bini, et al., 2017) The effect of pH on the release of cycloheximide-loaded gelatin nanoparticles has been studied as the result, release kinetic showed significantly high at acidic condition comparing with cycloheximide-loaded gelatin nanoparticles in alkaline phosphate buffer solution. (Verma, et al., 2005) Fluorescein was released out of high matrix density gelatin nanoparticles at slow and sustained rate over 18 hours. (Ahsan & Rao, 2017)
OBJECTIVES

1. To improve bioavailability, absorption, and drug delivery system of *Centella asiatica* by Gelatin one-step desolvation and Gelatin two-step desolvation methods
2. To study and compare bioavailability and drug delivery system of *Centella asiatica* extracted from conventional *Centella asiatica* via Gelatin one-step desolvation and Gelatin two-step desolvation methods
3. To study and compare physical properties of *Centella asiatica* extract-loaded gelatin nanoparticle produced by Gelatin one-step desolvation method and two-step desolvation methods from conventional *Centella asiatica* including entrapment efficiency, loading capacity, solubility, stability, control-release mechanism, and kinetic
4. To study and compare Biological properties of *Centella asiatica* extract-loaded gelatin nanoparticle produced by Gelatin one-step desolvation method and two-step desolvation methods from conventional *Centella asiatica* including antimicrobial efficiency and its mechanism, antioxidant efficiency and its mechanism
MATERIALS AND METHODS

Preparation of C. asiatica crude extract

The mixture of C. asiatica with 95% ethanol in ratio of 1:10 (g/ml) is soaked at 30°C, 120 rpm, for 48 hours. Mixture is filtered through Whatmann filter paper no. 4 after 48 hours. The crude extract is evaporated at 45°C by rotary evaporators (BUCHI Rotavapor R-205) is stored at -20°C prior to use in preparation of C. asiatica-gelatin nanoparticles. (Rattanakom & Yasurin, 2015)

Preparation of Gelatin one-step desolvation C. asiatica nanoparticles

Gelatin is 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 are formed after adding C. asiatica crude extract at the ratio of 1:2, 1:3 and 1:4 and adding 30 ml acetone dropwise. 300 µL 5% v/v glutaraldehyde solution is added to stabilize of CGNPs and the solution is stirred gently. CGNP solution is centrifuged. CGNP residue is purified by centrifuging in sterile distilled water for three times. After purification, CGNP particles are freeze-dried to obtain the free-flowed powder of C. asiatica extract-loaded GNP. (Azimi, et al., 2014)

Preparation of Gelatin two-step desolvation C. asiatica nanoparticles

The encapsulation of C. asiatica in gelatin nanoparticles is adapted from Azimi, et al., 2014. 600 mg gelatin was added in 30 ml sterile distilled water under constant temperature at 40±1°C. The precipitation of high molecular weight gelatin is obtained by adding 30 ml acetone into gelatin solution. The HMWG is dissolved with 30 ml sterile distilled water at constant temperature and pH of 40±1°C, pH 3 and stirred gently. The C. asiatica extract-loaded gelatin nanoparticles is formed by adding C. asiatica at different ratio (1:2, 1:3, and 1:4) and 30 ml acetone into gelatin solution. The stabilization of CGNP is generated by adding 300 µL glutaraldehyde solution (5% v/v) and stirring gently. The CGNP solution is centrifuged. The purification of CGNP is performed by centrifuging with sterile distilled water three times. The freeze-dry is applied to transform the liquid CGNP into form of CGNP powder.
Encapsulation efficiency and loading capacity

Encapsulation efficiency and loading capacity are determined from the *C. asiatica* trapped in gelatin nanoparticles by dissolving 2 mg CGNP in 1 ml of methanol and the mixture is shaken at 37 °C for 24 hours to completely remove *C. asiatica* crude extract from GNP into methanol. The supernatant is collected from the centrifugation of solution at 13,000 rpm for 10 minutes. 100 µL supernatant is diluted up to 2 mL prior measuring under UV-vis spectrophotometer at λmax. The amount of *C. asiatica* crude extract encapsulated and loaded in gelatin-nanoparticles is calculated by the following formulae below (Xie, et al., 2011);

\[
\text{Encapsulation efficiency(\%)} = \frac{\text{Weight of } C. \text{ asiatica crude extract in nanoparticles}}{\text{Weight of total } C. \text{ asiatica crude extract}} \times 100
\]

\[
\text{Loading capacity(\%)} = \frac{\text{Weight of } C. \text{ asiatica crude extract in nanoparticles}}{\text{Weight of nanoparticles}} \times 100
\]

Solubility and stability of *C. asiatica*-gelatin nanoparticles

Excessive *C. asiatica* crude extract or *C. asiatica* gelatin nanoparticles are added into 20 ml of sterile distilled water. The mixture is mixed and incubated in shaking incubator at the rotating speed of 200 rpm at 37 °C for 24 hours. Incubated samples are filtered through a 0.22 µm Millipore membrane and filtrate is diluted appropriately. The absorbance of diluted samples is measured at λmax UV-vis spectrophotometer to determine the optical density.

Phosphate buffer is added with 1 mg/mL of *C. asiatica* gelatin nanoparticles and incubated at 37 °C with 200 rpm. The sample is taken out at designated time points (0, 0.5, 1, 2, 3, 4, 5, and 6 hours) and the absorbance is measured at λmax by UV-vis spectrophotometer. The calculation for stability of *C. asiatica* extract is shown below;

\[
\text{Stability of } C. \text{ asiatica extract (\%)} = \frac{C_t}{C_o} \times 100
\]

Note: \(C_0\) and \(C_t\) represent the concentrations of *C. asiatica* extract in PBS at 0 h and t h, respectively (t =0, 0.5, 1, 2, 3, 4, 5, and 6 h). (Xie, et al., 2011)
Release kinetic in Vitro

Release kinetic methodology was modified (Xie, et al., 2011). The release of *C. asiatica* crude extract from CGNPs were done by dissolving 20 mg of CGNP in 15 ml artificial gastric juice (0.01 M PBS pH 2.0) and intestinal juice without enzymes (0.01 M PBS pH 7.4). The mixture is incubated at 37 °C at 200 rpm. At designated time points (0, 2, 4, 6, 8, 10, and 12 hours), mixture is sampled and centrifuged at 3000 rpm for 10 min. The pellet is resuspended in 100 µL of methanol to determine the amount of *C. asiatica* crude extract released by measuring optical density (OD) by UV-vis spectrophotometer at λmax. All measurements were done in triplicate and three replications independently.

Antimicrobial activity

*C. asiatica* crude extract or *C. asiatica* gelatin nanoparticles are added on MHA plates which are swabbed with 100 µL of bacteria (approx. 1.5 x 10⁸ CFU/mL) is referred to the modified agar well diffusion method adapted from Rattanakom and Yasurin (2015). (Rattanakom & Yasurin, 2015) And the positive and negative controls are penicillin G and DMSO, respectively. After 24 hours, the clear zones are measured to determine how effective *C. asiatica* crude extract and *C. asiatica* gelatin nanoparticles can inhibit certain microorganisms including *Escherichia coli* ATCC25822, *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica* Typhi-murium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone.

Antioxidant efficiency

DPPH radical scavenging activity

Brand-William *et al.* (Brand-Williams, et al., 1995) reported DPPH method to measure antioxidant activities. 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 methanol DPPH solution (5 x 10⁻⁵ mol/L) is shaken thoroughly and kept in the dark room for 30 minutes. The mixture is measured by UV-vis spectrophotometer at 517 nm. The unit of µg/mL of gallic acid equivalent (GAE) per 1 mg sample is used to express results.
**Ferric reducing (FRAP) antioxidant power**

FRAP method is used to measure antioxidant activities of *C. asiatica* crude extract and *C. asiatica* gelatin nanoparticles adapted from Benzie and Strain. (Benzie & Strain, 1999) The mixture (30 µL *C. asiatica* crude extract or *C. asiatica* gelatin nanoparticles with 270 µL FRAP reagent) is incubated in dark room for 30 minutes. The absorbance of solution is measured by UV-vis spectrophotometer at 593 nm. The unit of mmol of FeSO₄ equivalent per 1 mg sample is used to express results.

**In Vitro in simulated gastrointestinal conditions**

The determination of bioactivity of *C. asiatica*-gelatin nanoparticles is modified from Xie, et al., 2011 and Verruck, et al., 2015 by dissolving 20 mg of *C. asiatica*-gelatin nanoparticles in mastication step after pH adjusted to 6.9 with addition of 1 mol/L NaHCO₃ and *C. asiatica*-gelatin nanoparticles are treated with 100 U/mL of α-amylase and 1 mmol/L CaCl₂ as saliva solution at the rate of 0.6 mL/min for 2 minutes, 200 rpm. (Choi, et al., 2007) The treated sample is continuously added with 1 mol/mL HCl until pH reached 2.0 to create the oesophagus-stomach condition in addition 0.05 ml/g of sample pepsin solution is added and the sample is stirred at 130 rpm for 90 minutes. The sample is added with 0.25 ml/g of sample pancreatin-bovine bile salts solution and the condition of sample is changed to pH 5 by adding of 1 mol/L NaHCO₃. Sample is stirred at 45 rpm for 20 minutes as the duodenum section. Lastly, pH of sample increases to 6.5 by adding 1 mol/L NaHCO₃ and stirred at 45 rpm for 90 minutes. The mixture in all sections are incubated at 37 °C constantly and taken out and centrifuged at 3000 rpm for 10 minutes. The pellet is collected and determine antioxidant and antimicrobial activities. Pellets from each gastrointestinal section are collected according to the table 2 to determine antioxidant and antimicrobial activities.

**Statistical analysis**

All experiments are conducted in three replications and statistical analysis is accomplished using ANOVA with Duncan's multiple range tests (p < 0.05) by SAS software version 9.4.
Table 2 Processing conditions used in each step of simulated gastrointestinal conditions (Adapted from Verruck et al., 2015)

<table>
<thead>
<tr>
<th>Step</th>
<th>Simulated conditions</th>
<th>Stirring (rpm)</th>
<th>Final pH</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth</td>
<td>α-amylase+CaCl₂</td>
<td>200</td>
<td>6.9</td>
<td>2</td>
</tr>
<tr>
<td>Oesophagus-Stomach</td>
<td>Pepsin+HCl</td>
<td>130</td>
<td>5.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Pancreatin+bile salts+NaHCO₃</td>
<td>45</td>
<td>5.0</td>
<td>20</td>
</tr>
<tr>
<td>Ileum</td>
<td>NaHCO₃</td>
<td>45</td>
<td>6.5</td>
<td>90</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Entrapment efficiency and loading capacity

Table 3 Entrapment efficiency, loading efficiency, and solubility of CGNPs and C. asiatica crude extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio of gelatin and crude</th>
<th>Entrapment efficiency (%)&lt;sup&gt;ns&lt;/sup&gt;</th>
<th>Loading capacity (%)</th>
<th>Solubility (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-step gelatin</td>
<td>1:2</td>
<td>36.77±4.16</td>
<td>18.39±2.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.40±22.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>desolvation nanoparticles</td>
<td>1:3</td>
<td>35.66±1.44</td>
<td>11.89±0.48&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>31.10±10.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>36.90±3.53</td>
<td>9.23±0.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.70±10.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Two-step gelatin</td>
<td>1:2</td>
<td>32.94±9.78</td>
<td>16.47±4.89&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.23±15.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>desolvation nanoparticles</td>
<td>1:3</td>
<td>37.70±8.99</td>
<td>12.57±3.00&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>36.03±15.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>34.00±7.53</td>
<td>8.50±1.88&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.36±8.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Crude</td>
<td></td>
<td>216.53±32.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The drug efficiency is related to the quantitation of bioactive compounds loaded into carriers. Entrapment and loading efficiencies are parameters used to measure the ability for the bioactive compounds to be trapped into the carrier system and the quantity of bioactive compounds loaded into carrier, respectively. As the result, entrapment efficiencies in all concentration of CGNPs showed no significant difference as shown in table 3 and figure 2. The similar proportion in entrapment efficiency could be explained by the calculation as the ratio of larger quantity of crude entrapped into the GNP s to the concentration of initial crude was higher as well. This could cause the percentage of entrapment efficiency to be the same for all concentrations and both CGNP methods. The percentage of entrapment efficiencies (32.94-37.70 %) were agreed with Saxena who generated cycloheximide-loaded gelatin nanoparticle by two-step desolvation method and the entrapment efficiencies were in the range between 26.2 to 41.0 %. (Saxena, et al., 2005) Whereas, loading capacities in table 3 and figure 3 were varied depending on the concentration of C. asiatica crude extract that has been used. The highest loading capacities were 18.39±2.08 % and 16.47±4.89 % from One-step gelatin desolvation nanoparticle and two-step gelatin desolvation nanoparticle, respectively. According to other researchers, they were discovered that loading capacities were varied. 7.1-18.5 % were the percent loading capacity of two-step desolvation gelatin nanoparticles loaded with cycloheximide. (Saxena, et al., 2005) 5.6-15.6 % were the results
**Entrapment efficiency (%)**

- 1:20SCGNP: 36.77
- 1:30SCGNP: 35.66
- 1:40SCGNP: 36.9
- 1:2TSCGNP: 32.94
- 1:3TSCGNP: 37.7
- 1:4TSCGNP: 34

*Figure 2* The entrapment efficiency of *C. asiatica*-loaded one-step and two-step gelatin desolvation nanoparticles.

**Loading efficiency (%)**

- 1:20SCGNP: 18.39
- 1:30SCGNP: 11.89
- 1:40SCGNP: 9.23
- 1:2TSCGNP: 16.47
- 1:3TSCGNP: 12.57
- 1:4TSCGNP: 8.5

*Figure 3* The loading efficiency of *C. asiatica*-loaded one-step and two-step gelatin desolvation nanoparticles.

26
from methotrexate-loaded gelatin nanoparticles using w/o emulsion method. (Cascone & Lazzeri, 2002) The relationship between loading capacity and concentration of crude extract could be explained by the interaction of hydrophobic effect and hydrogen bonding under synergistic. That means only specific bioactive compounds that are available in crude extract and compatible with functional groups on gelatin structure can be attached to the matrix of gelatin. (Bennick, 2002; Yi, et al., 2006) The improvement of percent entrapment efficiency could be done by extending the time for particles to entrap more active compounds and adjust the addition time of acetone into the system. The concentration of glutaldehyde, pH, and temperature were factors that also influence in entrapment efficiency, and loading capacity of sample-loaded in gelatin nanoparticles as reported by Azimi. (Azimi, et al., 2014)

**Solubility and stability**

The binding strength of gelatin and bioactive compounds is depending on the compatibility of functional groups related to polarity and hydrophilicity. Gelatin showed an excellent carrier to bind with bioactive compounds due to its ampholytic polymer property consisting of anionic, cationic and hydrophobic regions within one molecule of gelatin. (Kommareddy, et al., 2005; Mohanty, et al., 2005) The evaluation of solubility was performed under controlled condition in distilled water at neutral pH and temperature of 37 °C for 24 hours. The result of solubility showed that CGNPs were barely dissolved in water comparing to *C. asiatica* crude extract at 31.10 to 45.40 µg/ml whereas the solubility of crude extract was 216.53±32.46 µg/ml as shown in table 3 and figure 4. Therefore, gelatin desolvation nanoparticles could help to protect from undesired condition and deliver hydrophilic bioactive compounds to attach and penetrate cell membranes of human and pathogenic bacteria with slow release rate in which, their cell membranes allow only hydrophobic compounds to access. (Kommareddy, et al., 2005) In figure 5, *C. asiatica* extract-loaded gelatin nanoparticles were investigated for stability in phosphate buffer solution pH 7.4 with an hour interval for 6 hours. One-step and two-step desolvation gelatin nanoparticles were very stable over 6 hours of study. The achievement of good stability needed to consider for these various factors, including pH, temperature, polarity, and concentration of carriers, prior the preparation of gelatin nanoparticles. Hence, gelatin nanoparticles are the hydrogel which allows water to diffuse into hydrophilic matrix made great stability for *C. asiatica* crude extracts by
protecting it from hydrolysis and biotransformation. (Xie, et al., 2011) Gelatin nanoparticles reveal a possibility to extend shelf-life for C. asiatica crude extract.

Figure 4 Percentage of solubility of C. asiatica crude extract and C. asiatica-loaded one-step and two-step gelatin desolvation nanoparticles

Figure 5 Stability of C. asiatica extract-loaded gelatin nanoparticles in PBS (pH 7.4) at 37 °C
In-Vitro gastrointestinal system

Antibacterial activity

Hydrophilicity and drug regulated release are beneficial properties from the application of nanoparticles with bioactive hydrophobic compounds could enhance their effectiveness for the drug throughout In-vitro digestive system. (Ghosh, et al., 2013; Rajendran, et al., 2013) 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. (Azimi, et al., 2014) 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. (Rattanakom & Yasurin, 2015) 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 desolation nanoparticles showed significantly increasing trend comparing with ethanolic crude extract (P<0.05). The OSCGNP showed the highest effective at the concentration of 300 µg/ml in oesophagus-stomach section against E. coli ATCC25822 in figure 2 and B. subtilis in figure 4 as indicated by the inhibition zone of 0.73±0.05 and 0.92±0.08 cm, respectively. In addition, S. aureus in figure 5, S. enterica Enteritidis (human) in figure 7, and S. enterica 4, 5, 12: i: - (human) US clone in figure 8 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 at concentration of 100 µg/ml. C. asiatica extracted crude could poorly inhibit foodborne pathogens because of its bioactive hydrophilic compounds as shown in previous study. C. asiatica impossibly adheres and penetrates pathogenic bacteria’s cells due to the property of cell membrane of pathogenic bacteria are 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. (Azimi, et al., 2014) From research claim above, bacterial cells are effectively
Figure 8 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.

Figure 9 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 10 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 11 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.
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. (Madigan & Martinko, 2005; Brown, et al., 2015) However, the mechanisms to explain how natural active compounds, especially terpenoids, interact with bacterial cells are not fully understood as stated by various researchers. The assumption of mechanism to interact with bacterial cells for terpenoids by disrupting the cell membranes. Complexation with cell wall or extracellular proteins, inactivation of enzymes that involve in bacterial cell reproduction, or blockage of adhesins are the mechanisms that have been discovered with active compounds in the group of flavonoids due to the polar molecules whereas terpenoids are lipophilic compounds. (Cowan, 1999) Therefore, location in gastrointestinal tract

Figure 12 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 value.

OSCGNP could affect 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. (Madigan & Martinko, 2005; Brown, et al., 2015) However, the mechanisms to explain how natural active compounds, especially terpenoids, interact with bacterial cells are not fully understood as stated by various researchers. The assumption of mechanism to interact with bacterial cells for terpenoids by disrupting the cell membranes. Complexation with cell wall or extracellular proteins, inactivation of enzymes that involve in bacterial cell reproduction, or blockage of adhesins are the mechanisms that have been discovered with active compounds in the group of flavonoids due to the polar molecules whereas terpenoids are lipophilic compounds. (Cowan, 1999) 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.

**Antioxidant activity**

Active compounds are commonly found in herbal products in large quantity. (Yanishlieva, et al., 2006) These active compounds can help to inactivate free radical generating either from food consumption or stress. (Beer, et al., 2002) Scavenging method is widely chosen to measure antioxidant activity in which free radical compounds are caught by active compound. (Huang, et al., 2005) *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*. (Hatano, et al., 1989; Laranjinha, et al., 1995; Acker, et al., 1998) DPPH radical scavenging were used for evaluating antioxidant activity of OSCGNP, and ethanolic crude extracts. (Brand-Williams, et al., 1995) The results were expressed as the gallic acid equivalent (GAE) in the unit of µg GAE/ml per 10 mg of OSCGNP 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 9, ethanolic crude extract showed significantly higher activity than OSCGNP (P < 0.05). Lower antioxidant activity of OSCGNP would refer 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 OSCGNP in DPPH radical scavenging and active compounds are tightly encapsulated into gelatin desolvation nanoparticles in which active compounds are regulated to release at the specific area. Furthermore, the weight of crude entrapped in GNP did not equivalent to the weight of pure *C. asiatica* crude extract. Therefore, the induction of oxidative stress could be succeeded by the application of gelatin nanoparticles in which the weight of entrapped crude in GNP was equivalent to the amount of *C. asiatica* crude extract. The initiative antioxidant activity of crude extract in the mouth section was very low and prevention of oxidative stress showed the increasing trend after crude extract was exposed in the gastric juice in stomach section until pH 3.8. The maximum scavenging activity of crude extract was observed during the sample tested under pH 3.8. From other research works on
the effect of pH, the recovery of active compounds was observed in acidic condition lower than pH 3.0. On the other hands, the reduction of oxidative stress decreased when pH changed over pH of 3.0. (Angela & Meireles, 2008) Ruenroengklin discovered the pH-dependent antioxidant activity of phenolic compounds in litchi fruit pericarp, the optimum pH was in the range of 3.0 to 4.0 showed relatively high radical scavenging activity. Whereas the antioxidant activity was gradually dropped in extremely acid condition or at pH 1.0-2.0 (Ruenroengklin, et al., 2008) The radical scavenging activity of bioactive compounds and total phenolic compounds in tomato wine treated at three different pH, 3.20, 3.40, and 4.11, showed significantly higher at pH 3.20 with 89.28 % scavenging activity and total phenolic compounds of 344.67 mg GAE/L comparing with 65.97 % and 335.47 mg GAE/L at pH 3.40 and 66.71 % and 322.97 mg GAE/L at pH 4.11. (Owusu, et al., 2015) Moreover, the study of pH affecting antioxidant properties on common medicinal herbs was investigated as the result, the radical scavenging activity of bioactive compounds was showing less effective in alkaline condition than in lower pH solution. (Bayliak, et al., 2016) The autoxidation of phenolic compounds was presented in alkaline solution which influenced the induction of oxidative stress as reported by several researchers. (Sakihama, et al., 2002; Maeta, et al., 2007; Halliwell, 2008; Dai & Mumper, 2010) Therefore, literature of various researches presented the approval on pH-dependent antioxidant activity especially at the optimal pH between 3.0 and 4.0 showing significantly more effective to inhibit oxidative stress. Low reduction of oxidative stress could be explained by the instability of many bioactive compounds at extremely acidic and alkaline pH. 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. (Kesombukao & Yasurin, 2016) Surprisingly, DPPH radical scavenging presented relatively high in all types and ratio of OSCGNP comparing with crude extract in late stomach section. However, there is no significant difference in statistic for antioxidant activity in late stomach section between OSCGNP 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 OSCGNP was 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.
Therefore, one-step gelatin desolvation nanoparticles could help to delay the release of active compounds at certain area in gastrointestinal system.

![Graph showing 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.]

**Release kinetic**

The application of gelatin nanoparticles in drugs and bioactive compounds of herbal products are successfully discovered that the particulate colloidal carriers have an ability to regulate the drug release at specific conditions. The kinetic release of drug loaded in gelatin nanocarriers has been used to evaluate the released quantity and time required for drug to release out from carriers. (Akhter, et al., 2012) The kinetic release of *C. asiatica* crude extract-loaded gelatin nanoparticles promised to regulate the release rate of bioactive compounds in phosphate
buffer at pH 7.4 at constant rate for up to 12 hours for both one-step and two-step gelatin nanoparticles as shown in figure 14. This low release of *C. asiatica* crude extract was due to the lower solubility of gelatin in the range of isoelectric point of pH 7.0 to 9.0 or zwitterion form with zero net charge in which there was no occurrence of an ion interaction. (Lin, et al., 2002) However, the acidic condition in gastric juice at pH 2.0 could denature the protein structure of gelatin which caused the structure to unfold and bioactive compounds were released at higher rate comparing to the release rate of CGNPs in PBS pH 7.4. The denaturation of gelatin could be explained by the imbalance charge on gelatin molecule happened when the pH was out of isoelectric point. (Lin, et al., 2002)

![Figure 14 Release rate of *C. asiatica* from One-step and Two-step CGNPs in vitro in PBS (in artificial gastric juice at pH 2.0 and artificial intestinal juice at pH 7.4) at 37°C over period of 12 hours.](image)
Table 4: The inhibition zone of CGNPs and crude extract against 7 different microorganisms in the unit of centimeter

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ratio (Crude:Gelatin)</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition Zone of 7 Microorganisms (cm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B. cereus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B. subtilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SUS</td>
</tr>
<tr>
<td>Gelatin One-Step Desolvation Nanoparticles</td>
<td>1:2</td>
<td>100</td>
<td>0.66±0.04A, bcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.81±0.27 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>0.72±0.09A, bcd</td>
</tr>
<tr>
<td>Gelatin Two-Step Desolvation Nanoparticles</td>
<td>1:3</td>
<td>100</td>
<td>0.73±0.02 A, bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.70±0.22 A, bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>0.69±0.12 A, bc</td>
</tr>
<tr>
<td>Crude</td>
<td>1:4</td>
<td>100</td>
<td>0.63±0.09A, bcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.64±0.10 A, bcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>0.59±0.10A, bcd</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>1:4</td>
<td>100</td>
<td>0.64±0.17A, bcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.65±0.13 A, bcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>0.53±0.08A, bcd</td>
</tr>
</tbody>
</table>

Note: Superscript in capital letters (A, B, C) and small letters (a, b, c) represented significantly different value in a row and a column at p<0.05, respectively.

ST stands for S. enterica Typhimurium, SE stands for S. enterica Enteritidis, and SUS stands for S. enterica 4,5,12:i:- (human) US clone.
Figure 17 Inhibition zone in millimeter of *S. enterica* Enteritidis from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G.

Figure 18 Inhibition zone in millimeter of *S. enterica* 4:5:2i:- US clone from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G.
Figure 19 Inhibition zone in millimeter of *S. aureus* from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G.

Figure 20 Inhibition zone in millimeter of *B. subtilis* from three different concentrations of one-step and two-step gelatin nanoparticles, crude extract and penicillin G.
Antibacterial activity

The application of nanoparticles with herbal crude extracts could enhance their efficacy on small size, hydrophilicity, and drug regulated release. (Ghosh, et al., 2013; Rajendran, et al., 2013) One-step and two-step gelatin desolvation methods were used to generate CGNPs from ethanolic C. asiatica crude extract and gelatin with the ratio concentration of 1:2, 1:3, and 1:4. (Azimi, et al., 2014) The determination of antibacterial activity on CGNPs and crude extract was done by well agar diffusion method. (Rattanakom & Yasmin, 2015) 100, 200, 300 µg/ml of CGNPs and crude extract 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 larger inhibition zone was discovered when 6 pathogenic bacteria except S. enterica Enteritidis (human) were treated with at least 200 µg/ml of CGNPs comparing with ethanolic crude extract. However, S. aureus was remarkably inhibited by all types of CGNPs and concentrations. Moreover, inhibition zone significantly increased by almost 3 times...
comparing with ethanolic crude extract as shown in table 4 (p<0.05). A research work has discovered that triterpenes in C. asiatica is effective to inhibit the growth of bacteria due to bacterial membrane disturbance. (Kalita & Saikia, 2012) Trombetta has mentioned several ways for the terpenoids to interfere with microorganism cells by partition of aqueous phase into membrane structure as the result, membrane expansion, membrane disturbance, respiration inhibition, alteration of ion transport process. (Trombetta, et al., 2005) However, the mechanisms to explain how natural active compounds, especially terpenoids, interact with bacterial cells are not fully understood as stated by various researchers. The assumption of mechanism to interact with bacterial cells for terpenoids by disrupting the cell membranes. Complexation with cell wall or extracellular proteins, inactivation of enzymes that involve in bacterial cell reproduction, or blockage of adhesins are the mechanisms that have been discovered with active compounds in the group of flavonoids due to the polar molecules whereas terpenoids are lipophilic compounds. (Cowan, 1999) Nevertheless, gelatin which is an economical, readily, and harmless material can accessibly entrap bioactive compounds to form Nano-sized particles and beneficially help those compounds to attach to bacterial cells. The slow release rate of a compound trapped in gelatin desolvation nanoparticles has been proven. (Azimi, et al., 2014) From above basis, gelatin nanoparticles can raise up the absorption rate into bacterial cells comparing to C. asiatica crude extract. The CGNP likely 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 structure of gram-negative and gram-positive bacteria are different in the way that gram-negative bacteria is constructed from inner membrane, thin layer made of peptidoglycan, and outer membrane whereas gram-positive has an inner membrane covered with thick layer of peptidoglycan. (Madigan & Martinko, 2005; Brown, et al., 2015) From the result, S. aureus and B. subtilis were highly inhibited due to its ability to generate the enzyme gelatinase which helps C. asiatica to regularly release during CGNP adhered to bacterial cells. (Achaya, 2014; Balan, et al., 2012; Tran & Nagano, 2002) Therefore, types of gelatin desolvation nanoparticles, concentration, and the ratio between ethanolic C. asiatica crude extracts were independent to antibacterial activity of CGNP against all pathogenic bacteria. The highest inhibition zone of CGNP was 1.65±0.57 cm against S. aureus using gelatin one-step desolvation method on ratio 1:4, 200 µg/ml. Moreover, the result showed that larger inhibition zone was discovered when 6 pathogenic bacteria except S. enterica
Enteritidis (human) were treated with at least 200 µg/ml of CGNPs comparing with ethanolic crude extract. However, *S. aureus* was remarkably inhibited by all types of CGNPs and concentrations. The antibacterial activity of CGNP gave almost 3 times higher than 95% ethanolic *C. asiatica* crude extracts. The higher inhibition zone of CGNPs over *C. asiatica* crude extract was beneficial from gelatin nanoparticles to slowly release triterpenes and other bioactive compounds that naturally found in *C. asiatica* crude extracts into cell membranes of pathogenic bacteria. The improvement of antibacterial activity of ethanolic *C. asiatica* crude extracts against foodborne pathogens can be achieved by the application of one-step and two-step gelatin desolvation nanoparticles.

**Antioxidant activity**

Table 5 Ferric reducing antioxidant potential and DPPH radical scavenging of CGNPs and *C. asiatica* crude extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratio of gelatin and crude</th>
<th>FRAP (mmol Fe$^{2+}$/mg equivalent to crude)</th>
<th>DPPH (µg GAE/ml per 1 mg equivalent to crude)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-step gelatin desolvation 1:2</td>
<td>0.97±0.10 b</td>
<td>0.84±0.14 b</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:3</td>
<td>0.79±0.09 b</td>
<td>1.05±0.41 b</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>0.70±0.06 b</td>
<td>0.91±0.20 b</td>
<td></td>
</tr>
<tr>
<td>Two-step gelatin desolvation 1:2</td>
<td>0.92±0.15 b</td>
<td>1.22±0.16 b</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:3</td>
<td>0.90±0.19 b</td>
<td>0.53±0.36 b</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>0.70±0.10 b</td>
<td>1.01±0.27 b</td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>1.33±0.31 a</td>
<td>4.95±1.74 a</td>
<td></td>
</tr>
</tbody>
</table>

The inhibition of free radical reactions and cellular damage is the mechanism of antioxidant substances. Antioxidants can be mainly categorized by enzymatic and hydrophilic properties. In enzymatic category, enzymatic antioxidants associate with some minerals such as copper, iron, manganese, and zinc in order to alter dangerous oxidative products to water molecules involving several stages and non-enzymatic antioxidants help to interfere in a sequence of reaction of free radicals. For category in hydrophilicity, antioxidants can be divided into 2 forms which are water-soluble antioxidants and lipid-soluble antioxidants. Moreover, different species of antioxidants have different process to inhibit free radicals by scavenging or absorbing free radical molecules.
Therefore, DPPH radical scavenging assay and FRAP assay were used to test for antioxidant activities in this study. DPPH radical scavenging assay is used to measure the reducing ability of antioxidants toward DPPH. The antioxidant effect is proportional to the disappearance of DPPH in a methanolic solution when added with CGNPs or *C. asiatica* crude ethanolic extracts. The FRAP assay is different from the DPPH assay as there are no free radicals involved. The assay determine ability of CGNPs or *C. asiatica* crude ethanolic extracts to reduce from ferric iron (Fe$^{3+}$) to ferrous iron (Fe$^{2+}$). (Silva & Sirasa, 2016) *C. asiatica* crude ethanolic extracts showed significantly greater ferric reducing antioxidant power with $1.33\pm0.31$ mmol Fe$^{2+}$/mg dried weight than both one-step and two-step CGNPs at all ratios (p<0.05). CGNPs prepared with one-step desolvation method at ratio 1:2 showed highest FRAP $0.97\pm0.10$ mmol Fe$^{2+}$/mg dried weight among CGNPs. Moreover, *C. asiatica* crude ethanolic extracts also showed higher radical scavenging than both one-step and two-step CGNPs in DPPH radical scavenging activity (p<0.05). CGNP prepared with two-step desolation method at ratio 1:2 showed highest DPPH radical scavenging activity with $1.22\pm0.16$ µg GAE/ mg sample among CGNPs. The occurrence of lower antioxidant activity of one-step and two-step gelatin nanoparticles was from the weight of *C. asiatica* crude ethanolic extracts did not equal to the weight of crude extract in GNPs. As the percent entrapment shown in table 3, only one third of crude extract was entrapped into GNPs. Therefore, the ability to reduce oxidative stress on both one-step and two-step CGNPs would show higher activity than the crude when the weight of CGNPs were weighed equivalent to the amount of crude extract. In addition, there are no significantly different between CGNPs preparation method, gelatin one-step and two-step desolvation methods (p<0.05). So, CGNPs prepared by using one-step desolvation method at ratio 1:4 is most effective in an economical way of the CGNPs production focusing on antioxidant activity because it consumed least times and material and showed no significantly different in antioxidant activity compared with others. According to previous study on *C. asiatica* Extract-Loaded BSA nanoparticles, the nanoparticles has slow release rate up to 6 hours and its antioxidant activity showed less or equal compare to crude extract. (Kesornbuakao & Yasurin, 2016) The result of this study showed that CGNPs also have less antioxidant activity compare to crude extract. Therefore, the antioxidant activity may need longer reaction time to release more active compounds of CGNPs in order to get higher antioxidant activity.
CONCLUSION

The application of gelatin desolvation nanoparticles can improve physical and biological properties of herbal plant extracts involving in bioavailability and drug delivery system. CGNPs were the least soluble in water than crude extract therefore, the hydrophobic characteristic of CGNPs can help to improve for biological active compounds to enter cell membranes with controllable release rate of bioactive compounds. These results were agreed with antibacterial activity of CGNPs that showed almost 3 times higher than crude extract whereas CGNPs showed significantly low antioxidant activity on both DPPH and FRAP (p<0.05). The higher inhibition zone of CGNPs over *C. asiatica* crude extract was beneficial from gelatin nanoparticles to slowly release triterpenes and phenolic compounds into cell membranes of pathogenic bacteria. Furthermore, it helps to regulate the release of biological active compounds at targeted area and stabilize bioactive constituents to release at constant rate. Thus, CGNP at concentration ratio of 1:4 is the most effective in term of economical production and less time consumption because it used less crude extract. The production of *C. asiatica* crude extract-loaded gelatin desolvation nanoparticles would promise to improve bioavailability and drug delivery system of *C. asiatica* crude extract. After all, CGNPs can be applied in cosmetic products with oil-based type according to its good stability in neutral to alkaline solutions, slow release of active compounds out of GNP at specific conditions. The application of CGNPs in skincare products can guarantee the excellent shelf-life storage with promising to maintain the quantity and quality of bioactive compounds that compose of various health benefits toward human satisfaction including prevention of oxidative stress from free radicals and inhibition of wide range of pathogenic bacteria.
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52


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APPENDIX A
Calculation and standard curve

Entrapment efficiency and loading capacity

Procedure
Encapsulation efficiency and loading capacity are determined from the *C. asiatica* trapped in gelatin nanoparticles by dissolving 2 mg CGNP in 1 ml of methanol and the mixture is shaken at 37 °C for 24 hours to completely remove *C. asiatica* crude extract from GNP into methanol. The supernatant is collected from the centrifugation of solution at 13,000 rpm for 10 minutes. 100 µL supernatant is diluted up to 2 mL prior measuring under UV-vis spectrophotometer at λmax. The amount of *C. asiatica* crude extract encapsulated and loaded in gelatin-nanoparticles is calculated by the following formulae below (Xie, et al., 2011):

Encapsulation efficiency(%) = \[
\frac{\text{Weight of } C. \text{ asiatica crude extract in nanoparticles}}{\text{Weight of total } C. \text{ asiatica crude extract}} \times 100
\]

Loading capacity(%) = \[
\frac{\text{Weight of } C. \text{ asiatica crude extract in nanoparticles}}{\text{Weight of nanoparticles}} \times 100
\]

Standard curve

Table 6 Average absorbance values of *C. asiatica* crude extracts from 3 replications for plotting standard curve at λmax

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absolute amount (µg)</th>
<th>Absorbance rep1</th>
<th>Avg.absorbance</th>
<th>Absorbance rep2</th>
<th>Avg.absorbance</th>
<th>Absorbance rep3</th>
<th>Avg.absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0002 g/ml</td>
<td>20</td>
<td>0.322</td>
<td>0.303</td>
<td>0.349</td>
<td>0.325</td>
<td>0.456</td>
<td>0.432</td>
</tr>
<tr>
<td>0.0004 g/ml</td>
<td>40</td>
<td>0.736</td>
<td>0.755</td>
<td>0.673</td>
<td>0.721</td>
<td>0.644</td>
<td>0.635</td>
</tr>
<tr>
<td>0.0006 g/ml</td>
<td>60</td>
<td>1.074</td>
<td>1.145</td>
<td>1.121</td>
<td>1.113</td>
<td>0.977</td>
<td>0.928</td>
</tr>
<tr>
<td>0.0008 g/ml</td>
<td>80</td>
<td>1.596</td>
<td>1.450</td>
<td>1.497</td>
<td>1.514</td>
<td>1.369</td>
<td>1.317</td>
</tr>
<tr>
<td>0.0010 g/ml</td>
<td>100</td>
<td>1.818</td>
<td>1.808</td>
<td>1.796</td>
<td>1.807</td>
<td>1.962</td>
<td>1.962</td>
</tr>
</tbody>
</table>

59
Standard curve rep 1

Figure 22 Standard curve of absolute amount of C. asiatica crude extract against absorbance value at 416 nm

Standard curve rep 2

Figure 23 Standard curve of absolute amount of C. asiatica crude extract against absorbance value at 412 nm
Standard curve rep 3

Figure 24 Standard curve of absolute amount of *C. asiatica* crude extract against absorbance value at 413 nm

**Calculation**

From equations in individual standard curve:

\[ y = 0.0188x - 0.0314 \]  
for 1st replication

\[ y = 0.0189x - 0.0671 \]  
for 2nd replication

\[ y = 0.0183x - 0.0322 \]  
for 3rd replication

Where, \( y \) is the absorbance and \( x \) represents the absolute amount of *C. asiatica* crude extract in sample.

**Table 7 Absorbance value of One-step Gelatin desolvation nanoparticles at \( \lambda_{\text{max}} \)**

<table>
<thead>
<tr>
<th>Replication</th>
<th>1:2</th>
<th>1:3</th>
<th>1:4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>0.441</td>
<td>0.433</td>
<td>0.438</td>
</tr>
<tr>
<td>2</td>
<td>0.309</td>
<td>0.341</td>
<td>0.333</td>
</tr>
<tr>
<td>3</td>
<td>0.333</td>
<td>0.341</td>
<td>0.348</td>
</tr>
</tbody>
</table>

61
Table 8 Absorbance value of Two-step Gelatin desolvation nanoparticles at \( \lambda \text{max} \)

<table>
<thead>
<tr>
<th>Replication</th>
<th>Absorbance for Two-step gelatin desolvation at ( \lambda \text{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>0.248</td>
</tr>
<tr>
<td>2</td>
<td>0.267</td>
</tr>
<tr>
<td>3</td>
<td>0.427</td>
</tr>
</tbody>
</table>

Table 9 Microgram of *C. asiatica* crude extract in One-step and Two-step gelatin nanoparticles 100 µl of 2 mg/ml

<table>
<thead>
<tr>
<th>Replication</th>
<th>Microgram of crude extract in GNPs 100 µl of 2 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-step gelatin desolvation</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>24.933</td>
</tr>
</tbody>
</table>

Table 10 Conversion of microgram to gram of *C. asiatica* crude extract in 1 mg One-step and Two-step gelatin nanoparticles

<table>
<thead>
<tr>
<th>Replication</th>
<th>Gram of crude extract in 1 mg GNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-step gelatin desolvation</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>0.000125</td>
</tr>
<tr>
<td>2</td>
<td>0.000104</td>
</tr>
<tr>
<td>3</td>
<td>0.000102</td>
</tr>
</tbody>
</table>

Table 11 The percentage of entrapment efficiency of One-step and Two-step gelatin nanoparticles

<table>
<thead>
<tr>
<th>Replication</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-step gelatin desolvation</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>41.55</td>
</tr>
<tr>
<td>2</td>
<td>34.81</td>
</tr>
<tr>
<td>3</td>
<td>33.96</td>
</tr>
</tbody>
</table>

Table 12 The percentage of loading capacity of One-step and Two-step gelatin nanoparticles

<table>
<thead>
<tr>
<th>Replication</th>
<th>Loading capacity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-step gelatin desolvation</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>1</td>
<td>20.78</td>
</tr>
<tr>
<td>2</td>
<td>17.41</td>
</tr>
<tr>
<td>3</td>
<td>16.98</td>
</tr>
</tbody>
</table>
Table 13 The mean±SD of entrapment efficiency and loading capacity

<table>
<thead>
<tr>
<th>Methods</th>
<th>CGNPs with different ratio between crude and gelatin</th>
<th>Entrapment efficiency (%)</th>
<th>Loading capacity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-step</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gelatin 1:2</td>
<td>36.77 ± 4.16</td>
<td>18.39 ± 2.08 a</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:3</td>
<td>35.66 ± 1.45</td>
<td>11.89 ± 0.48 bc</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:4</td>
<td>36.90 ± 3.53</td>
<td>9.22 ± 0.88 c</td>
<td></td>
</tr>
<tr>
<td>Two-step</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gelatin 1:2</td>
<td>32.94 ± 9.78</td>
<td>16.47 ± 4.89 ab</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:3</td>
<td>37.70 ± 8.99</td>
<td>12.57 ± 3.00 bc</td>
<td></td>
</tr>
<tr>
<td>nanoparticles 1:4</td>
<td>34.00 ± 7.53</td>
<td>8.50 ± 1.88 c</td>
<td></td>
</tr>
</tbody>
</table>

Solubility and stability

Procedure

Excessive C. asiatica crude extract or C. asiatica gelatin nanoparticles are added into 20 ml of sterile distilled water. The mixture is mixed and incubated in shaking incubator at the rotating speed of 200 rpm at 37 °C for 24 hours. Incubated samples are filtered through a 0.22 µm Millipore membrane and filtrate is diluted appropriately. The absorbance of diluted samples is measured at λmax UV-vis spectrophotometer to determine the optical density.

Phosphate buffer is added with 1 mg/mL of C. asiatica gelatin nanoparticles and incubated at 37 °C with 200 rpm. The sample is taken out at designated time points (0, 0.5, 1, 2, 3, 4, 5, and 6 hours) and the absorbance is measured at λmax by UV-vis spectrophotometer. The calculation for stability of C. asiatica extract is shown below:

\[
\text{Stability of C. asiatica extract (\%) = \frac{C_t}{C_o} \times 100}
\]

Note: C₀ and Cₜ represent the concentrations of C. asiatica extract in PBS at 0 h and t h, respectively (t = 0, 0.5, 1, 2, 3, 4, 5, and 6 h). (Xie, et al., 2011)
**Standard curve**

Using equations from previous section;

\[ y = 0.0188x - 0.0314 \] for 1st replication  
\[ y = 0.0189x - 0.0671 \] for 2nd replication  
\[ y = 0.0183x - 0.0322 \] for 3rd replication

Where, \( y \) is the absorbance and \( x \) represents the absolute amount of *C. asiatica* crude extract in sample.

**Calculation**

Table 14 Absorbance value of one-step gelatin desolvation nanoparticles from three replications at \( \lambda_{\text{max}} \)

<table>
<thead>
<tr>
<th>Replication</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
<td>1:3</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>1</td>
<td>0.026 0.040 0.013</td>
<td>0.022 0.003 0.002</td>
<td>0.026 0.034 0.020</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.068 0.075 0.053</td>
<td>0.014 0.003 0.023</td>
<td>0.022 0.031 0.030</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.045 0.056 0.026</td>
<td>0.030 0.015 0.019</td>
<td>0.032 0.044 0.020</td>
<td></td>
</tr>
</tbody>
</table>

Table 15 Absorbance value of two-step gelatin desolvation nanoparticles and crude extract from three replications at \( \lambda_{\text{max}} \)

<table>
<thead>
<tr>
<th>Replication</th>
<th>Two-step gelatin desolvation</th>
<th>Two-step gelatin desolvation</th>
<th>Crude</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
<td>1:3</td>
<td>1:4</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>1:3</td>
<td>1:4</td>
</tr>
<tr>
<td>1</td>
<td>0.005 0.002 0.003</td>
<td>0.001 0.008 0.019</td>
<td>0.041 0.052 0.029</td>
</tr>
<tr>
<td>2</td>
<td>0.020 0.046 0.004</td>
<td>0.040 0.025 0.031</td>
<td>0.022 0.034 0.030</td>
</tr>
<tr>
<td>3</td>
<td>0.047 0.040 0.049</td>
<td>0.023 0.040 0.027</td>
<td>0.031 0.025 0.040</td>
</tr>
</tbody>
</table>

Table 16 Average of absorbance value of one-step and two-step gelatin desolvation nanoparticles and crude extract at \( \lambda_{\text{max}} \)

<table>
<thead>
<tr>
<th>Replication</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
<th>One-step gelatin desolvation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.016 0.009 0.027</td>
<td>0.003 0.009 0.041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.065 0.013 0.028</td>
<td>0.023 0.032 0.029</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>0.042 0.021 0.032</td>
<td>0.045 0.030 0.032</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

64
Release kinetic in Vitro

Procedure

Release kinetic methodology was modified (Xie, et al., 2011). The release of C. asiatica crude extract from CGNPs were done by dissolving 20 mg of CGNP in 15 ml artificial gastric juice (0.01 M PBS pH 2.0) and intestinal juice without enzymes (0.01 M PBS pH 7.4). The mixture is incubated at 37 °C at 200 rpm. At designated time points (0, 2, 4, 6, 8, 10, and 12 hours), mixture is sampled and centrifuged at 3000 rpm for 10 min. The pellet is resuspended in 100 µL of methanol to determine the amount of C. asiatica crude extract released by measuring optical density (OD) by UV-vis spectrophotometer at λmax. All measurements were done in triplicate and three replications independently.

Calculation

Table 18 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for first replication

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration of crude in PBS (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OS12</td>
</tr>
<tr>
<td>0</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>53.21</td>
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<tr>
<td>4</td>
<td>33.20</td>
</tr>
<tr>
<td>6</td>
<td>46.28</td>
</tr>
<tr>
<td>8</td>
<td>72.04</td>
</tr>
<tr>
<td>10</td>
<td>41.97</td>
</tr>
<tr>
<td>12</td>
<td>76.49</td>
</tr>
</tbody>
</table>

Table 17 Solubility of one-step and two-step gelatin nanoparticles and crude extract in unit of µg/ml

<table>
<thead>
<tr>
<th>Replication</th>
<th>Microgram of crude extract in 1 ml solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One-step gelatin desolvation</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>2</td>
<td>70.071</td>
</tr>
<tr>
<td>3</td>
<td>40.729</td>
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</table>
Table 19 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for second replication

<table>
<thead>
<tr>
<th>Time</th>
<th>OS12</th>
<th>OS13</th>
<th>OS14</th>
<th>TS12</th>
<th>TS13</th>
<th>TS14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>3.55</td>
<td>3.55</td>
<td>3.55</td>
<td>3.55</td>
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</tr>
<tr>
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<td>43.85</td>
<td>18.82</td>
<td>18.68</td>
</tr>
<tr>
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<td>24.45</td>
<td>20.20</td>
<td>14.94</td>
<td>18.40</td>
<td>14.40</td>
<td>11.15</td>
</tr>
<tr>
<td>6</td>
<td>43.11</td>
<td>22.99</td>
<td>15.40</td>
<td>21.49</td>
<td>13.32</td>
<td>19.35</td>
</tr>
<tr>
<td>8</td>
<td>52.05</td>
<td>24.13</td>
<td>29.62</td>
<td>27.41</td>
<td>20.34</td>
<td>23.13</td>
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<tr>
<td>10</td>
<td>56.27</td>
<td>24.20</td>
<td>18.56</td>
<td>18.58</td>
<td>20.76</td>
<td>18.96</td>
</tr>
<tr>
<td>12</td>
<td>53.14</td>
<td>32.10</td>
<td>21.98</td>
<td>40.68</td>
<td>26.05</td>
<td>11.05</td>
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</tbody>
</table>

Table 20 Concentration of crude extract in one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for third replication

<table>
<thead>
<tr>
<th>Time</th>
<th>OS12</th>
<th>OS13</th>
<th>OS14</th>
<th>TS12</th>
<th>TS13</th>
<th>TS14</th>
</tr>
</thead>
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<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
<td>1.76</td>
</tr>
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<td>14.89</td>
<td>38.32</td>
<td>25.71</td>
<td>13.56</td>
</tr>
<tr>
<td>4</td>
<td>15.48</td>
<td>18.81</td>
<td>19.21</td>
<td>20.30</td>
<td>27.77</td>
<td>13.29</td>
</tr>
<tr>
<td>6</td>
<td>17.57</td>
<td>21.87</td>
<td>21.30</td>
<td>28.95</td>
<td>27.39</td>
<td>11.74</td>
</tr>
<tr>
<td>8</td>
<td>28.70</td>
<td>35.24</td>
<td>21.23</td>
<td>53.23</td>
<td>46.42</td>
<td>17.72</td>
</tr>
<tr>
<td>10</td>
<td>16.04</td>
<td>20.78</td>
<td>18.15</td>
<td>23.67</td>
<td>35.95</td>
<td>14.93</td>
</tr>
<tr>
<td>12</td>
<td>33.47</td>
<td>37.62</td>
<td>23.58</td>
<td>51.61</td>
<td>44.29</td>
<td>16.19</td>
</tr>
</tbody>
</table>

Table 21 %release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for first replication

<table>
<thead>
<tr>
<th>Time</th>
<th>OS12</th>
<th>OS13</th>
<th>OS14</th>
<th>TS12</th>
<th>TS13</th>
<th>TS14</th>
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</thead>
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<td>1.11</td>
<td>0.56</td>
<td>0.84</td>
<td>1.11</td>
</tr>
<tr>
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<td>17.74</td>
<td>11.70</td>
<td>14.33</td>
<td>15.04</td>
<td>5.55</td>
<td>7.89</td>
</tr>
<tr>
<td>4</td>
<td>11.07</td>
<td>11.77</td>
<td>10.95</td>
<td>7.62</td>
<td>4.97</td>
<td>7.32</td>
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<td>8.04</td>
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<td>15.49</td>
<td>21.50</td>
<td>6.97</td>
<td>10.44</td>
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<td>21.63</td>
<td>22.46</td>
<td>16.35</td>
<td>7.01</td>
<td>11.43</td>
</tr>
</tbody>
</table>
Table 22 %release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for second replication

<table>
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<th>Time</th>
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<th>OS13</th>
<th>OS14</th>
<th>TS12</th>
<th>TS13</th>
<th>TS14</th>
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<td>1.78</td>
<td>2.37</td>
<td>1.18</td>
<td>1.78</td>
<td>2.37</td>
</tr>
<tr>
<td>2</td>
<td>11.49</td>
<td>8.11</td>
<td>8.89</td>
<td>14.62</td>
<td>9.41</td>
<td>12.46</td>
</tr>
<tr>
<td>4</td>
<td>8.15</td>
<td>10.10</td>
<td>9.96</td>
<td>6.13</td>
<td>7.20</td>
<td>7.43</td>
</tr>
<tr>
<td>6</td>
<td>14.37</td>
<td>11.49</td>
<td>10.27</td>
<td>7.16</td>
<td>6.66</td>
<td>12.90</td>
</tr>
<tr>
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<td>17.35</td>
<td>12.07</td>
<td>19.74</td>
<td>9.14</td>
<td>10.17</td>
<td>15.42</td>
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<td>18.76</td>
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<td>6.19</td>
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<td>14.65</td>
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</table>

Table 23 %release of one-step and two-step gelatin nanoparticles in phosphate buffer solution at pH2.0 for third replication

<table>
<thead>
<tr>
<th>Time</th>
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<th>OS13</th>
<th>OS14</th>
<th>TS12</th>
<th>TS13</th>
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</thead>
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<td>1.17</td>
<td>0.59</td>
<td>0.88</td>
<td>1.17</td>
</tr>
<tr>
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<td>10.35</td>
<td>11.28</td>
<td>9.93</td>
<td>12.77</td>
<td>12.86</td>
<td>9.04</td>
</tr>
<tr>
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<td>5.16</td>
<td>9.40</td>
<td>12.81</td>
<td>6.77</td>
<td>13.89</td>
<td>8.86</td>
</tr>
<tr>
<td>6</td>
<td>5.86</td>
<td>10.93</td>
<td>12.20</td>
<td>9.65</td>
<td>13.69</td>
<td>7.83</td>
</tr>
<tr>
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<td>9.57</td>
<td>17.62</td>
<td>14.15</td>
<td>17.74</td>
<td>23.21</td>
<td>11.81</td>
</tr>
<tr>
<td>10</td>
<td>5.35</td>
<td>10.39</td>
<td>12.10</td>
<td>7.89</td>
<td>17.97</td>
<td>9.95</td>
</tr>
<tr>
<td>12</td>
<td>11.16</td>
<td>18.81</td>
<td>15.72</td>
<td>17.20</td>
<td>22.15</td>
<td>10.79</td>
</tr>
</tbody>
</table>
APPENDIX B
SAS output
SAS code for ANOVA analysis

data name;
input Treatment$ rep value;
cards;
120S 1 41.55
120S 2 34.81
120S 3 33.96
130S 1 35.03
130S 2 37.31
130S 3 34.64
140S 1 33.40
140S 2 40.46
140S 3 36.83
12TS 1 26.04
12TS 2 28.64
12TS 3 44.13
13TS 1 31.66
13TS 2 48.03
13TS 3 33.41
14TS 1 25.31
14TS 2 38.70
14TS 3 37.98;
proc anova data=name;
class Treatment rep;
model value=Treatment rep;
means Treatment;
means Treatment/ duncan;
ods rtf;
run;
ods rtf close;
SAS results
The ANOVA Procedure

Entrapment efficiency

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>ANOVA SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>5</td>
<td>51.3410278</td>
<td>10.2682056</td>
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<td>0.9320</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>114.0535111</td>
<td>57.0267556</td>
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<td>0.2977</td>
</tr>
</tbody>
</table>

Loading efficiency

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>ANOVA SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.0069</td>
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<td>0.5269</td>
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</table>

Solubility

<table>
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<tr>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
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<td>13699.85662</td>
<td>40.96</td>
<td>&lt;0.0001</td>
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<td>rep</td>
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<td>658.69092</td>
<td>329.34546</td>
<td>0.98</td>
<td>0.4018</td>
</tr>
</tbody>
</table>

Stability

Stability at 0.5 hour

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>ANOVA SS</th>
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<th>F Value</th>
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Stability at 1 hour

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SAS results
The ANOVA Procedure

### Stability at 2 hours

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### Stability at 6 hours

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### Stability of one-step gelatin nanoparticle at the ratio of 1:2

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<td>Treatment</td>
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SAS results
The ANOVA Procedure

Stability of one-step gelatin nanoparticle at the ratio of 1:3

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<td>Treatment</td>
<td>7</td>
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Stability of one-step gelatin nanoparticle at the ratio of 1:4

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<tr>
<td>Treatment</td>
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<td>116.6849333</td>
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Stability of two-step gelatin nanoparticle at the ratio of 1:2

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<tr>
<td>Treatment</td>
<td>7</td>
<td>142.1209167</td>
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Stability of two-step gelatin nanoparticle at the ratio of 1:3

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Stability of two-step gelatin nanoparticle at the ratio of 1:4

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In-Vitro release kinetic

Release kinetic at 0 hour

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<tbody>
<tr>
<td>Treatment</td>
<td>11</td>
<td>3.58835556</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>6.75795556</td>
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# SAS results
## The ANOVA Procedure

### Release kinetic at 2 hours

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### Release kinetic at 4 hours

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### Release kinetic at 6 hours

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### Release kinetic at 8 hours

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### Release kinetic at 10 hours

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### Release kinetic at 12 hours

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SAS results
The ANOVA Procedure

Release kinetic at pH 2.0 of one-step gelatin nanoparticle at the ratio 1:2

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Release kinetic at pH 2.0 of one-step gelatin nanoparticle at the ratio 1:3

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Release kinetic at pH 2.0 of one-step gelatin nanoparticle at the ratio 1:4

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Release kinetic at pH 2.0 of two-step gelatin nanoparticle at the ratio 1:2

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Release kinetic at pH 2.0 of two-step gelatin nanoparticle at the ratio 1:3

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Release kinetic at pH 2.0 of two-step gelatin nanoparticle at the ratio 1:4

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**SAS results**
The ANOVA Procedure

Release kinetic at pH 7.4 of one-step gelatin nanoparticle at the ratio 1:2

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Release kinetic at pH 7.4 of one-step gelatin nanoparticle at the ratio 1:3

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<tbody>
<tr>
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<td>37.62553333</td>
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Release kinetic at pH 7.4 of one-step gelatin nanoparticle at the ratio 1:4

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Release kinetic at pH 7.4 of two-step gelatin nanoparticle at the ratio 1:2

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Release kinetic at pH 7.4 of two-step gelatin nanoparticle at the ratio 1:3

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Release kinetic at pH 7.4 of two-step gelatin nanoparticle at the ratio 1:4

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Simulated gastrointestinal system

Antibacterial activity

SAS results according to pathogenic bacterial strains

*Escherichia coli* ATCC25822

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*Bacillus cereus*

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*Bacillus subtilis*

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*Staphylococcus aureus*

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*Salmonella enterica* Typhimurium U302 (DT104b)

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**SAS results**
The ANOVA Procedure

*Salmonella enterica Enteritidis (human)*

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*Salmonella enterica 4,5,12:i:- (human) US clone*

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SAS results according to concentrations of CGNP and steps of simulated gastrointestinal system

*In Mouth section at pH 6.9,*

**For 100 µg/ml CGNP**

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**For 200 µg/ml CGNP**

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**For 300 µg/ml CGNP**

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**SAS results**

The ANOVA Procedure

### For 100 µg/ml crude extract

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### For 200 µg/ml crude extract

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### For 300 µg/ml crude extract

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### In Stomach section at pH 5.5,

#### For 100 µg/ml CGNP

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#### For 200 µg/ml CGNP

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#### For 300 µg/ml CGNP

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**SAS results**

The ANOVA Procedure

**For 100 µg/ml crude extract**

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**For 200 µg/ml crude extract**

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**For 300 µg/ml crude extract**

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**In Stomach section at pH 4.6,**

**For 100 µg/ml CGNP**

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**For 200 µg/ml CGNP**

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**For 300 µg/ml CGNP**

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### SAS results

The ANOVA Procedure

#### For 100 µg/ml crude extract

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#### For 200 µg/ml crude extract

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#### For 300 µg/ml crude extract

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In Stomach section at pH 3.8,

#### For 100 µg/ml CGNP

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#### For 200 µg/ml CGNP

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#### For 300 µg/ml CGNP

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### SAS results

The ANOVA Procedure

For 100 µg/ml crude extract

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For 200 µg/ml crude extract

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For 300 µg/ml crude extract

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In Stomach section at pH 2.8,

For 100 µg/ml CGNP

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For 300 µg/ml CGNP

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### SAS results
The ANOVA Procedure

#### For 100 µg/ml crude extract

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#### For 200 µg/ml crude extract

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#### In Stomach section at pH 2.3,

#### For 100 µg/ml CGNP

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#### For 200 µg/ml CGNP

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#### For 300 µg/ml CGNP

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### SAS results

The ANOVA Procedure

**For 100 µg/ml crude extract**

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**For 200 µg/ml crude extract**

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**For 300 µg/ml crude extract**

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In Stomach section at pH 2.0,

**For 100 µg/ml CGNP**

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**For 200 µg/ml CGNP**

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**For 300 µg/ml CGNP**

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SAS results
The ANOVA Procedure

For 100 µg/ml crude extract

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For 200 µg/ml crude extract

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For 300 µg/ml crude extract

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In Duodenum section at pH 5.0,

For 100 µg/ml CGNP

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For 200 µg/ml CGNP

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For 300 µg/ml CGNP

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### SAS results

**The ANOVA Procedure**

#### For 100 µg/ml crude extract

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#### For 200 µg/ml crude extract

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#### For 300 µg/ml crude extract

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**In Ileum section at pH 6.5,**

#### For 100 µg/ml CGNP

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#### For 200 µg/ml CGNP

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#### For 300 µg/ml CGNP

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### SAS results
#### The ANOVA Procedure

**For 100 µg/ml crude extract**

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<td>0.07904762</td>
<td>0.01317460</td>
<td>1.50</td>
<td>0.2085</td>
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<tr>
<td>rep</td>
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<td>0.00095238</td>
<td>0.00095238</td>
<td>0.11</td>
<td>0.7441</td>
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**For 200 µg/ml crude extract**

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<th>Pr &gt; F</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.11476190</td>
<td>0.01912698</td>
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<td>0.0719</td>
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<tr>
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<td>0.00023810</td>
<td>0.00023810</td>
<td>0.03</td>
<td>0.8708</td>
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**For 300 µg/ml crude extract**

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<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.21309910</td>
<td>0.03551652</td>
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<td>0.0339</td>
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<td>0.01576577</td>
<td>1.19</td>
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### Antioxidant activity

SAS results according to steps of simulated gastrointestinal system

**In Mouth section at pH 6.9,**

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<tr>
<td>Treatment</td>
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<td>158.3654476</td>
<td>26.3942413</td>
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<td>rep</td>
<td>2</td>
<td>4.6600286</td>
<td>2.3300143</td>
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**In Stomach section at pH 5.5,**

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>308.6151810</td>
<td>51.4358635</td>
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<td>rep</td>
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<td>20.9602571</td>
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### SAS results

The ANOVA Procedure

#### In Stomach section at pH 4.6,

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>596.2247810</td>
<td>99.3707968</td>
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<td>0.0003</td>
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<td>rep</td>
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<td>64.8910381</td>
<td>32.4455190</td>
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#### In Stomach section at pH 3.8,

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>920.5033143</td>
<td>153.4172190</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>30.5597238</td>
<td>15.2798619</td>
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#### In Stomach section at pH 2.8,

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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>576.7804571</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>6.1325810</td>
<td>3.0662905</td>
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<td>0.1913</td>
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#### In Stomach section at pH 2.3,

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<th>Pr &gt; F</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>474.8435619</td>
<td>79.1405937</td>
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<td>0.0012</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>11.0318000</td>
<td>5.5159000</td>
<td>0.57</td>
<td>0.5825</td>
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#### In Stomach section at pH 2.0,

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>198.3905810</td>
<td>33.0650968</td>
<td>0.46</td>
<td>0.8275</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>74.5202952</td>
<td>37.2601476</td>
<td>0.51</td>
<td>0.6107</td>
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#### In Duodenum section at pH 5.0,

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>106.0385238</td>
<td>17.6730873</td>
<td>3.53</td>
<td>0.0300</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>8.6907714</td>
<td>4.3453857</td>
<td>0.87</td>
<td>0.4447</td>
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**Note:** The table values are rounded for simplicity. Actual results may vary due to rounding or computational precision.
SAS results
The ANOVA Procedure

In Ileum section at pH 6.5,

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<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>63.09284762</td>
<td>10.51547460</td>
<td>1.07</td>
<td>0.4307</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>3.20420000</td>
<td>1.60210000</td>
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<td>0.8513</td>
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SAS results according to Types of CGNPs and crude extract

For one-step gelatin nanoparticle at the ratio of 1:2

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<tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>466.5526667</td>
<td>58.319083</td>
<td>6.80</td>
<td>0.0006</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>21.7790889</td>
<td>10.8895444</td>
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<td>0.3077</td>
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For one-step gelatin nanoparticle at the ratio of 1:3

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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>299.5854000</td>
<td>37.4481750</td>
<td>1.82</td>
<td>0.1472</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>98.2500222</td>
<td>49.1250111</td>
<td>2.38</td>
<td>0.1241</td>
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For one-step gelatin nanoparticle at the ratio of 1:4

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<th>F Value</th>
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</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>130.7214074</td>
<td>16.3401759</td>
<td>2.63</td>
<td>0.0472</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>0.2283185</td>
<td>0.1141593</td>
<td>0.02</td>
<td>0.9818</td>
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For two-step gelatin nanoparticle at the ratio of 1:2

<table>
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<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>287.2421333</td>
<td>35.9052667</td>
<td>6.30</td>
<td>0.0009</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>20.9156222</td>
<td>10.4578111</td>
<td>1.84</td>
<td>0.1916</td>
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</table>

For two-step gelatin nanoparticle at the ratio of 1:3

<table>
<thead>
<tr>
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<th>Mean Square</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>239.7494963</td>
<td>29.9686870</td>
<td>1.33</td>
<td>0.2978</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>16.7869852</td>
<td>8.3934926</td>
<td>0.37</td>
<td>0.6949</td>
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</table>
SAS results
The ANOVA Procedure

For two-step gelatin nanoparticle at the ratio of 1:4

<table>
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<th>Mean Square</th>
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<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>207.0846667</td>
<td>25.885583</td>
<td>4.18</td>
<td>0.0072</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>40.5920667</td>
<td>20.296033</td>
<td>3.28</td>
<td>0.0640</td>
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For crude extract

<table>
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<tbody>
<tr>
<td>Treatment</td>
<td>8</td>
<td>770.4921852</td>
<td>96.311523</td>
<td>4.95</td>
<td>0.0032</td>
</tr>
<tr>
<td>rep</td>
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<td>240.9968074</td>
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<td>0.0102</td>
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Antibacterial activity

SAS results according to individual pathogenic bacteria strain

*Escherichia coli* ATCC25822

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<tbody>
<tr>
<td>Treatment</td>
<td>22</td>
<td>1.94248116</td>
<td>0.08829460</td>
<td>6.09</td>
<td>&lt;.0001</td>
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<tr>
<td>rep</td>
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<td>0.08751594</td>
<td>0.04375797</td>
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<td>0.0591</td>
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*Bacillus cereus*

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<th>F Value</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>22</td>
<td>1.35123188</td>
<td>0.06141963</td>
<td>12.09</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>rep</td>
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<td>0.03464638</td>
<td>0.01732319</td>
<td>3.41</td>
<td>0.0420</td>
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*Bacillus subtilis*

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<th>F Value</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>22</td>
<td>6.66691594</td>
<td>0.30304163</td>
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<td>0.02226377</td>
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**SAS results**

*The ANOVA Procedure*

For one-step gelatin nanoparticle at the ratio of 1:4,

**At 100 µg/ml**

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<tr>
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<th>DF</th>
<th>Anova SS</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.08509524</td>
<td>0.01418254</td>
<td>1.22</td>
<td>0.3603</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>0.20309524</td>
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<td>0.0045</td>
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</table>

**At 200 µg/ml**

<table>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>2.45412381</td>
<td>0.40902063</td>
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<td>rep</td>
<td>2</td>
<td>0.55055238</td>
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<td>0.0820</td>
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**At 300 µg/ml**

<table>
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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.14776190</td>
<td>0.02462698</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>0.22446667</td>
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For two-step gelatin nanoparticle at the ratio of 1:2,

**At 100 µg/ml**

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<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>1.98118095</td>
<td>0.33019683</td>
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<td>0.1261</td>
</tr>
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<td>rep</td>
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<td>0.19785714</td>
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<td>0.5466</td>
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**At 200 µg/ml**

<table>
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<th>F Value</th>
<th>Pr &gt; F</th>
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<tbody>
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<td>Treatment</td>
<td>6</td>
<td>1.18798095</td>
<td>0.19799683</td>
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<tr>
<td>rep</td>
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<td>0.48003810</td>
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<td>4.24</td>
<td>0.0404</td>
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### SAS results
The ANOVA Procedure

At 300 µg/ml

<table>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>2.05749524</td>
<td>0.34291587</td>
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<td>0.3519</td>
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<td>0.25586190</td>
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<td>0.4225</td>
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For two-step gelatin nanoparticle at the ratio of 1:3,

At 100 µg/ml

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<th>Pr &gt; F</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
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<td>0.84379048</td>
<td>0.14063175</td>
<td>1.93</td>
<td>0.1561</td>
</tr>
<tr>
<td>rep</td>
<td>2</td>
<td>0.46886667</td>
<td>0.23443333</td>
<td>3.22</td>
<td>0.0759</td>
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At 200 µg/ml

<table>
<thead>
<tr>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>1.13116190</td>
<td>0.18852698</td>
<td>1.39</td>
<td>0.2935</td>
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<td>rep</td>
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<td>0.29608571</td>
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<td>0.3659</td>
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</table>

At 300 µg/ml

<table>
<thead>
<tr>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>1.04499048</td>
<td>0.17416508</td>
<td>1.84</td>
<td>0.1737</td>
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<tr>
<td>rep</td>
<td>2</td>
<td>0.78740000</td>
<td>0.39370000</td>
<td>4.16</td>
<td>0.0425</td>
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</table>

For two-step gelatin nanoparticle at the ratio of 1:4,

At 100 µg/ml

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Anova SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.01793333</td>
<td>0.00298889</td>
<td>1.00</td>
<td>0.4682</td>
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<tr>
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<td>15.87</td>
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## Antioxidant activity

### DPPH

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<th>Pr &gt; F</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
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<td>42.47786667</td>
<td>7.07964444</td>
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<td>&lt;.0001</td>
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<td>2.01</td>
<td>0.1770</td>
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### FRAP

<table>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>0.82678095</td>
<td>0.13779683</td>
<td>5.36</td>
<td>0.0067</td>
</tr>
<tr>
<td>rep</td>
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<td>0.06378095</td>
<td>0.03189048</td>
<td>1.24</td>
<td>0.3240</td>
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