# Folate conjugated chitosan nanoparticles containing curcumin for functional food applications

SYMARINY OUCH

ID: 5828009

A special project submitted to the School of Biotechnology, Assumption University in part of fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology

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| **Title** | **:** | **Folate conjugated chitosan nanoparticles containing curcumin for functional food applications** |
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| **Advisor** | **:** | **Dr. Waralee Watcharin** |
| **Level of Study** | **:** | **Bachelor of Science** |
| **Department** | **:** | **Food Technology** |
| **Faculty** | **:** | **Biotechnology** |
| **Academic year** | **:** | **2019** |



.....Waralee W.....

**Dr. Waralee Watcharin**
**(Advisor)**

## Acknowledgement

This project could not be successfully completed without the guidance and support from the people who I sincerely feel grateful towards. Above all, I would like to show my gratitude and thank my senior project advisor Dr. Waralee Watcharin for her guidance, her enormous help and for sharing her worthy experiences from the beginning until the end of this accomplishment.

I would also like to acknowledge Dr. Tatsawan Tipvarakankool whose guidance and assistance in the Freeze Drying contributed immensely to the success on this project. I also would like to thank Assist. Prof. Dr. Veerawat Teeranachaideekul, Mahidol University for particle size measurement and valuable suggestions.

I would like to thank the laboratory staff whose have helped me and co-operated with me during my project work and show my appreciation to Phathit Reungwech, my best friend and all of my friends who directly and indirectly helped me to complete this project.

At last but not least gratitude goes to my family members especially, my father, Mr. Syphalla Ouch, for encouragement, love, and great support throughout my four years of study Bachelor Degree of Science.

Symariny Ouch

## Abstract

Turmeric (*Curcuma longa*) is known for potent medicinal properties because of its high proportion of curcumin composition. Curcumin is a hydrophobic polyphenol that has advantages in a variety of pharmacological activities such as antioxidant, anti-inflammatory, antitumor, antimicrobial, and anticancer. Due to its low bioavailability, nanoencapsulation of curcumin into chitosan nanoparticles is used as an enhancement of the bioactive compound curcumin. The chitosan nanoparticles were prepared with the ratio of chitosan (CS): tripolyphosphate (TPP) as 1:1 and 1:2, optimum pH at pH 3 and pH 5, and chitosan concentrations of 0.5% and 1% w/v. Encapsulation of curcumin using chitosan nanoparticles were synthesized by an adapted ionic gelation method using 3 different optimization methods: A. Adding curcumin after nanoparticle formation, B. Adding curcumin before nanoparticle formation by mixing curcumin with TPP, and C. Adding curcumin before nanoparticle formation by mixing curcumin with CS. The chitosan nanoparticles were then undergo folate-conjugation before curcumin encapsulation for its further bioavailability and targeted cells. Folate-conjugated chitosan nanoparticles (FCS-NPs) were synthesized by carbodiimide chemistry method and subsequently encapsulated with curcumin to yield Folate conjugated chitosan nanoparticles containing curcumin (C-FCS-NPs). The encapsulation and loading efficiency, and particle content were used to examine the efficiency of curcumin encapsulated-chitosan nanoparticles (C-CS-NPs). The particle size and polydispersity index (PDI) were measured by Zetasizer 3000. The antioxidant activity and phenolic content were evaluated by DPPH scavenging assay and total phenolic content assay. The chelating ability was studied on ferrous ions.

The result showed that the ratio 1:1 of CS:TPP in whether 0.5% or 1% chitosan at pH 3 had promoted the highest nanoparticle formation. The optimization method of C-CS-NPs as A, B and C showed no significant difference in percent encapsulation efficiency at $94.08\pm0.15_a$, $94.38\pm0.41_a$, and $94.55\pm0.39_a$, respectively ($p<0.05$). Whereas C-CS-NPs using 0.5%CS and C-CS-NPs using 1%CS were found to have no significant different effect on the percent encapsulation efficiency at $89.04\pm0.20_a$ and $89.28\pm0.66_a$, respectively ($p<0.05$). Comparing C-FCS-NPs and C-CS-NPs, both percent encapsulation and loading efficiency of C-FCS-NPs were found to be significantly higher than that of C-CS-NPs at $97.29\pm0.69$ and $5.98\pm0.04$, respectively. For particle content determination, C-CS-NPs (using CS 0.5%) and C-CS-NPs (using CS 1%) were found to be the highest particle content compare to other samples.

Nevertheless, C-FCS-NPs was found to have the least particle content. Where particle size was determined to 369.8nm with PDI at 0.054 and 482.4nm with PDI at 0.208 for CS-NPs and C-FCS-NPs, respectively. In addition, comparing between C-FCS-NPs and the other two formulated nanoparticles, percent radical scavenging of C-FCS-NPs was equal to the double of the sum of CS-NPs and C-CS-NPs, at 29.98±1.569. Also, in ranking from highest to lowest value of total phenolic content in sample formulations was C-FCS-NPs > C-CS-NPs > CS-NPs. For chelating ability, chitosan had a major contribution of chelating iron ions; all three samples were found to have high percentage of chelating ability though C-FCS-NPs showed the highest chelating ability at 98.95±0.55%. These results indicated that encapsulation of curcumin using folate conjugated-chitosan nanoparticles could provide advantages in the full usage of functional food applications.



Keywords: Curcumin, Folate, Chitosan nanoparticles, Functional food applications

# Content

## List of tables

## List of figures

## Introduction

In both medicinal and biopharmaceutical science, turmeric (*Curcuma longa*) is a spice that has been received much interest for its potent medicinal properties as the source of curcumin (Hewling & Kalman, 2017). Curcumin is a hydrophobic polyphenol that has advantages in a variety of pharmacological activities such as antioxidant, anti-inflammatory, antitumor, antimicrobial, and anticancer (Nugroho and Nafisah, 2017). In the function of antioxidant, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is a scavenger of oxygen species including hydroxyl radicals, superoxide anions and singlet oxygen and interferes with lipid peroxidation (Yadav *et al.*, 2012).

In spite of these healthful properties, curcumin has a low bioavailability in which its absorption in the GI tract is a significant factor. To enhance the curcumin's therapeutic potential, several encapsulation methods were studied (Hewling & Kalman, 2017). Nanoencapsulation of curcumin could be a strategy to increase its bioavailability and reduce the dose required for a desired effect in therapeutic use (Yadav *et al.*, 2012).

In nanoencapsulation of curcumin, curcumin will be encapsulated into chitosan nanoparticles. According to potential of chitosan to interact with the negatively charge of the mucosal cells in the gastrointestinal tract. Chitosan helps to extend the residence time while deliver curcumin into human body (Tiyaboonchai *et al.*, 2003). Chitosan is a second most abundant polysaccharide on earth, produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans and cell walls of fungi (Rinaudo, 2016). From further study on the usage of chitosan nanoparticles, it is best for controlled drug release and found to have minimal drug side effects (Elgadir et al., 2015). The bioactive compound curcumin can be tested or evaluated using the spectroscopic method; nanoparticles were determined by dynamic light scattering through a dilution (Souza *et al.*, 2013).

On the other hand, folate is one of the naturally occurring form of vitamin B9 which found in foods including leafy vegetables, beans, and eggs. Folate can be synthesized, as well as normally found in supplements and fortified foods, and it is known as "Folic acid". Folic acid has numerous health benefits. Namely, it helps regulate the amount of sulfur-containing amino acid in the body, a great vitamin for brain health and preventing Alzheimer's disease, reduced risk of anemia and high blood pressure (Chen *et al.*, 2016). Most importantly, it is known to be very significant for pregnant women which helps developing new cells for the baby and reducing the risk of birth defects, specifically Neural Tube Defects (NTDs).

For folic acid's incorporating application, it is a functional drug vehicle of curcumin compound in order to address the issue of poor water solubility and improve its targeted accumulation at tumor site. One of the techniques used is folic acid-conjugate micelle in which thin film hydration method is adopted (Hao *et al.*, 2017). However, using folic acid-conjugate as a ligand incorporated with chitosan could retain its original physiochemical properties of chitosan and target the folate receptor to up-regulate on the surface of many cancer cells (Li *et al.*, 2011). The nanotechnology of encapsulating curcumin into chitosan with the further of folic acid-conjugate would enhanced the bioavailability and retention time of curcumin in human body. It would also be used as a functional food to improve health benefits.

Therefore, this research is aim to maximize the efficiency of curcumin by developing a certain morphology, size, and encapsulation capability as well as to evaluate the bioactivities (i.e. antimicrobial and antioxidant) of the encapsulated curcumin.

## Objectives

1. To prepare curcumin-encapsulated chitosan nanoparticles and optimize the encapsulation method of curcumin into chitosan nanoparticles.
2. To prepare folate-conjugate curcumin-encapsulated chitosan nanoparticles.
3. To evaluate the characteristics and encapsulation capability of the prepared chitosan nanoparticles.
4. To evaluate chelating ability, antioxidant properties, and antimicrobial activities of the prepared chitosan nanoparticles.

## Literature review

There are many sources that focus on the functionality of encapsulated curcumin via chitosan nanoparticles, however, different sources have their own application, for example, not only the stable encapsulated curcumin could be delivered and cured colon cells and breast cancer cells in humans, but it can be also used for treatment of arsenic toxicity. In this review, many ideas, information, methods, and techniques have been generated to produce a stable and efficient encapsulated curcumin, by discussing all advantages and disadvantages of all sources that are useful for this research.

### I. Chitosan and its application

#### 1.1 Chitosan

In medical field, chitosan is an interesting compound which has been used broadly with the production of encapsulation or a drug carrier. It was produced by deacetylated of chitin_ existed in fungal cell walls and crustacean shells. Chitosan can be obtained *via* two ways; one is obtained from deacetylated chitin using sodium hydroxide and the other one is obtained from deacetylated chitin using enzymes under favorable conditions. Though, deacetylated chitin under alkaline condition is a better choice of converting chitin into chitosan (Elgadir A. et al, 2014).

Chitosan is biodegradable polymer that functions as an adhesive, antibacterial and antifungal agent. One source stated that, "the elemental composition of the chitosan polymer is carbon (44.11%), hydrogen (6.84%) and nitrogen (7.97 %) and the viscosity of average molecular weight of chitosan is ~5.3x10$_5$ Daltons" (Soutter W., 2013). The degree of deacetylation has an impact on chitosan molecular weight which then affect many properties of its compound in carrying other raw materials into biological cells. Those properties included solubility, viscosity (Rege PR. et al., 1999), reactivity of proteinaceous material coagulation, and heavy metal ion chelation (Gamage A. et al., 2007), and physical properties of films formulated using chitosan such as tensile strength, elasticity, elongation, and moisture absorption (Elgadir A. et al, 2014).

Chitosan has a unique functional property after a slight modification. As it is soluble in an aqueous acidic solution; the amino group could possibly protonate and giving itself a positive charge. Within this property, cationic chitosan compound can interact with other anionic molecules through electrostatic interaction. Usually, chitosan nanoparticles would

formed by interacting with a polyanion such tripolyphosphate (TPP) into chitosan solution (Elgadir A. et al, 2014).

### 1.2 Antibacterial Property of Chitosan

Chitosan nanoparticles and copper-loaded nanoparticles exhibited antibacterial property against many bacteria including *E. coli*, *S. choleraesuis*, *S. typhimurium*, and *S. aureus*. The result showed disruption of cell membranes and the leakage of cytoplasm of *S. choleraesuis* when it exposed to chitosan nanoparticle *(Qi L. at el, 2004).* This could clearly show the advantage of using chitosan nanoparticle in this research. Not only the curcumin that existed in turmeric plant has antimicrobial property, but also the carrier of the main compound has antibacterial as well.

### 1.3 Applications of Chitosan Nanoparticles

Chitosan nanoparticles has been used in variety of purpose as it has been described from the first start. One research demonstrated chitosan nanoparticles in loading insulin for the enhancement of nasal absorption (Rocio F, 1999). It has antibacterial agent, and it is the best carriers of other foreign materials that beneficial to biological cells. Chitosan nanoparticles has also been used as adjuvant for vaccines such hepatitis B. Furthermore, it has been proved to prevent infection in wounds and faster the wound-healing process by promoting the growth of skin cells (Soutter W., 2013).

## II. Curcumin

### 2.1 Turmeric compositions

In turmeric, its main composition is curcuminoid compounds, turmeric oil, oleoresin, and other components. The main interesting components are turmeric oil and curcuminoid, which contains antimicrobial, anti-inflammatory, antioxidant and insect repellent properties. These properties allow turmeric oil to be used in food, cosmetic, and pharmaceutical applications (G. Asghari at el., 2009). Significantly in vitro, turmeric oil could inhibit the growth of pathogenic molds and dermatophytes though it is not irritating skin, also this essential oil showed antimicrobial property against *B. subtilis* plus *S. aureus* (P. Lertsutthiwong, 2011).

### 2.2 Curcumin Properties and Advantages

Curcumin is a polyphenolic compound, mostly a ketone group where it is soluble in ethanol as well as acetone but not soluble in water. Curcumin can be extracted from the dried rhizome of turmeric. Curcumin can target various biochemical pathways; it is a promise for the treatment of many diseases including colorectal cancer, breast cancer, biliary disorders, anorexia, inflammation, hepatic disorders, wound healing, sinusitis and rheumatism (A. Shehzad at el., 2010). From its chemical structure, it possesses the antioxidant, anti-inflammatory, anti-platelet, cholesterol lowering, antibacterial and antifungal effects (Ikpeama at el., 2014).

## III. Folate and its bioavailability activity

Folate (Vitamin B9) is an essential vitamin which body needs; it is widely known as an important vitamin for pregnant women in order to produce red blood cells and synthesizing certain amino acids. Also, it is recognized as health-beneficial in the prevention of neural tube defects, anemia, cardiovascular disease, poor cognitive performance, and some forms of cancers. Due to its beneficial properties, folate was used to study its bioavailability and research shown for human and pig that folates synthesized by colon bacteria is bioavailable. Passive diffusion of folate across the cell membrane is limited and happens only at high doses. Minor extent folate is also absorbed in the colon; and it is suggested that colonic absorption may contribute significantly to total folate absorption (Veronica O. and Cornelia W., 2018).

## IV. Nanotechnology

### 4.1 Nanotechnology and Chitosan-TPP preparation

Nanotechnology refers to the use of nano-size materials by which its tiny dimension could perform as a vehicle for drug delivery in food and biomedical applications. Due to its effective transport property, many plant extracts were applied to nanotechnology for the best enhancement and the release of plant's active compounds, improve activity, reduce required dose, and decrease side effects (K.J Gohil J.A. Patel and A.K. Gajjar et al., 2010). Nanotechnology is used here because turmeric oil is unstable, volatile and insoluble in water, and this has restricted its use as a therapeutic agent. The problems with curcumin bioavailability seemingly have not lowered researchers' enthusiasm to pursue its anticancer property. The bioavailability studies of curcumin in colon cancer showed its poor absorption and quick elimination from the body (A. Shehzad at el., 2010). However, chitosan nanoparticles are prepared as a carrier or delivering agent for this essential oil to reach certain

place of biological cells. The curcumin's melting point, ($C_2H_{52}OO_6$) is 184.2$_o$C, and thus temperature is one of the factors impacting the preparation of chitosan nanoparticles (Ikpeama at el., 2014). Cationic chitosan can perform a stable interaction with anionic materials such gene, drug, protein and small molecule via electrostatic interaction. Significantly, cation chitosan would be best to allow to them to interact with polyanion such tripolyphosphate (TPP); this contributes to nanoparticle's function in delivering interested compound (Soutter W., 2013).

**4.2 Curcumin Chitosan-Encapsulated Methods**

Many methods have been proposed for encapsulation of turmeric essential oils, including melt emulsification, emulsification-diffusion, double emulsification, polymer-coating, layer-by-layer, coacervation, and ionotropic gelation (P. Lertsutthiwong at el., 2011). The interaction of cationic chitosan and polyanionic TPP is called electrostatic interaction and the method creates this corporation is called ionic gelation. The main advantage of ionic gelation is due to the structure of chitosan and curcumin themselves. It was originally taken from the idea of phospholipid bilayer such liposome_ a spherical vesicle that have at least one lipid bilayer (Narayanan at el., 2009).

The encapsulation of turmeric oil can be prepared into nano-capsules by three single steps including oil in water emulsification, gelification and solvent removal. O/W emulsification was made from dispersing ethanolic solution of turmeric oil into alginate solution contain Tween 80 as an emulsifier and followed up by sonication. The mixture was equilibrated overnight and removal of solvent under reduced pressure (P. Lertsutthiwong at el., 2011).

**4.3 Folate-conjugate chitosan**

Recent studies showed the significant of incorporation of targeted ligands with chitosan not only to keep the original physiochemical properties of chitosan but also to targeted tumor cells (Li et al., 2011). With variety of ligands, folate is an interesting compound for targeting cell membrane and promoting the nanoparticles endocytosis to deliver the attached therapeutic agents into the tumor sites. With folate receptor, it is even more important to do folate-conjugate with its high affinity property of folate to bind to its receptor at the site. Research studies synthesized folate-conjugate chitosan based on the mechanism of carbodiimide chemistry (Dube, Francis, Leroux, & Winnik, 2002).

Carbodiimide chemistry is a functional group with a formula RN=C=NR, exclusively synthetic and used in peptide synthesis. Plus, the structure of carbodiimide is very stable.

Carbodiimide compounds that are most readily available and commonly used are water-soluble 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for its aqueous crosslinking method. EDC reacts with carboxylic acid groups to form an active o-acylisourea intermediate which it is easily replaced by nucleophilic attack from primary amino groups in the mixture. Moreover, EDC crosslinking is at best in acidic condition (pH 4.5) and need to be performed in buffers devoid of extraneous carboxyls and amines (Thermofisher.com, 2019). Carbodiimide chemistry is used widely in nanotechnology such catechin-loaded folate conjugated chitosan nanoparticles that promised to target the tumor cell and release the attach drug at the right sites (Liu et al., 2019).

## Materials, Equipment, and Methods

### 1. Materials

Chitosan; MW = 178.19 kDa, from shrimp shells, practical grade (CS), sodium tripolyphosphate (TPP), DPPH, and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Where 1-ethyl-3-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and dimethyl sulfoxide (DMS) were obtained from Thermo Fisher Scientific-UK. Curcumin extract was obtained from Government Pharmaceutical Organization (GPO). Folate, ethanol, and acetic acid were AR grade and used as received. Curcumin was extracted by soaking turmeric powder in ethanol solution. Whereas deionized water was obtained from double-distilled water.

### 2. Equipment

Freeze dryer (Model: Epsilon FD-8; LAB freeze dryer), hot air oven (Model: UM 500-MTEC), incubator (37$_o$C) (Model: BTS.1/94-Jouan), ultra-low temperature freezer (Model: U410-New Brunswick), pH meter (Model: SED-12500V-Speedmark), spectrophotometer (Model: 1200-UNICO).

### 3. Methods

#### 3.1 Preparation of chitosan nanoparticles (CS-NPs)

Ionic gelation technique was used to assemble the chitosan (CS) and tripolyphosphate (TPP) with the help of acetic acid in dissolving chitosan (Rajesh S. Nair *et. al*, 2018). In order to optimize nanoparticle preparation procedure, preliminary experiments were done. The preliminary experiments were varied on concentration of chitosan solution (0.5% and 1% m/v), ratio between chitosan and TPP (1:1 and 1:2) and pH of chitosan solution (pH 3 and pH 5). Chitosan (0.5% and 1% m/v) solution was prepared in dilute acetic acid (0.5% v/v), and the pH was kept as 3.0 and adjusted to 5.0 using aqueous NaOH. The 10ml solution was stirred (500–1000 rpm on a magnetic stirrer, and volumes of the cross-linker (TPP, 0.5% w/v) solution were varied by adding TPP dropwise using syringe with needle to this mixture to achieve volume ratios of chitosan and TPP of between 1:1 and 1:2. The suspension obtained was further stirred for 60 min at room temperature and then allowed it to stand for 5-10 minutes before collecting the cloudiness part for purification process. The collected part was purified using snake skin membrane (MWCO 10,000 Da) in 1L distilled water stirring overnight. The chitosan nanoparticles were then collected into Eppendorf tube (1ml each) and was

subsequently added distilled water with the cryoprotectant (Mannitol, 0.1% v/v), lyophilized at -50°C (store at -80°C overnight), and kept at 2–8°C after lyophilization for further characterization

Lyophilized conditions were done as follow:

- Pre-freezing (Thermal treatment) at atmospheric pressure at -80°C overnight; then isothermal at -30°C, for 3 hours soaking
- Primary drying (Sublimation) under vacuum at 5°C; 0.194°C/min (RAMP 3 hours), for 5 hours soaking
- Primary drying (Sublimation) under vacuum at 15°C; 0.055°C/min (RAMP 3 hours), for 5 hours soaking
- Secondary drying (Desorption) under vacuum at 35°C; 0.111°C/min (RAMP 3 hours), until sample was taken out.

### 3.2 Encapsulation of curcumin into chitosan nanoparticles

#### Preparation of curcumin solutions

Curcumin solutions were prepared by dissolving curcumin in 10% ethanol and 50% ethanol at curcumin concentration of 0.5mg/ml. For this study, the absorbance of curcumin solutions was measured at 430nm and compared using calibration curve of standard curcumin concentrations of 0.1-0.5mg/ml.

#### Optimization of encapsulation method

In order to optimize the best encapsulation efficiency, there were three treatments that have been done to obtain the best encapsulation efficiency. The three treatment were done as follows: A-adding curcumin after nanoparticle formation, B-adding curcumin before nanoparticle formation by mixing curcumin with TPP, and C-adding curcumin before nanoparticle formation by mixing curcumin with CS. Treatment A was prepared by adding 10 ml of 0.5% (m/v) TPP solution dropwise using syringe with needle into 10 ml of 0.5% (m/v) chitosan solution on a magnetic stirrer (500-1000 rpm). After that, 1ml of (0.5 mg/ml) curcumin extract solution was added dropwise. Treatment B was prepared by mixing 10ml of 0.5% (m/v) TPP with 1ml of (0.5mg/ml) curcumin extract solution and then added this solution dropwise using syringe with needle into 10ml of 0.5% (m/v) chitosan solution that was placed in a 20ml vial bottle, on a magnetic stirrer (500–1000 rpm). Treatment C was prepared by mixing 10ml of 0.5% (m/v) chitosan solution with 1ml of (0.5mg/ml) curcumin extract solution on a magnetic stirrer (500–1000 rpm), and then, varied volumes of the cross-linker (TPP, 0.5%

w/v) solution by added dropwise using syringe with needle to this mixture to achieve volume ratios of 1:1 between chitosan and TPP (Duse L. et. al, 2017).

The suspension obtained from treatment A, B, and C were further stirred for 60 min at room temperature and then allowed to stand for 5-10 minutes before collecting the cloudiness part for purification process. The collected part was purified using SnakeSkin® Dialysis Tubing (10,000 MWCO) in 1L distilled water stirring overnight. The curcumin chitosan nanoparticles were then collected into Eppendorf tube (1ml each) and was subsequently added 100µl distilled water with the cryoprotectant (Mannitol, 0.1% v/v), lyophilized at -50oC (store at -80oC overnight), and kept at 2–8°C after lyophilization for further characterization.

**Encapsulation of curcumin into chitosan nanoparticles (C-CS-NPs) using 0.5% and 1% CS**

From the previous study, treatment C (adding curcumin before nanoparticle formation by mixing curcumin with CS) was chosen as an adopted method for the encapsulation of curcumin into chitosan. Chitosan (0.5% and 1% m/v) solution was prepared in diluted acetic acid (0.5% v/v) and curcumin extract solution (0.5mg/ml) was prepared by dissolving curcumin extract in 50% (v/v) ethanol. However, 1:10 volume ratio of curcumin extract to chitosan solution had been used to do the encapsulation (Lokesh P. et al, 2015). The solution was prepared by mixing 10ml of chitosan solution (0.5% and 1% m/v) with 1ml of curcumin extract solution (0.5mg/ml) on a magnetic stirrer (500–1000 rpm), and then, varied volumes of the cross-linker (TPP, 0.5% w/v) solution by added dropwise using syringe with needle to this mixture to achieve volume ratios of 1:1 between chitosan and TPP. The suspension obtained was further stirred for 60 min at room temperature and then allowed to stand for 5-10 minutes before collecting the cloudiness part for purification process. The collected part was purified using snake skin dialysis membrane in 1L distilled water stirring overnight. The curcumin-chitosan nanoparticles were then collected into Eppendorf tube (1ml each) and 100µl distilled water with the cryoprotectant (Mannitol, 0.1% v/v) was subsequently added, and lyophilized at -50oC (store at -80oC overnight), with the same conditions as used in the preparation method of chitosan nanoparticles and kept at 2–8°C for further characterization.

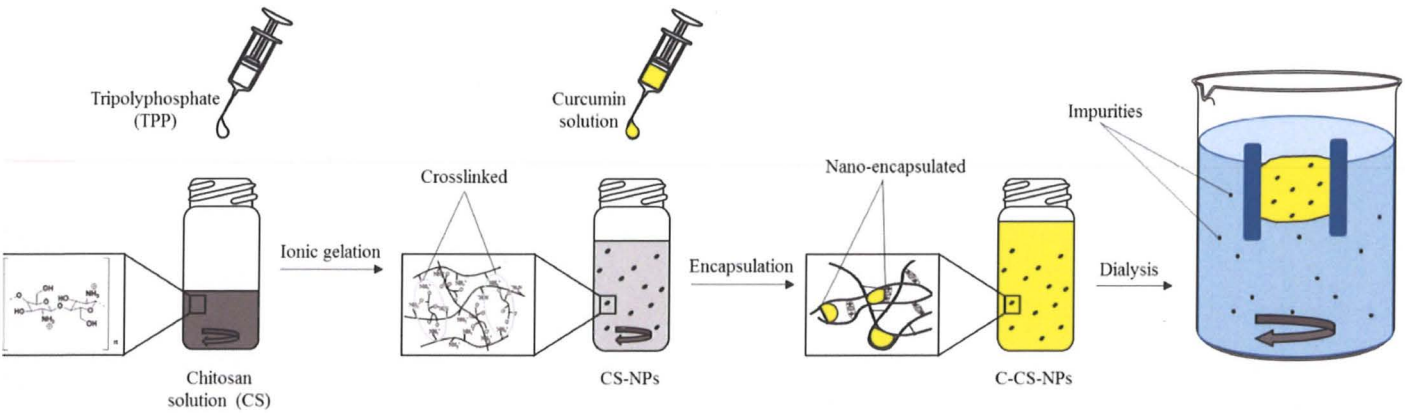



*Figure 1: Procedure of encapsulation of curcumin into chitosan nanoparticles (C-CS-NPs)*

### 3.3 Synthesis of folate-conjugated chitosan nanoparticles (FCS-NPs)

The carbodiimide chemistry was used to synthesize folate-conjugated chitosan (Dubé, Francis, Leroux, & Winnik, 2002). Chitosan was weighed 0.1g and dissolved in 20ml of acetate buffer (pH 4.8), forming 0.5% (m/v) CS solution. Folic acid and EDC were weighed 0.1g and 0.02g, respectively to achieve a mass ratio of 5:1 and mixture solution was prepared by dissolving them simultaneously in 4.5ml of anhydrous dimethyl sulfoxide until folic acid was well dissolved. This mixture solution was then added to the CS solution and stirred at room temperature for 16 h. The obtained coagulation was purified using snake skin dialysis membrane against 0.1M phosphate buffer (pH 7.4) for a day and followed by distilled water for another 2 days. Lastly, the prepared yellow colored folate-conjugated chitosan was collected to analyzed and lyophilized at -50ₒC. The lyophilized step was done by storing the sample at -80ₒC overnight and freeze drying at -50ₒC with the same conditions as used in the preparation method of chitosan nanoparticles and kept at 2–8°C for further characterization (Buyun Liu *et. al*, 2018).

### 3.4 Encapsulation of curcumin into folate-conjugated chitosan nanoparticles (C-FCS-NPs)

After conjugation of folate to chitosan nanoparticles, the encapsulation of curcumin into folate-conjugate nanoparticles (C-FCS-NPs) was performed by dissolved 60mg freeze dried FCS-NPs in 10ml of diluted acetic acid (0.5% v/v) which is equivalent to 10ml of folate-conjugate nanoparticles (FCS-NPs) after dialysis suspended in 10ml of diluted acetic acid (0.5% v/v) (Buyun Liu *et. al*, 2018). Then, Treatment C was also adopted in the research by mixing 10ml of dissolved folate-conjugate solution with 1ml of (0.5mg/ml) curcumin extract

solution on a magnetic stirrer (500–1000 rpm), and then, varied volumes of the cross-linker (TPP, 0.5% w/v) solution by added dropwise using syringe with needle to this mixture to achieve volume ratios of 1:1 between chitosan and TPP (Duse L. et. al, 2017). The suspension obtained was further stirred for 60 min at room temperature and then allowed to stand for 5-10 minutes before collecting the cloudiness part for purification process. The collected part was purified using snake skin dialysis membrane in 1L distilled water stirring overnight. The curcumin encapsulation into folate-conjugate chitosan nanoparticles were then collected into Eppendorf tube (1ml each) and was subsequently added 100µl distilled water with the cryoprotectant (Mannitol, 0.1% v/v), lyophilized at -50$_o$C (store at -80$_o$C overnight), with the same conditions as used in the preparation method of chitosan nanoparticles and kept at 2–8°C for further characterization (Rajesh S. Nair *et. al*, 2018).

### 3.5 Determination of particle content

Particle content of samples, CS-NPs (using CS 0.5%), CS-NPs (using CS 1%), C-CS-NPs (A), C-CS-NPs (B), C-CS-NPs (C), and C-FCS-NPs, were determined by weighing the 1ml freeze dried sample. Each sample was done quadruple and averaged the weight in milligram per 1ml sample.

### 3.5 Determination of particle size

Dynamic light scattering was used to determine the mean diameter of the prepared samples; CS-NPs and C-FCS-NPs. In this study, 10 µl of sample was diluted with 1ml Milli-Q water and then were characterized using Zetasizer 3000 (Malvern, UK). The samples were prepared from three independent experiments and measured in triplicate.

### 3.6 Determination of encapsulation efficiency and loading efficiency of curcumin

The encapsulation efficiency and loading efficiency of C-CS-NPs and C-FCS-NPs were determined as follows: C-CS-NPs and C-FCS-NPs suspensions were allowed to stand for 5-10 minutes. The absorbance at 430 nm of free curcumin of C-CS-NPs and C-FCS-NPs in the supernatant were measured 5 times and 10 times, respectively. The standard curve of curcumin was done as described previously (Prasad, Kavita, Chandrashekhar, & Manohar, 2018) with some modifications. Curcumin 30mg extract were accurately weighed and transfer into 30ml of (50% v/v) ethanol-containing beaker to obtain a concentration of 1mg/ml of stock solution.

From stock solution, 2, 4, 6, 8, 10 ml of solutions were withdrawn and diluted to 10ml with 50% (v/v) ethanol in order to obtain concentrations of 0.2, 0.4, 0.6, 0.8, 1 mg/ml, respectively. However, the 60oC dried-overnight and undissolved curcumin were then weighed to find out the actual concentration to be 0.5467mg/ml as the real stock solution. Hence, the normalized concentrations of curcumin in the standard curve were 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml. Then, these standard solutions of curcumin were measured using UV-VIS spectrophotometer at a wavelength of 430nm with the used of ethanol as the blank (Rajesh, Andrew, Nashiru, & Chee-Onn, 2018). The amount of free curcumin in the supernatant was calculated by comparison with the standard curve of curcumin ranging from 0.1-0.5mg/ml. The encapsulation efficiency and loading efficiency were determined according to formula as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{total amount of curcumin}} \times 100$$

$$\text{Loading efficiency (\%)} = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{Mass of NPs after freeze dried}} \times 100$$

### 3.7 Determination of Antioxidant activity

#### DPPH method of antioxidant assay

DPPH or 1,1-diphenyl-2-picryl-hydrazyl assay was prepared by adding 1ml of 0.1mM solution of DPPH in methanol and 1ml of different formulation. Freeze dried nanoencapsulation samples, CS-NPs, C-CS-NPs, and C-FCS-NPs, were used in this experiment. It was required to add 1ml of 0.5%(v/v) acetic acid into one Eppendorf tube of sample. Then, the Eppendorf tubes were vortex until the samples were dissolved, however, if there's particle remained, centrifuge at 7000rpm for 5 minutes would be required. The supernatant of sample was micropipette for 1ml and transferred into test tube with the addition of 1ml of prepared DPPH solution and shake vigorously for a few min, then, let the solution stands for 30minutes at room temperature in the dark. The antioxidant determination experiment was done in triplicate. The absorbance was measured at 517 nm using spectrophotometer by using methanol as blank and acetic acid with DPPH as control. The capability of scavenging the DPPH radical was determined by using the following formula.

$$\text{DPPH scavenging effect (inhibition)} = \left(\frac{Ao - A1}{Ao}\right) \text{x } 100$$

$A_0$ = the absorbance of the control reaction

$A_1$ = absorbance in presence of sample

### 3.8 Determination of Total Phenolic Content Assay

In total phenolic content assay, Folin-Ciocalteu reagent was reduced by phenolic compounds that existed in the prepared samples which resulted a blue complex formation. Freeze dried nanoencapsulation samples, CS-NPs, C-CS-NPs, and C-FCS-NPs, were used in this experiment. It was required to add 1ml of 0.5% (v/v) acetic acid into one Eppendorf tube of sample. Then, the Eppendorf tubes were vortex until the samples were dissolved, however, if there's particle remained, centrifuge at 7000rpm for 5 minutes would be required. In this study, 0.1ml nanoencapsulation sample was mixed homogeneously with 1.25ml of 0.1N Folin-Ciocalteu reagent solution. Immediately, 1 ml of 0.7M $Na_2CO_3$ was added to the mixture and allowed to sit for an hour at room temperature (25-30$_o$C). Then, the mixture was read at 765nm using UV-Vis spectrophotometer and the measurement was compared to a calibration curve of standard gallic acid solution. The standard curve of gallic acid was prepared according to (AOAC SMPR 2015.009) with the concentration range of 6.25ppm-100ppm. The total phenolic content was expressed as milligrams of gallic acid equivalents per ml of sample (mg GAE/ml).

### 3.9 Determination of Chelating ability

Ferrous ions binding ability of the chelators was determined by the method of Dinis *et al.*, (1994) except that ferrous sulphate was used instead of ferrous chloride. Each sample formulations including CS-NPs, C-CS-NPs, and C-FCS-NPs (10mg/ml, 1ml) in 0.5% acetic acid was mixed with 3.7ml of methanol and ferrous chloride (2mM, 0.1ml). The mixture was then further reacted with ferrozine (5mM, 0.2ml) for 10 minutes at room temperature. The absorbance of the solution was measured at 562 nm. The control was the mixture of 0.5% acetic acid and reagent solution. The higher the absorbance at 562 nm that is due to the ferrous ion-ferrozine complex, the weaker the ferrous iron binding strength of the chelator.

The chelating ability was calculated as:

$$\text{Chelating ability (\%)} = \frac{\Delta A_{562}\ of\ control - \Delta A_{562}\ of\ sample}{\Delta A_{562}\ of\ control} \times 100$$

### 3.10 Antimicrobial activity

Antimicrobial activity of nanoencapsulation of curcumin into chitosan were done using disk-diffusion agar method. This method was using two types of pathogenic bacteria such gram-positive (*Escherichia coli*) and gram-negative bacteria (*Staphylococcus aureus*). Before assay was started, materials that need to be used must be sterile by autoclave at 121$_o$C for 30

minutes. The bacteria were cultured by place one loop of bacteria into 500ml nutrient broth at room temperature (30oC). Using aseptic technique, a sterile swab was placed into nutrient broth culture that was prepared 24 hours before the assay started. Swab was gently pressed or rotated against the inside of tube to get rid of excess broth then was streak them onto nutrient agar uniformly. Using a flame-sterilized forceps, disk or filter paper was soaked with sterile water, (10mg/ml) ampicillin, and samples, then disk was placed onto agar gently. Water and ampicillin were used for negative and positive control, respectively. There were five samples used to determine their antimicrobial property, namely, chitosan nanoparticles, curcumin encapsulated chitosan nanoparticles, folate-conjugate nanoparticles, curcumin encapsulated folate-conjugate chitosan nanoparticles, and pure curcumin (0.5467mg/ml). Plates were incubated overnight at an incubation temperature of 37 °C and the diameter of inhibition zone were measured to determine the effectiveness of the samples against each bacterium (Hossein K., Rasool S., Sorrosh N., Alireza A., and Keirollah G, 2018). The experiment was done in three replications independently.

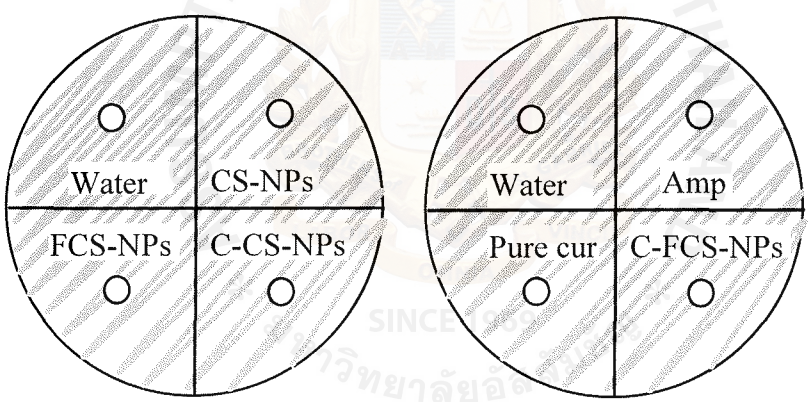



*Figure 2: Example of disk-diffusion method*

### 3.11 Experimental design and Statistics analysis

Independent t-test were used to analyze the best aspect for the best outcome of the encapsulation of curcumin into chitosan nanoparticles (including curcumin solution and chitosan concentration study); using R-Program version R 2.15.3. Whereas Randomized Complete Block Design (RCBD; ANOVA) and Duncan's Multiple Range test were used to analyze encapsulation optimization method, DPPH method of antioxidant assay, Total Phenolic Content assay, and antimicrobial activity of each sample formulations regarding each study; using R-Program version R 2.15.3.

## Result and Discussion

### 3.1 Preparation of chitosan nanoparticles (CS-NPs)

In this study, CS-NPs were prepared by ionic gelation method using chitosan concentrations of 0.5% and 1% (w/v), ratio of chitosan to tripolyphosphhate of 1:1 and 1:2, at two different pH (pH 3 and pH 5) as shown in *Table 1*. The intensity of turbidity was measured from each variation to be analyzed for the best result of CS-NPs.

*Table 1: Formulation of CS-NPs at concentrations of 0.5%CS and 1%CS, different ratio of CS to TPP at pH 3 and pH 5.*

| | **pH 3** | |
|---|---|---|
| | **Concentration of chitosan** | |
| | 0.5% CS | 1% CS |
| **Ratio of CS:TPP (ml)** | | |
| 1:1 | +++ | ++++ |
| 1:2 | +++ | ++++ |
| | **pH 5** | |
| 1:1 | + | ++++ |
| 1:2 | + | ++ |

*** Remark: (+) = intensity of turbidity

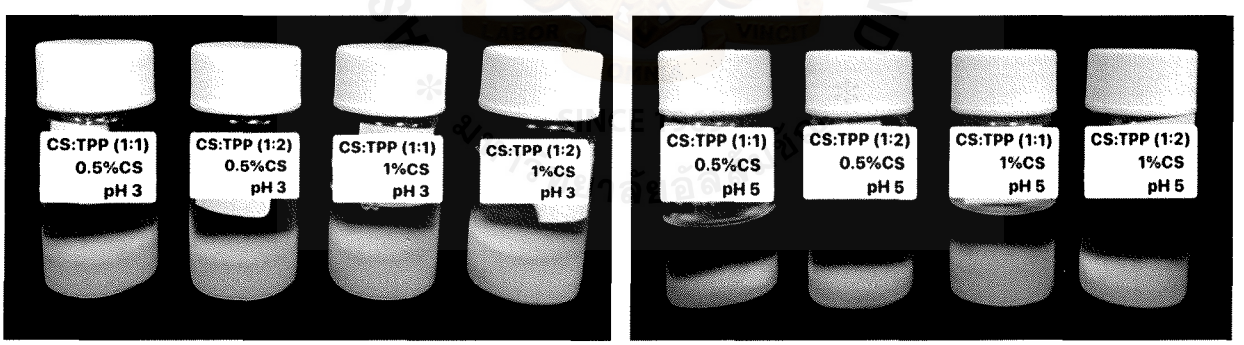



*Figure 3: Formulation of CS-NPs at concentrations of 0.5%CS and 1%CS, different ratio of CS to TPP at pH 3 and pH 5*

According to *Table 1* and *Figure 3,* at pH3, 0.5% chitosan and 1% chitosan with 1:1 and 1:2 ratio of CS:TPP generates indistinguishable result; high intensity of turbidity and cloudiness. Whereas, at pH 5, only 1:1 ratio of CS:TPP in 1% chitosan generates the highest intensity of turbidity and cloudiness. That have been said, the ratio 1:1 of CS:TPP in whether 0.5% or 1% chitosan in pH 3 had promoted the highest potential for nanoparticle formation. Theoretically, chitosan's backbone is hydrophobic in which it can self-associate to form particle and gel in high pH. However, at pH 3-4.5, TPP was added to the CS solution, there is

an occurrence of crosslinking between negative charge TPP and the positive charge CS. This interaction produced the formation of nano-sized polyelectrolyte complex under magnetic stirrer (Hakim B., 2018). Based on some research, it also had been proved that the correlation between pH of the solution and degree of protonation of chitosan can be explained by the Henderson-Hasselbalch equation; as the pH of the solution rises from 4.7 to 8.0, the degree of protonation was found to decrease from 100 to 0% (Rajesh S., 2018).

### 3.2 Encapsulation of curcumin into chitosan nanoparticles

#### Preparation of curcumin solutions

Curcumin solutions were prepared by dissolving curcumin in 2 conditions: 10% ethanol and 50% ethanol, at curcumin concentration of 0.5mg/ml. In this study, the absorbance of curcumin solutions was measured at 430nm and compared using calibration curve of standard curcumin concentrations of 0.1-0.5mg/ml. The result was further analyzed using Statistics software R-Program version R 2.15.3, by independent t-test.

*Table 2: Absorbance of curcumin solution in ethanol at 10% and 50% ethanol concentration.*

| Ethanol Concentrations (Treatments) | Abs 430nm |
|---|---|
| 50% ethanol | 3.93±0.0574* |
| 10% ethanol | 0.6562±0.0536 |

* indicates that the values within the same column are significantly different ($p<0.05$).

In *Table 2,* at optical density of 430nm, curcumin solution with 50% ethanol gives absorbance intensity of 3.93±0.0574 whereas curcumin solution with 10% ethanol gives 0.6562±0.0536. It shows that 50% ethanol dissolved more curcumin into the solution and this contributes to a higher degree of encapsulation. As more curcumin dissolved, nanoparticles could entrap more of the dissolved curcumin solution as well. Thus, the method of using 50% ethanol in preparing curcumin solution is adopted for the further study.

#### Optimization of encapsulation method

In encapsulating curcumin into chitosan nanoparticles, there were three treatments that have been done to optimize the best encapsulation efficiency as follows: A-adding curcumin

after nanoparticle formation, B-adding curcumin before nanoparticle formation by mixing curcumin with TPP, and C-adding curcumin before nanoparticle formation by mixing curcumin with CS. In this study, the absorbance of free curcumin was measured at 430nm and compared using calibration curve of standard curcumin concentrations of 0.1-0.5mg/ml. The results were further analyzed using Statistics software R-Program version R 2.15.3, by undergo RCBD experimental design and multiple comparison in Duncan.

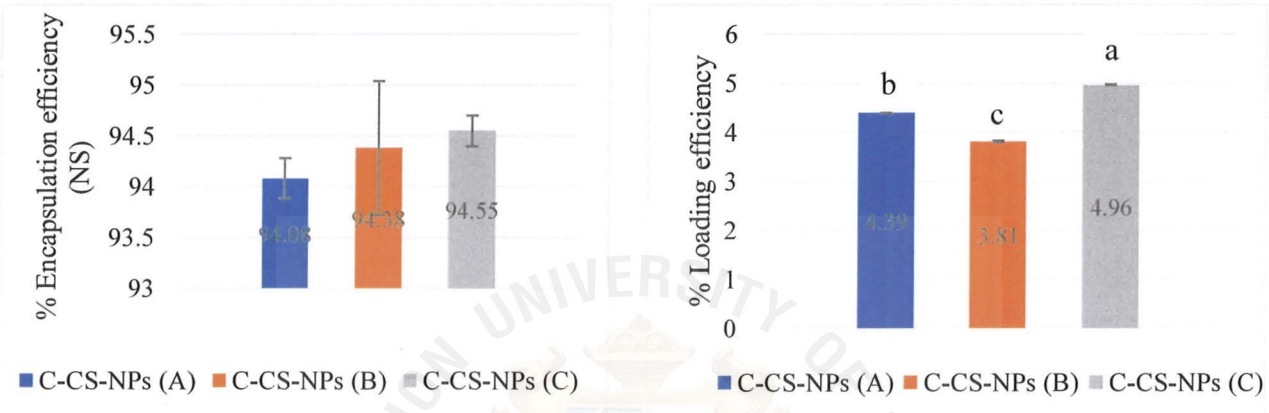



*Figure 4: Comparison of percentage of encapsulation and loading efficiency of C-CS-NPs (A), C-CS-NPs (B), and C-CS-NPs (C)*

*Table 3: Comparison of percent encapsulation and loading efficiency of curcumin in CS-NPs using A, B, and C treatments, at initial curcumin concentration of 0.5467mg/ml in 50% ethanol. Multiple comparison done by Duncan*

| Treatments | % Encapsulation efficiency (NS) | % Loading efficiency |
|---|---|---|
| C | 94.55±0.3942 | 4.958±0.0207a |
| A | 94.08±0.1516 | 4.39±0.0071b |
| B | 94.38±0.4064 | 3.804±0.0164c |

NS indicates that the values within the same column are not significantly different ($p<0.05$).
a mean ± SD
b the same letter in the same row means no significant difference ($p<0.05$)

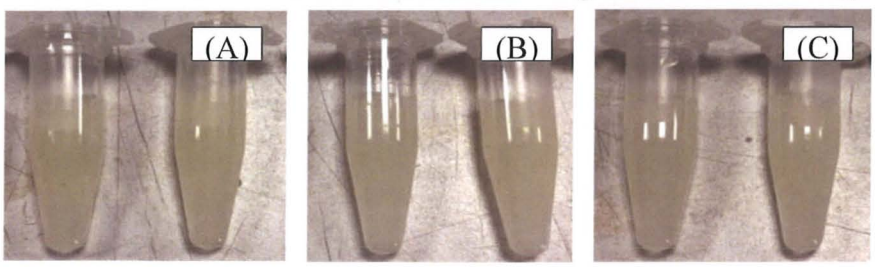



*Figure 5: Curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using A (adding curcumin after nanoparticle formation), B (adding curcumin before nanoparticle formation by mixing curcumin with TPP), C (adding curcumin before nanoparticle formation by mixing curcumin with CS).*

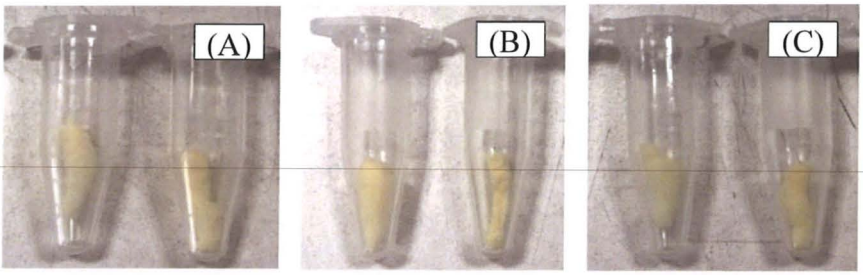



*Figure 6: Freeze dried curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using A (adding curcumin after nanoparticle formation), B (adding curcumin before nanoparticle formation by mixing curcumin with TPP), C (adding curcumin before nanoparticle formation by mixing curcumin with CS*

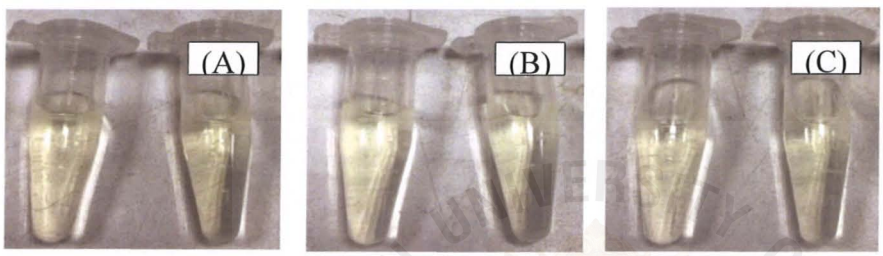



*Figure 7: Supernatant of curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using A (adding curcumin after nanoparticle formation), B (adding curcumin before nanoparticle formation by mixing curcumin with TPP), C (adding curcumin before nanoparticle formation by mixing curcumin with CS)*

The encapsulation efficiency and loading efficiency are parameters that used to measure the ability of bioactive compounds to be entraped and quantity of bioactive compounds to be loaded into carrier system, respectively (Ming S., 2019).

From *Figure 4* and *Table 3,* C-CS-NPs (A), C-CS-NPs (B), and C-CS-NPs (C) showed no significant difference in percent encapsulation efficiency at 94.08±0.1516, 94.38±0.4064, and 94.55±0.3942, respectively ($p<0.05$). Whereas in percent loading efficiency, there is significant between all three treatments of encapsulation method ($p<0.05$). The C-CS-NPs (C) showed the highest percent loading efficiency at $4.958\pm0.0207_a$, followed by C-CS-NPs (A) at $4.39\pm0.0071_b$, and C-CS-NPs (B) at $3.804\pm0.0164_c$. Ranking from highest to lowest order for percent loading efficiency, are C-CS-NPs (C) > C-CS-NPs (A) > C-CS-NPs (B). Since C-CS-NPs (C) shown the excellent result in both encapsulation and loading efficiency, therefore C-(adding curcumin before nanoparticle formation by mixing curcumin with CS) is the optimized encapsulation method for the further study.

In addition, the reason why C-CS-NPs (C) has the highest loading efficiency compare to other two encapsulation methods because curcumin was added to chitosan before

nanoparticles were formed. As hydrophobic bioactive curcumin compound was added into amphiphillic chitosan, the hydrophobic backbone of chitosan had already loaded a certain amount of the bioactive curcumin into its self-association. Thus, when TPP is added after, the nanoparticles had formed and aid in loading more of curcumin into the chitosan carriers. Since encapsulation method C produced a higher quantity to be loaded into chitosan carriers, this method is used as an adopted preparation for the next study.

In *Figure 5, 6, 7,* they have shown a closely color intensity and the same cloudiness intensity in the encapsulation method A, B, and C. Where in the freeze-dried form, encapsulation method A, B, and C showed similar quantity of particle content. This could be one of a visual evidence that the encapsulation method A, B, and C are not much of a different.

**Encapsulation of curcumin into chitosan nanoparticles (C-CS-NPs) using 0.5% and 1% CS**

Based on previous study, treatment C (adding curcumin before nanoparticle formation by mixing curcumin with CS) was chosen as an adopted method for the encapsulation of curcumin into chitosan. For this study, chitosan (0.5% and 1% w/v) solutions were used to study the optimal chitosan concentration with the ratio of CS:TPP as 1:1 at pH 3. The encapsulation and loading efficiency were used as the parameters to analyze their ability of drug entrapped and quantity of drug loaded into nanoparticles, respectively. The result was further analyzed using Statistics software R-Program version R 2.15.3, by independent t-test.

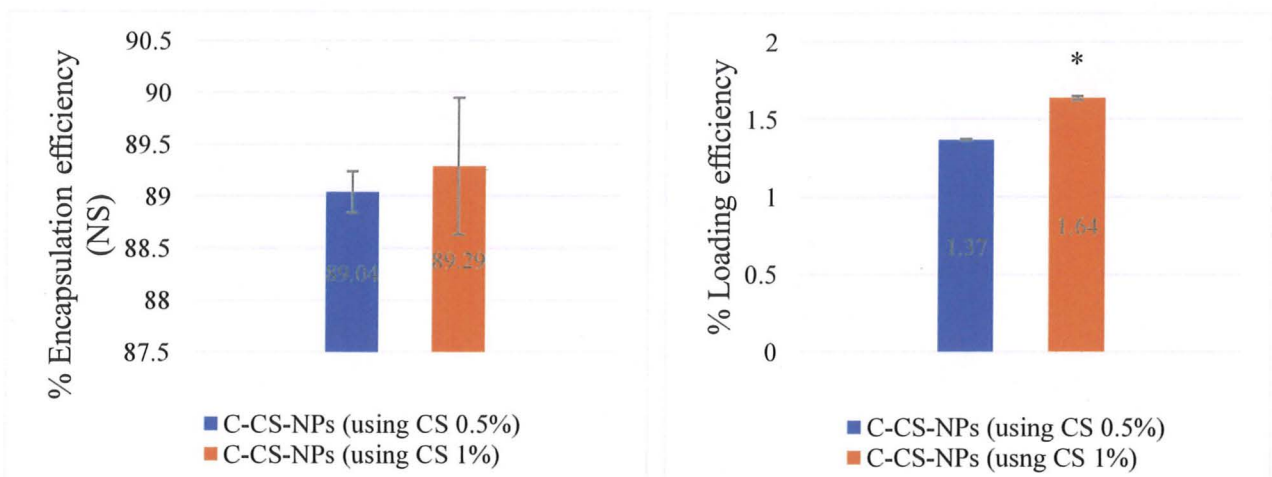



*Figure 8: Comparison of percentage of encapsulation and loading efficiency of C-CS-NPs (using CS 0.5% and 1%, respectively)*

*Table 4: Comparison of percent encapsulation and loading efficiency of curcumin in CS-NPs using 0.5% and 1% CS, at initial curcumin concentration of 0.5467mg/ml in 50% ethanol.*

| Sample formulation (Treatments) | % Encapsulation efficiency (*NS*) | % Loading efficiency |
|---|---|---|
| 2 (C-CS-NPs using 1%CS) | 89.28±0.66 | 1.64±0.0122* |
| 1 (C-CS-NPs using 0.5%CS) | 89.04±0.20 | 1.37±0.0030 |

*NS* indicates that the values within the same column are not significantly different ($p<0.05$).
* indicates that the values within the same column are significantly different ($p<0.05$).

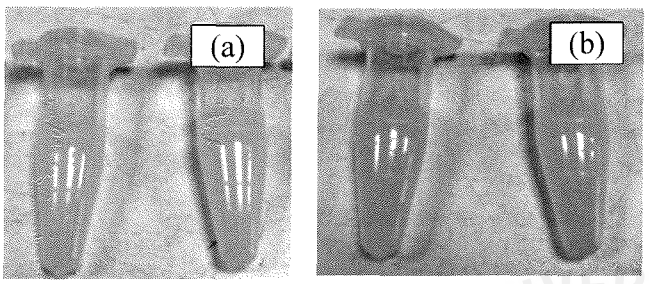



*Figure 9: Preparation of curcumin encapsulated-chitosan nanoparticles C-CS-NPs using (a) 0.5% chitosan and (b) 1% chitosan*

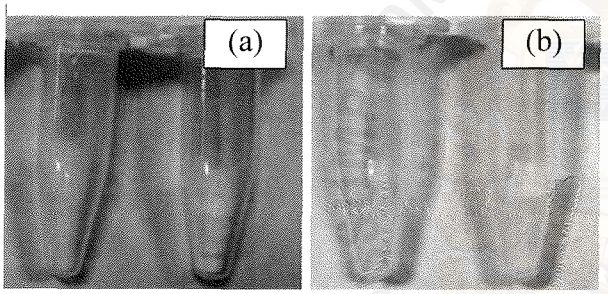



*Figure 10: Freeze dried curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using (a) 0.5% and (b) 1% chitosan*

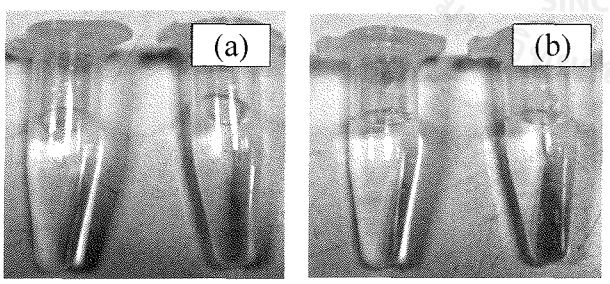



*Figure 11: Supernatant of encapsulation of curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using (a) 0.5% and (b) 1% chitosan*

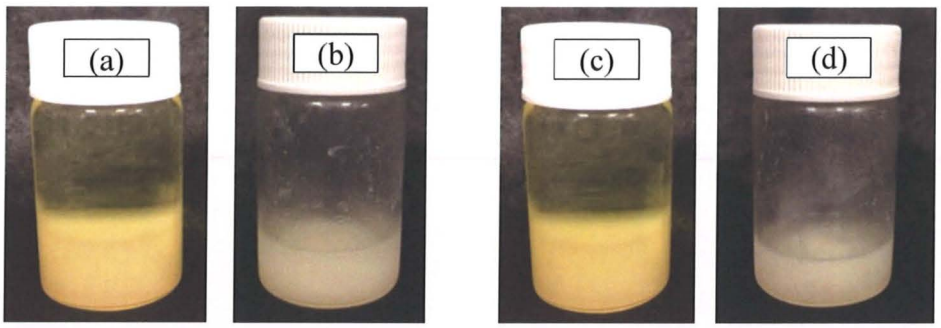



*Figure 12: Comparison of curcumin encapsulated-chitosan nanoparticles (C-CS-NPs) using; 0.5% chitosan: (a) before dialysis and (b) after dialysis, and 1% chitosan: (c) before dialysis and (d) after dialysis.*

Based on *Figure 8* and *Table 4,* the two sample formulations of C-CS-NPs using 0.5%CS and C-CS-NPs using 1%CS had no significant different effect on the percent encapsulation efficiency ($p<0.05$). The percent encapsulation efficiency of C-CS-NPs using 0.5%CS and C-CS-NPs using 1%CS were found to be 89.04±0.20 and 89.28±0.66, respectively. However, the result of percent loading efficiency was found to have significantly different between C-CS-NPs using 0.5%CS and C-CS-NPs using 1%CS at 1.37±0.0030 and 1.64±0.0122*, respectively ($p<0.05$). It was found that C-CS-NPs using 0.5%CS and C-CS-NPs using 1%CS had generated similar result in drug entrapment ability, hence C-CS-NPs using 0.5%CS is used for the next study as it used less material with similar generated result.

The higher loading efficiency of C-CS-NPs using 1%CS can be explained that the higher chitosan matrix favored the more drug loaded into nanoparticles than C-CS-NPs using 0.5%CS. Whereas, the none significant difference of encapsulation efficiency might be interpreted that the limit amount of TPP used might exceed the capacity of chitosan nanoparticles to encapsulate the curcumin compound. In addition, some researchers have confirmed that encapsulation efficiency may vary according to the concentration of chitosan and TPP used (Rajesh S., 2018). In this work, we have maintained the TPP concentration constant and the amount of chitosan concentration was varied among the formulations; it had shown not much different between the two formulations.

In *Figure 9, 10, 11,* they have shown a closely color intensity and the same cloudiness intensity in C-CS-NPs using 0.5%CS and 1%CS. Where in the freeze-dried form, encapsulation of curcumin using 0.5%CS showed a slight different in yellow color shade than encapsulation of curcumin using 1%CS. This could be one of a visual evidence that the

encapsulation of curcumin into chitosan nanoparticles (C-CS-NPs) using 0.5% and 1% CS are not much of a different.

### 3.3-3.4 Encapsulation and loading efficiency of curcumin into folate-conjugated chitosan nanoparticles (C-FCS-NPs)

Folate-conjugated chitosan were synthesized based on carbodiimide chemistry (Dubé D, Francis M, Leroux JC, & Winnik FM, 2002). The curcumin was subsequently encapsulated into Folate-conjugated chitosan (FCS-NPs) and further comparing to the previous sample formulation of C-CS-NPs using 0.5% CS, at initial curcumin concentration of 0.5467mg/ml in 50% ethanol at pH 3 to evaluate the percent encapsulation and loading efficiency.

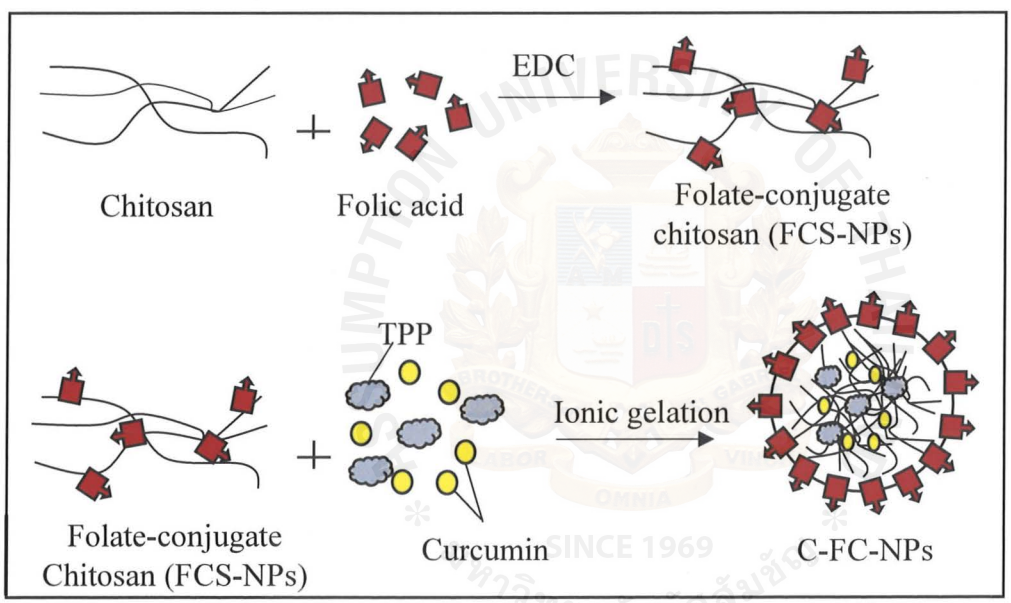



*Figure 13: Mechanism of C-FCS-NPs preparation based on carbodiimide chemistry*

*Table 5: Comparison of percent encapsulation and loading efficiency of curcumin in CS-NPs and FCS-NPs using 0.5% CS, at initial curcumin concentration of 0.5467mg/ml in 50% ethanol.*

| Sample Formulations (Treatments) | % Encapsulation efficiency | % Loading efficiency |
|---|---|---|
| C-CS-NPs | 94.55±0.3942 | 4.958±0.0207 |
| C-FCS-NPs | 97.29±0.6922 | 5.98±0.0424 |

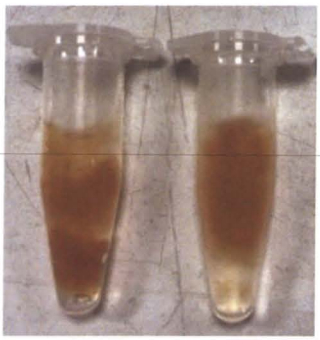


*Figure 14: Curcumin and folate-conjugated chitosan nanoparticles (C-FCS-NPs)*

*Figure 15: Freeze dried curcumin and folate-conjugated chitosan nanoparticles (C-FCS-NPs)*

*Figure 16: Supernatant of Curcumin and folate-conjugated chitosan nanoparticles (C-FCS-NPs)*

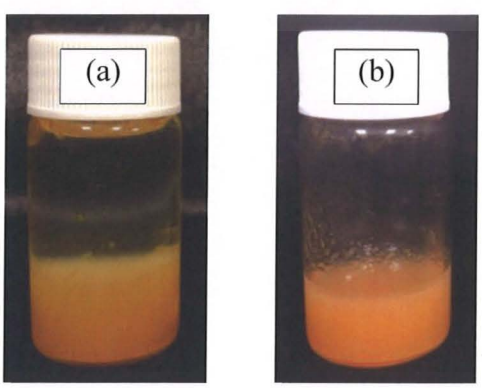



*Figure 17: Curcumin and folate-conjugated chitosan nanoparticles (C-FCS-NPs); (a) before dialysis and (b) after dialysis.*

From *Figure 13,* FCS-NPs was prepared *via* EDC-mediated reaction of folic acid and primary amine function linked to the polymer. Chemically, EDC was used to bring about the

reaction between COOH group of folic acid and $NH_2$ group of chitosan (Wang *et al.,* 2009). Then, FCS-NPs was brought to the ionic gelation reaction with TPP and curcumin; to be the carrier of curcumin and functionally endow tumor-targeted activity (Li *et al.,* 2011). With this stable structure, this study compared C-FCS-NPs with regular C-CS-NPs to observe their abilities of drug entrapment and quantity of drug loading.

In *Table 5,* C-FCS-NPs have been compared with C-CS-NPs using 0.5%CS in percent encapsulation and loading efficiency. Both percent encapsulation and loading efficiency of C-FCS-NPs were found to be significantly higher than C-CS-NPs' at 97.29±0.6922 and 5.98±0.0424, respectively. While percent encapsulation and loading efficiency of C-CS-NPs were only at 94.55±0.3942 and 4.958±0.0207, respectively. In *Figure 14, 15, 16, & 17,* the overall appearances of C-FCS-NPs are shown in suspension form, freeze dried form, and supernatant of C-FCS-NPs, as well as C-FCS-NPs before and after dialysis, respectively.

Based on research studies, the higher percent encapsulation and loading efficiency of C-FCS-NPs than C-CS-NPs was due to the fact of two electrostatic interactions; one is-cationic amino group of chitosan with anionic carboxyl group of folic acid, and two is- cationic chitosan with polyanionic TPP (Huijuan S. *et al.,* 2013). In spite, C-CS-NPs had only one electrostatic interaction of cationic chitosan with polyanionic TPP. With this reason, the more electrostatic interactions had contributed to bioactive compounds to be more entrapped and more loaded into the nanoparticles. Besides C-FCS-NPs favored the drug entrapment and drug loaded, in some research had also proved that folate-conjugate chitosan nanoparticles can become a promising targeted delivery system of polyphenolic compounds (Liu B, 2018).

### 3.5 Determination of particle content

Particle content of each sample formulations were obtained from weighing each sample in 1ml freeze dried sample in Eppendorf tube. These weights were used for the evaluation of percent loading efficiency in all sample formulations as mentioned in *Table 6.*

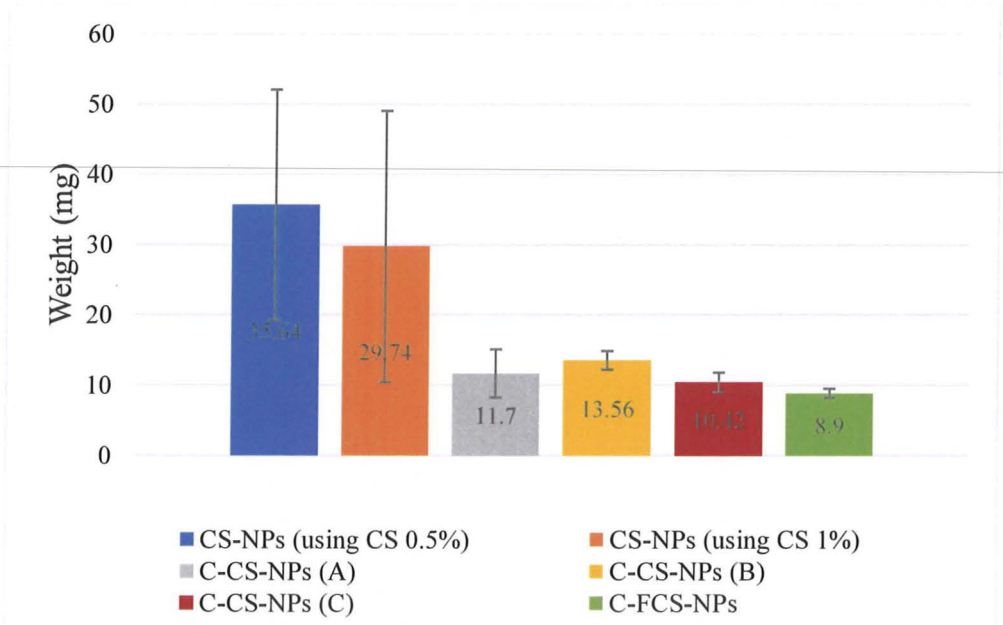



*Figure 18: Comparison of particle content in milligram (dried weight of particle in 1 ml) of CS-NPs (using CS 0.5%), CS-NPs (using CS 1%), C-CS-NPs (A), C-CS-NPs (B), C-CS-NPs (C), and C-FCS-NPs.*

*Table 6: Particle content of each sample formulations*

| **Treatments** | **Average±SD (mg/ml)** |
|---|---|
| CS-NPs (using CS 0.5%) | 35.64±16.38 |
| CS-NPs (using CS 1%) | 29.74±19.28 |
| C-CS-NPs (A) | 11.7±3.43 |
| C-CS-NPs (B) | 13.56±1.33 |
| C-CS-NPs (C) | 10.42±1.39 |
| C-FCS-NPs | 8.9±0.64 |

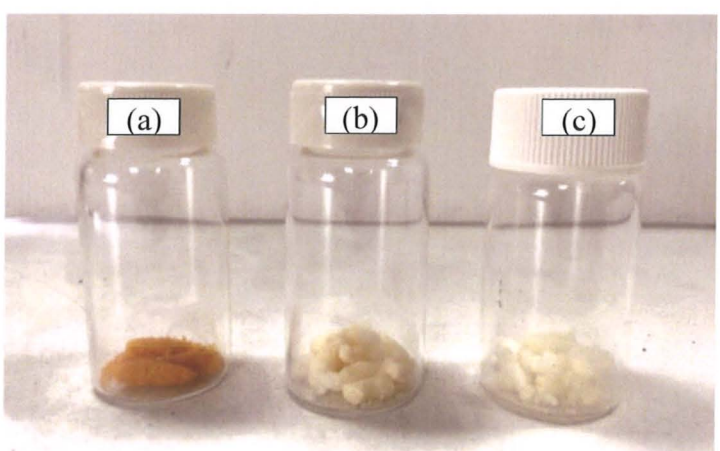



*Figure 19: Freeze dried form of C-FCS-NPs (a), C-CS-NPs (b) and CS-NPs (c) using 0.5%CS, and method C for encapsulation of curcumin*

Based on *Figure 18 and Table 6*, particle content in milligram of C-CS-NPs (using CS 0.5%), C-CS-NPs (using CS 1%), C-CS-NPs (A), C-CS-NPs (B), C-CS-NPs (C), and C-FCS-NPs were found to be 35.64±16.3884, 29.74±19.2874, 11.7±3.4334, 13.56±1.3351, 10.42±1.3905, and 8.9±0.6481, respectively. Therefore, CS-NPs (using CS 0.5%) and CS-NPs (using CS 1%) were found to be the highest particle content compare to other samples. Nevertheless, C-FCS-NPs was found to have the least particle content. Where *Figure 19* shows the overall appearance such color and particle differences between freeze dried form of C-FCS-NPs, C-CS-NPs, and CS-NPs.

From the result, it showed that the particle content has significant effect on the percent loading efficiency of all treatments. In the analysis of percent loading efficiency with the particle content, it is shown to be inversely proportional to each other indicating that the lower the mass of particle content the higher the percent of loading efficiency of the sample, vice versa.

### 3.6 Determination of particle size and polydispersity

Particle size of sample formulations; CS-NPs and C-FCS-NPs were obtained from outsource measurement by dynamic light scattering and were characterized using Zetasizer 3000 (Malvern, UK). It was done in triplicates.

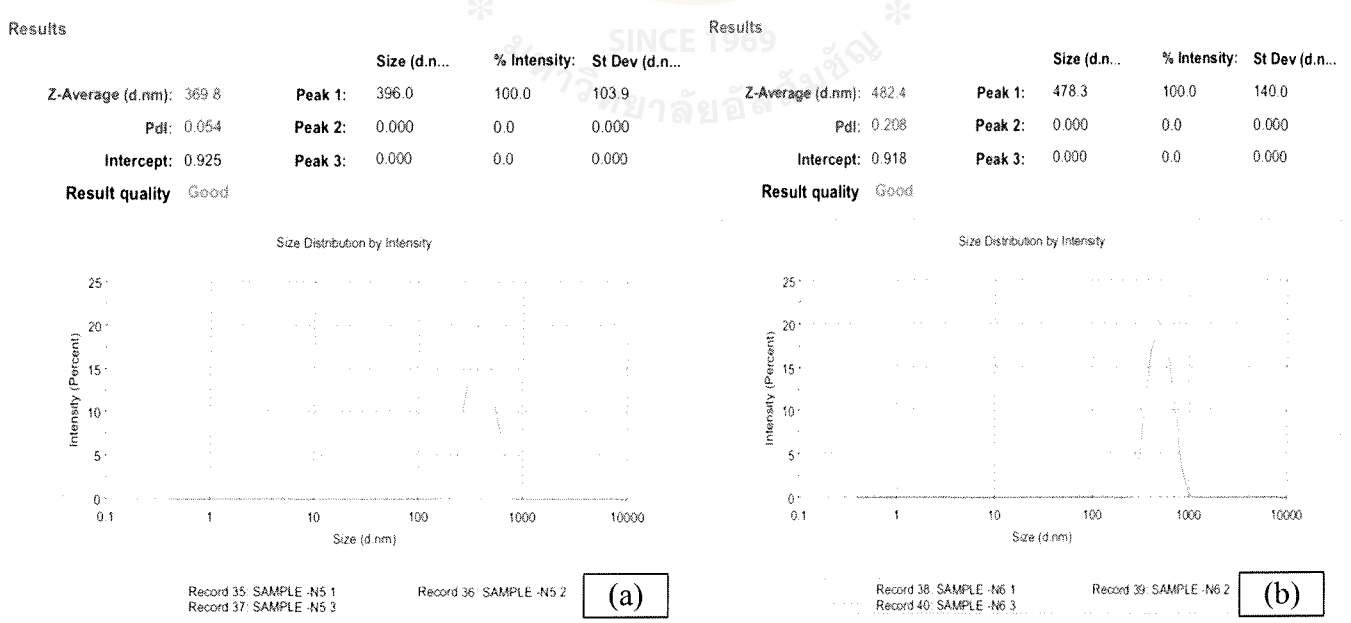



*Figure 20: Result of particle size of (a) CS-NPs and (b) C-FCS-NPs that were measured by Dynamic light scattering*

From Figure 20, the particle size of CS-NPs and C-FS-NPs were measured in triplicate and the mean size were found to be 369.8nm and 482.4nm, respectively. Some research was as well found to have similar size at about 300nm; and had proved that curcumin encapsulation had minimal effect of nanoparticle size. In spite, chitosan had contributed a major effect on nanoparticle size. Both of the samples also had generated a good result of size distribution among the three measurements as shown in the three peaks; to have about same size, which is shown in the polydispersity index (PDI). PDI is a dimensionless measure of particle size distribution (Hasan M. et al., 2019). The result above shown the PDI of CS-NPs and C-FCS-NPs of 0.054 and 0.208; it is indicating that particles had a controlled size distribution and a narrow dispersity; meaning they both had a homogenous size distribution.

### 3.7 Determination of Antioxidant activity

#### DPPH method of antioxidant assay

The antioxidant activity of the sample compounds is the ability that compounds could take up free radical and scavenging capacity of antioxidants toward DPPH. This assay is regarding to a theory that a hydrogen donor is an antioxidant of sample compounds, in which DPPH-free radical (DPPH•) could accept and becomes a DPPH stable compound (Wicks M. *et at.,* 2006). Within the experiment, DPPH reagent turns purple to yellow was caused by the absorption of hydrogen from an antioxidant. It can be measured by scavenging or DPPH trapping method at the absorbance of 517nm in regarding to the number of hydrogen atoms absorbed (Huang *et al.*, 2006). In this research, three samples (CS-NPs, C-CS-NPs, and C-FCS-NPs) were studied on its antioxidant activity using DPPH scavenging method. The antioxidant of sample formulations was statistically analyzed to see the difference between each sample. The results were further interpreted using Randomized Complete Block Design (RCBD) with Duncan's multiple range test in R-Program version R 2.15.3.

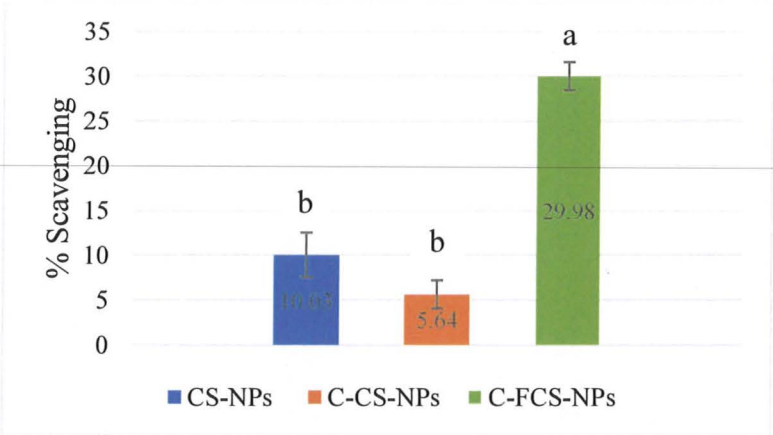



*Figure 21: Comparison of antioxidant activity of CS-NPs, C-CS-NPs and C-FCS-NPs using DPPH scavenging protocol*

*Table 7: Comparison of percent scavenging in CS-NPs, C-CS-NPs, and C-FCS-NPs, using 0.5% CS, at initial curcumin concentration of 0.5467mg/ml in 50% ethanol. Multiple comparison of % DPPH scavenging done by Duncan*

| Sample formulation (Treatments) | % Radical Scavenging |
|---|---|
| 3 (C-FCS-NPs) | 29.98±1.569a |
| 1 (CS-NPs) | 10.04±2.49b |
| 2 (C-CS-NPs) | 5.641±1.573b |

a mean ± SD

b the same letter in the same row means no significant difference ($p<0.05$)

Regarding to *Figure 19* and *Table 7*, DPPH scavenging capacity was expressed in percent radical scavenging. The percent radical scavenging of C-FCS-NPs have found to be significantly different from percent radical scavenging of CS-NPs and C-CS-NPs ($p<0.05$), at 29.98±1.569a. While the percent radical scavenging of CS-NPs and C-CS-NPs were at 10.04±2.49b and 5.641±1.573b, respectively. Comparing between C-FCS-NPs and the other two formulations, percent radical scavenging of C-FCS-NPs was equal to the double of the sum of CS-NPs and C-CS-NPs. This means C-FCS-NPs has a higher antioxidant activity and higher percent radical scavenging capacity.

### 3.8 Total Phenolic Content Assay

Three formulation samples (CS-NPs, C-CS-NPs, and C-FCS-NPs) were studied for their total phenolic content using total phenolic content assay at absorbance of 765nm. The total phenolic content of sample formulations was statistically analyzed to see the difference between each sample. The results were further interpreted using Randomized Complete Block

Design (RCBD) with Duncan's multiple range test in R-Program version R 2.15.3. Antioxidant activity in total phenolic content assay is the colorimetric measurement in finding the amount of phenolic content in the studied sample though research showing that Folin-Ciocalteu reagent used in total phenolic content assay was not specific. Phenolic compounds have redox propertied capable to act as antioxidants (Ming S., 2019).

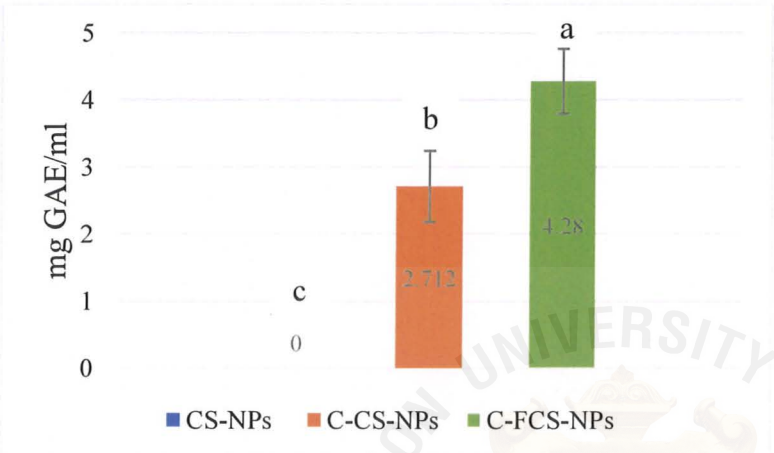



*Figure 22: Comparison of total phenolic content of CS-NPs, C-CS-NPs and C-FCS-NPs*

*Table 8: Total phenolic content of each sample formulation. Multiple comparison of total phenolic content done by Duncan*

| Sample Formulation (Treatments) | Total phenolic content (mg GAE/ml) |
|---|---|
| 3 (C-FCS-NPs) | $4.28 \pm 0.481_a$ |
| 2 (C-CS-NPs) | $2.712 \pm 0.528_b$ |
| 1 (CS-NPs) | $0_c$ |

a mean ± SD

b the same letter in the same row means no significant difference ($p<0.05$)

The results of total phenolic content were obtained from the comparison of standard gallic acid as phenolic compound and were expressed as mg GAE/ml dried weight. In *Figure 20* and *Table 8*, the highest antioxidant activity represented by the amount of phenolic content of C-FCS-NPs was $4.28 \pm 0.481_a$ mg GAE/ml, which was significantly different from C-CS-NPs and CS-NPs at $2.712 \pm 0.528_b$ and $0_c$, respectively ($p<0.05$). In ranking from highest to lowest value of total phenolic content in sample formulations was C-FCS-NPs > C-CS-NPs > CS-NPs.

In the two assays that were used in measuring the antioxidant activity by DPPH and measuring phenolic content by Total phenolic content assay, showing in the same trend that

the C-FCS-NPs had a higher antioxidant activity and total phenolic content than the other two formulations (C-CS-NPs and CS-NPs). These results indicated a significant increase of antioxidant property after the encapsulation of curcumin into folate-conjugated chitosan nanoparticles. This could be supported by the reasons that this formulation has all the possible antioxidants including chitosan, curcumin, and folic acid. As for chitosan, previous studies had proved that the chitosan chains have active hydroxyl and amino groups that can react with the free radicals, the scavenging activity of chitosan was due to the reaction between free radicals and protonated amino groups (Xie *et al.,* 2001).

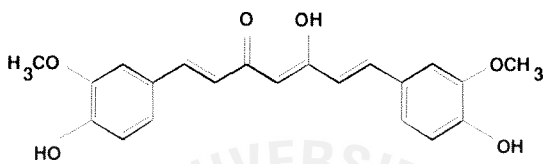



*Figure 23: Chemical structure of curcumin*

As for curcumin, it is known to protect bio-membranes from peroxidative damage which occurred mostly in lipid peroxidation and causes the damage of cell membranes. Most of the antioxidants have either a phenolic functional group or a β-diketone group. However, curcumin is a unique antioxidant, which contains many of functional groups, including the β-diketone group, carbon–carbon double bonds, and phenyl rings containing varying amounts of hydroxyl and methoxy substituents (*Figure 21*). Theoretical calculations by the density functional theory (DFT) demonstrated that the enol form of curcumin is significantly more stable than the diketo form and that the bond dissociation enthalpy (BDE) of the phenolic O:H bond is significantly lower than the BDE of the central O:H bond, suggesting that the hydrogen atom abstraction takes place in the phenolic group (K. I. Priyadarsini *et al.,* 2003).

This had supported the contradiction in the result above that total phenolic content assay showed antioxidant property of C-CS-NPs higher than of CS-NPs, whereas in DPPH assay showed antioxidant property of C-CS-NPs lower than of CS-NPs. As curcumin abstracted hydrogen atom in the phenolic group, has contributed to a higher result of antioxidant property in total phenolic content assay.

As for folic acid, it is known as one of the essential vitamins which has similar antioxidant property as curcumin; that folic acid was proved to have a significant inhibition property in microsomal lipid peroxidation (Joshi R, 2001). Also, folic acid has another benefit toward functional food system in a way that it is appealed to the targeted cell with its folate

receptors (Buyun L., 2018). This could further support the result of the C-FCS-NPs having the highest antioxidant property in this study.

### 3.9 Chelating ability

Three formulation samples (CS-NPs, C-CS-NPs, and C-FCS-NPs) were studied for their chelating ability on ferrous ions using Ferrozine reagent by measuring its absorbance at 562nm spectrophotometrically. The chelating ability of sample formulations was calculated based on specified formula in the methodology and statistically analyzed to see the difference between each sample. The results were further interpreted using Randomized Complete Block Design (RCBD) with Duncan's multiple range test in R-Program version R 2.15.3.

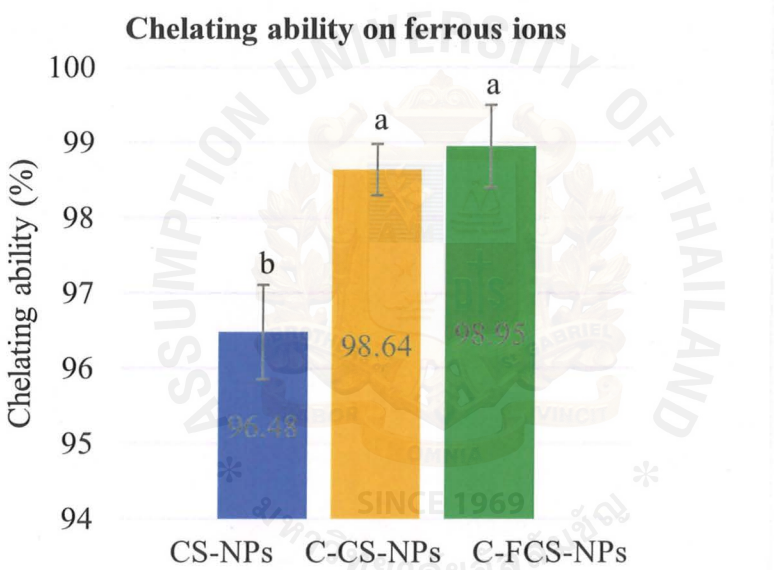



*Figure 24: Comparison of chelating ability on ferrous ions of CS-NPs, C-CS-NPs, and C-FCS-NPs*

*Table 9: % Chelating ability of each sample formulation. Multiple comparison of % chelating ability done by Duncan*

| Sample Formulation (Treatments) | Chelating ability (%) |
|---|---|
| 3 (C-FCS-NPs) | 98.95±0.55a |
| 2 (C-CS-NPs) | 98.64±0.34a |
| 1 (CS-NPs) | 96.47±0.63b |

a mean ± SD

b the same letter in the same row means no significant difference ($p<0.05$)

Based on *Figure 24* and *Table 9,* the percent chelating ability of C-FCS-NPs was found to be highest at 98.95±0.55a among other sample formulation though there is no significant different between it and C-CS-NPs (at 98.64±0.34a) ($p<0.05$). On the other hand, the percent chelating ability of CS-NPs (at 96.47±0.63b) was found to be significantly different from both C-CS-NPs and C-FCS-NPs ($p<0.05$). In spite that CS-NPs was found to be the lowest but its percent chelating ability is still considerably high. This is due to the fact that chitosan has an ability to chelating heavy metal through specific interaction of amine group to the heavy metal (Muzzarelli, 1973). Where percent chelating ability of C-CS-NPs and C-FCS-NP were found to be slightly higher than CS-NPs because some research had proved that curcumin also had an ability to chelate iron as well (Jiao Y. *et al.,* 2006). Another paper was also showed that folic acid could as well chelate heavy metals such zinc, copper and iron (Miline DB. *et al.,* 1984).

### 3.10 Antimicrobial activity

With the used of nanotechnology, drugs encapsulating with nanostructure system promote the sustained release of active compounds, decrease in require dose, reduce side effects, and prolong their bioactivity (Ghosh *et al.,* 2013). Within that, cells could take up drug efficiently than the micromolecules, thus nanovehicles would be used as an effective transport and delivery system.

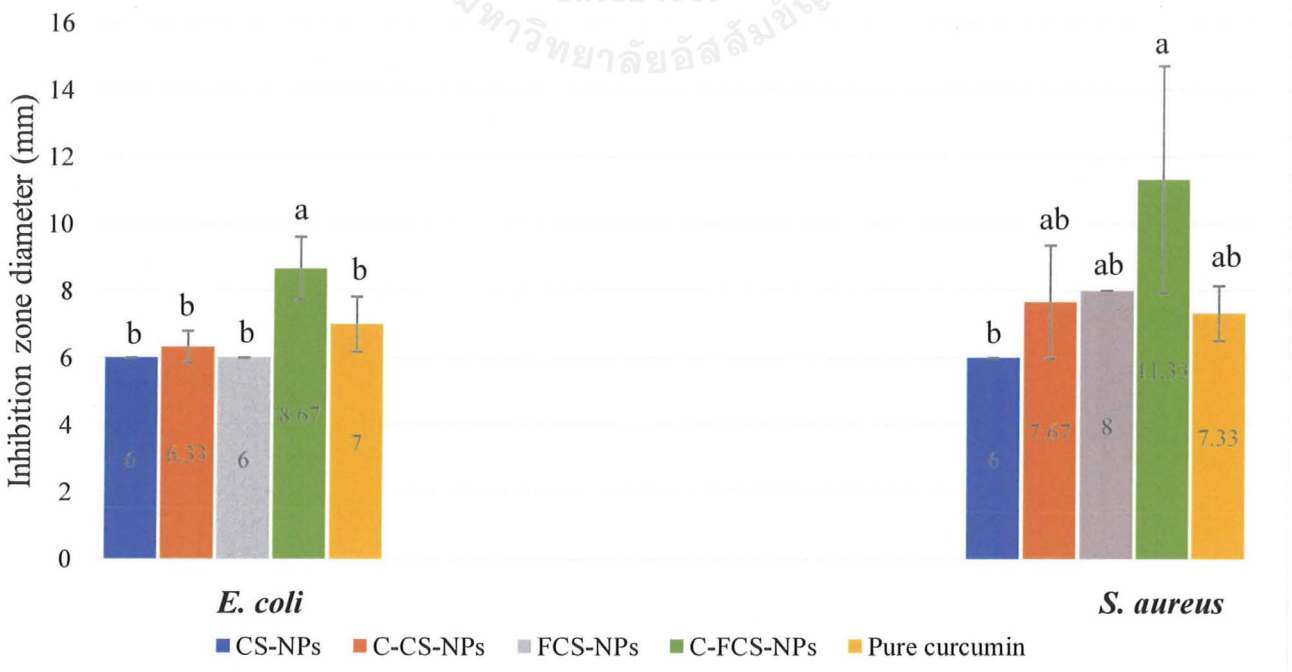



*Figure 25: Comparison of antimicrobial activity of CS-NPs, C-CS-NPs, FCS-NPs, C-FCS-NPs, and Pure curcumin on E. coli and S. aureus.*

*Table 10: Antimicrobial results of E. coli for each sample formulation. Multiple comparison of E. coli inhibition done by Duncan*

| Sample Formulation (Treatments) | Inhibition diameter (mm) | Ratio of clear zone diameter over paper disc's diameter |
|---|---|---|
| 4 (C-FCS-NPs) | $8.67 \pm 0.00_a$ | 8.67/6 |
| 5 (Pure curcumin) | $7 \pm 0.47_b$ | 7/6 |
| 2 (C-CS-NPs) | $6.33 \pm 0.00_b$ | 6.33/6 |
| 1 (CS-NPs) | $6 \pm 0.94_b$ | 6/6 |
| 3 (FCS-NPs) | $6 \pm 0.82_b$ | 6/6 |

a mean ± SD
b the same letter in the same row means no significant difference ($p<0.05$)
Note: paper disc's diameter is 6mm

*Table 11: Antimicrobial results of S.aureus for each sample formulation. Multiple comparison of S. aureus inhibition done by Duncan*

| Sample Formulation (Treatments) | Inhibition diameter (mm) | Ratio of clear zone diameter over paper disc's diameter |
|---|---|---|
| 4 (C-FCS-NPs) | $11.33 \pm 0.00_a$ | 1.89 (11.33/6) |
| 3 (FCS-NPs) | $8 \pm 1.69_{ab}$ | 1.33 (8/6) |
| 5 (Pure curcumin) | $8 \pm 0.00_{ab}$ | 1.33 (8/6) |
| 2 (C-CS-NPs) | $7.67 \pm 3.39_{ab}$ | 1.28 (7.67/6) |
| 1 (CS-NPs) | $6 \pm 0.82_b$ | 1.0 (6/6) |

a mean ± SD
b the same letter in the same row means no significant difference ($p<0.05$)
Note: paper disc's diameter is 6mm

Five formulation samples (CS-NPs, C-CS-NPs, FCS-NPs, C-FCS-NPs, and pure curcumin) were studied their antimicrobial activity using disk-diffusion agar method. The antimicrobial activity was studied on both gram positive and gram-negative bacteria, *S. aureus* and *E. coli*, respectively. This method was analyzed through the measurement of inhibition diameter, known as clear zone, comparing with paper disc's diameter (6mm). Where the results of antimicrobial activity of formulation samples were further interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range test in R-Program version R 2.15.3.

In *Figure 22 and Table 9 & 10,* the antimicrobial activity of C-FCS-NPs is shown to be significantly higher than other sample formulations for both *E. coli* and *S. aureus*, at an inhibition diameter of $8.667 \pm 0.00_a$ and $11.33 \pm 0.00_a$ mm, respectively ($p<0.05$). Following by C-CS-NPs, FCS-NPs, and pure curcumin that have been grouped with C-FCS-NPs for *S. aureus* at an inhibition diameter of $7.667 \pm 3.39_{ab}$, $8 \pm 1.69_{ab}$, and $8 \pm 0.00_{ab}$ mm, respectively

($p<0.05$). However, these three sample formulations were grouped with the least antimicrobial activity sample, CS-NPs, for *E. coli* and *S. aureus* at an inhibition diameter of $6\pm0.94_b$ and $6\pm0.82_b$ mm, respectively ($p<0.05$).

The growth of bacteria was inhibited by curcumin in C-FCS-NPs and pure curcumin sample. Due to the ability of curcumin, curcumin could reduce the bundling of FtsZ in the cytokinetic Z-ring which it helps suppressed the bacterial cells. It also had been proved that by suppressing the FtsZ assembly leading to disruption of prokaryotic cell division (C. H. Liu, 2012). The gram-positive bacteria were easily invaded by the active compounds of curcumin than the gram-negative bacteria. This happened because gram-positive bacteria have an inner cell membrane which surrounded peptidoglycan cell wall (Lisa B. *et al.,* 2015). Whereas lipopolysaccharides of the outer cell envelope of gram-negative bacteria represent the outermost permeability barrier for a variety of antimicrobial compounds responsible for the unusually slow influx of lipophilic solutes such curcumin in gram-negative bacteria (H. Nikaido, 2003). Thus, curcumin compound in this study had antimicrobial property effecting on gram-positive bacteria like *S. aureus* more than gram-negative like *E. coli.*

## Conclusion

In chitosan nanoparticle, chitosan's backbone is hydrophobic in which it can self-associate to form nanoparticles at pH 3-4.5, TPP was added to the CS solution as an occurrence of crosslinking between negative charge TPP and the positive charge CS. This interaction produced the formation of nano-sized polyelectrolyte complex that could entrapped bioactive curcumin effectively. All chitosan nanoparticles have been successfully prepared by using 0.5% chitosan at pH 3 with the ratio of chitosan to TPP at 1:1. The C-CS-NPs using 1%CS had higher chitosan matrix favored the more drug loaded into nanoparticles than C-CS-NPs using 0.5%CS. While C-CS-NPs (A, B, and C) showed no significantly different in encapsulation efficiency ($p<0.05$) but C-CS-NP (C) showed significantly higher than others in percent loading efficiency. C-CS-NPs (C) was obtained by added curcumin to chitosan before nanoparticles were formed, thus nanoparticles had formed later-aiding in loading more of curcumin into the chitosan carriers. For C-FCS-NPs, folate conjugated chitosan showed a higher percent encapsulation and loading efficiency than C-CS-NPs. Furthermore, the particle content of sample formulations was found to be ranking from highest to lowest: CS-NPs (0.5%CS) > CS-NPs (1%CS) > C-CS-NPs (B) > C-CS-NPs (A) > C-CS-NPs (C) > C-FCS-

NPs. Where the particle size and polydispersity index of CS-NPs and C-FCS-NPs were 369.8nm, 0.054 PDI and 482.4nm, 0.208PDI, respectively, indicating a homogenous particles with uniform size distribution. The result of DPPH and total phenolic content assay were also agreed that C-FCS-NPs had higher antioxidant activity and total phenolic content than CS-NPs and C-CS-NPs due to the higher percent encapsulation of curcumin with the use of folic acid aiding in scavenging radical and inhibiting the lipid peroxidation reaction. For chelating ability, the percent chelating ability on iron ions were ranked from highest to lowest as C-FCS-NPs > C-CS-NPs > CS-NPs, due to the chitosan ability in the major contribution of chelating iron ions. Where disk-diffusion method was also supported that C-FCS-NPs had a higher antimicrobial activity as bioactive compound curcumin and folic acid could reduce the bundling of FtsZ in the cytokinetic Z-ring which resulting suppress bacterial cells.

The development of C-CS-NP and C-FCS-NPs provide a promising drug delivery system using chitosan nanoencapsulation to increase bioavailability of curcumin. With the advantages of curcumin incorporating with folate-chitosan nanoparticles, these could be further applied to use in both biopharmaceutical and functional food applications including nano-carriers for drug targeting, food products and supplements, as well as medical applications such as control release of folate and curcumin.

## References

Nugroho, Praptanti S. A, and Umi Nafisah. "Characterization Of Curcumin Encapsulation In Chitosan-Sodium Citrate Nanoparticles". Journal Of Applied Chemical Science, 2017, pp. 370-372. Jacsonline Group Publisher, doi:10.22341/jacs.on.00402p370.

Yadav, Abhishek et al. "Curcumin Encapsulated In Chitosan Nanoparticles: A Novel Strategy For The Treatment Of Arsenic Toxicity". Chemico-Biological Interactions, vol 199, no. 1, 2012, pp. 49-61. Elsevier BV, doi:10.1016/j.cbi.2012.05.011.

Souza, Marthyna P. et al. "Quercetin-Loaded Lecithin/Chitosan Nanoparticles For Functional Food Applications". Food And Bioprocess Technology, vol 7, no. 4, 2013, pp. 1149-1159. Springer Science And Business Media LLC, doi:10.1007/s11947-013-1160-2.

Liang, Jin et al. "Applications Of Chitosan Nanoparticles To Enhance Absorption And Bioavailability Of Tea Polyphenols: A Review". Food Hydrocolloids, vol 69, 2017, pp. 286-292. Elsevier BV, doi:10.1016/j.foodhyd.2017.01.041.

Chen, Hui et al. "Folic Acid Supplementation Mitigates Alzheimer'S Disease By Reducing Inflammation: A Randomized Controlled Trial". Mediators Of Inflammation, vol 2016, 2016, pp. 1-10. Hindawi Limited, doi:10.1155/2016/5912146.

Hao, Jifu et al. "Folic Acid-Functionalized Drug Delivery Platform Of Resveratrol Based On Pluronic 127/D-Α-Tocopheryl Polyethylene Glycol 1000 Succinate Mixed Micelles". International Journal Of Nanomedicine, Volume 12, 2017, pp. 2279-2292. Informa UK Limited, doi:10.2147/ijn.s130094.

Xu, Yongmei, and Yumin Du. "Effect Of Molecular Structure Of Chitosan On Protein Delivery Properties Of Chitosan Nanoparticles". International Journal Of Pharmaceutics, vol 250, no. 1, 2003, pp. 215-226. Elsevier BV, doi:10.1016/s0378-5173(02)00548-3.

Fan, Wen et al. "Formation Mechanism Of Monodisperse, Low Molecular Weight Chitosan Nanoparticles By Ionic Gelation Technique". Colloids And Surfaces B: Biointerfaces, vol 90, 2012, pp. 21-27. Elsevier BV, doi:10.1016/j.colsurfb.2011.09.042.

De Campos, Angela M. et al. "Chitosan Nanoparticles: A New Vehicle For The Improvement Of The Delivery Of Drugs To The Ocular Surface. Application To Cyclosporin A". International Journal Of Pharmaceutics, vol 224, no. 1-2, 2001, pp. 159-168. Elsevier BV, doi:10.1016/s0378-5173(01)00760-8.

Shehzad, A. et al. "Curcumin Therapeutic Promises And Bioavailability In Colorectal Cancer". Drugs Of Today, vol 46, no. 7, 2010, p. 523. Clarivate Analytics (US), doi:10.1358/dot.2010.46.7.1509560.

Lertsutthiwong, Pranee et al. "Preparation Of Turmeric Oil-Loaded Chitosan-Alginate Biopolymeric Nanocapsules". Materials Science And Engineering: C, vol 29, no. 3, 2009, pp. 856-860. Elsevier BV, doi:10.1016/j.msec.2008.08.004.

Narayanan, Narayanan K. et al. "Liposome Encapsulation Of Curcumin And Resveratrol In Combination Reduces Prostate Cancer Incidence In PTEN Knockout Mice". International Journal Of Cancer, vol 125, no. 1, 2009, pp. 1-8. Wiley, doi:10.1002/ijc.24336.

Ohrvik, Veronica E., and Cornelia M. Witthoft. "Human Folate Bioavailability". Nutrients, vol 3, no. 4, 2011, pp. 475-490. MDPI AG, doi:10.3390/nu3040475.

Liu, Buyun et al. "Synthesis, Characterization Of Catechin-Loaded Folate-Conjugated Chitosan Nanoparticles And Their Anti-Proliferative Effect". Cyta - Journal Of Food, vol 16, no. 1, 2018, pp. 868-876. Informa UK Limited, doi:10.1080/19476337.2018.1491625.

Wang, Peter Y. et al. "Carbodiimide Reagents For The Chemical Probing Of RNA Structure In Cells". RNA, vol 25, no. 1, 2018, pp. 135-146. Cold Spring Harbor Laboratory, doi:10.1261/rna.067561.118.

Thermofisher.com. (2019). *Carbodiimide Crosslinker Chemistry | Thermo Fisher Scientific - UK*. [online] Available at: https://www.thermofisher.com/th/en/home/lifescience/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/carbodiimide-crosslinker-chemistry.html

H, Bangun et al. "Preparation And Evaluation Of Chitosan-Tripolyphosphate Nanoparticles Suspension As An Antibacterial Agent". Journal Of Applied Pharmaceutical Science, vol 8, no. 12, 2018, pp. 147-156. Journal Of Applied Pharmaceutical Science, doi:10.7324/japs.2018.81217.

Dubé, Denis et al. "Preparation And Tumor Cell Uptake Of Poly(N-Isopropylacrylamide) Folate Conjugates". Bioconjugate Chemistry, vol 13, no. 3, 2002, pp. 685-692. American Chemical Society (ACS), doi:10.1021/bc010084g.

Wang, Han et al. "Folic Acid-Modified Dendrimer-Entrapped Gold Nanoparticles As Nanoprobes For Targeted CT Imaging Of Human Lung Adencarcinoma". Biomaterials, vol 34, no. 2, 2013, pp. 470-480. Elsevier BV, doi:10.1016/j.biomaterials.2012.09.054.

Li, Puwang et al. "Synthesis And Characterization Of Folate Conjugated Chitosan And Cellular Uptake Of Its Nanoparticles In HT-29 Cells". Carbohydrate Research, vol 346, no. 6, 2011, pp. 801-806. Elsevier BV, doi:10.1016/j.carres.2011.01.027.

Song, Huijuan et al. "Folic Acid-Chitosan Conjugated Nanoparticles For Improving Tumor-Targeted Drug Delivery". Biomed Research International, vol 2013, 2013, pp. 1-6. Hindawi Limited, doi:10.1155/2013/723158.

Pohanka, Miroslav. "Analytical Tools For The Determination Of Antioxidants And Antioxidant Capacity In Biological Samples, Principles And Applications". Current Organic Chemistry, vol 21, no. 20, 2017. Bentham Science Publishers Ltd., doi:10.2174/1385272821666170410145951.

Huang, Ming-Shan et al. "Centella Asiatica Extract Loaded BSA Nanoparticles Using The Organic And Conventional C. Asiatica To Improve Bioavailability Activity And Drug Delivery System". Applied Science And Engineering Progress, vol 13, no. 1, 2020. King Mongkut's University Of Technology North Bangkok, doi:10.14416/j.asep.2020.01.001.
Dinis, T. C. P.; Madeira, V. M. C.; Almeida, L. M. Arch. Biochem. Biophys. 1994, 315, 161.

Xie, Wenming et al. "Antioxidant Activity Of Water-Soluble Chitosan Derivatives". Bioorganic & Medicinal Chemistry Letters, vol 11, no. 13, 2001, pp. 1699-1701. Elsevier BV, doi:10.1016/s0960-894x(01)00285-2.

Priyadarsini, K.Indira et al. "Role Of Phenolic O-H And Methylene Hydrogen On The Free Radical Reactions And Antioxidant Activity Of Curcumin". Free Radical Biology And Medicine, vol 35, no. 5, 2003, pp. 475-484. Elsevier BV, doi:10.1016/s0891-5849(03)00325-3.

Liu, Chi-Hsien, and Hsin-Ying Huang. "Antimicrobial Activity Of Curcumin-Loaded Myristic Acid Microemulsions Against Staphylococcus Epidermidis". Chemical And Pharmaceutical Bulletin, vol 60, no. 9, 2012, pp. 1118-1124. Pharmaceutical Society Of Japan, doi:10.1248/cpb.c12-00220.

# Appendix

## Appendix A:

*Table 12: Code of different samples and its description.*

| Sample Code | Description |
|---|---|
| CS-NPs | Chitosan nanoparticles |
| C-CS-NPs | Curcumin encapsulated- chitosan nanoparticles |
| FCS-NPs | Folate-conjugated chitosan nanoparticles |
| C-FCS-NPs | Folate-conjugated chitosan nanoparticles containing curcumin |

*Table 13: Quantity of chemical used in this research*

| Chemical Used | Amount of Chemical | | Amount of solvent | | | % Conc. (m/v) | % Conc. (v/v) |
|---|---|---|---|---|---|---|---|
| | Mass (g) | Volume (ml) | Distilled $H_2O$ (ml) | Ethanol (ml) | 0.5% Acetic acid (ml) | | |
| Tripolyphosphate (TPP) | 2.5 | - | 500 | - | - | 0.5 | - |
| Chitosan | 0.1, 0.2 | - | - | 20 | - | 0.5, 1 | - |
| Acetic acid | - | 2.5 | 500 | - | - | - | 0.5 |
| Ethanol | - | 250 | 500 | - | - | - | 50 |
| NaOH | - | 125 | 500 | - | - | - | 25 |
| Curcumin | 0.001 | - | - | 1 | - | 0.1 | - |

*Table 14: Preparation of 0.1M acetate buffer pH 4.8 for 1 liter in volume*

| Component | Mass | Molarity |
|---|---|---|
| Sodium Acetate (MW: 82.03 g/mol) | 4.791g | 0.0584M |
| Acetic acid (MW: 60.05 g/mol) | 2.498g | 0.0416M |

Acetate buffer pH 4.8 was prepared and adopted from AAT Bioquest methodology (Bioquest International, 2019).

*Table 15: Preparation of 0.1M phosphate buffer pH 7.4 for 1 liter in volume*

| Component | Mass | Molarity |
|---|---|---|
| Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4$- $7H_2O$) (MW: 268.07g/mol) | 20.21g | 0.0754M |
| Sodium phosphate monobasic monohydrate ($NaH_2PO_4H_2O$) (MW: 137.99/mol) | 3.394g | 0.0246M |

Phosphate buffer pH 7.4 was prepared and adopted from total antioxidation activity methodology (J. AOAC International, 2012).

*Table 16: Weight of nanoparticles after freeze dried per ml used for %LE calculation*

| Treatments | Replications | | | | | Average±SD (g/ml) | Average±SD (mg/ml) |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| C-CS-NPs (using CS 0.5%) | 0.0371 | 0.0428 | 0.0562 | 0.0062 | 0.0359 | 0.03564±0.0164 | 35.64±16.3884 |
| C-CS-NPs (using CS 1%) | 0.0542 | 0.0487 | 0.0073 | 0.0098 | 0.0287 | 0.02974±0.0193 | 29.74±19.2874 |
| C-CS-NPs (A) | 0.0143 | 0.0055 | 0.0118 | 0.0115 | 0.0154 | 0.0117±0.0034 | 11.7±3.4334 |
| C-CS-NPs (B) | 0.0151 | 0.0148 | 0.0139 | 0.0121 | 0.0119 | 0.01356±0.0013 | 13.56±1.3351 |
| C-CS-NPs (C) | 0.0113 | 0.0089 | 0.0086 | 0.012 | 0.0113 | 0.01042±0.0014 | 10.42±1.3905 |
| C-FCS-NPs | 0.0092 | 0.0085 | 0.0098 | 0.0079 | 0.0091 | 0.0089±0.00006 | 8.9±0.6481 |

*Table 17: Stock preparation and dilution sequence for preparation of curcumin standard curve*

| Standard no. | Stock curcumin (0.5mg/ml) | Volume $dH_2O$ (ml) | Total volume (ml) | % dilution of stock curcumin | Final conc. (mg/ml) |
|---|---|---|---|---|---|
| Blank | 0 | 10 | 10 | 0 | 0 |
| 1 | 2 | 8 | 10 | 20 | 0.1 |
| 2 | 4 | 6 | 10 | 40 | 0.2 |
| 3 | 6 | 4 | 10 | 60 | 0.3 |
| 4 | 8 | 2 | 10 | 80 | 0.4 |
| 5 | 10 | 0 | 10 | 100 | 0.5 |

weight of vial + curcumin after drying = 15.9943g

weight of vial + curcumin before drying = 15.9774g

weight of curcumin after drying = 0.0169g

weight of used curcumin = 0.0333 – 0.0169 = 0.0164g/30ml = 16.4mg/30ml

⇨ weight of used curcumin = **0.5467 mg/ml (Stock solution)**

*Table 18: Standard curve of curcumin at absorbance of 430nm*

| | Final conc. of curcumin (mg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **0.1** | **0.2** | **0.3** | **0.4** | **0.5** |
| **Abs 430nm** | 0 | 0.399 | 0.888 | 1.202 | 1.528 | 1.997 |
| | 0 | 0.409 | 0.856 | 1.249 | 1.524 | 1.984 |
| | 0 | 0.413 | 0.889 | 1.197 | 1.601 | 1.988 |
| **Average±SD** | 0 | 0.407±0.0059 | 0.878±0.0153 | 1.216±0.0234 | 1.551±0.0354 | 1.990±0.0054 |

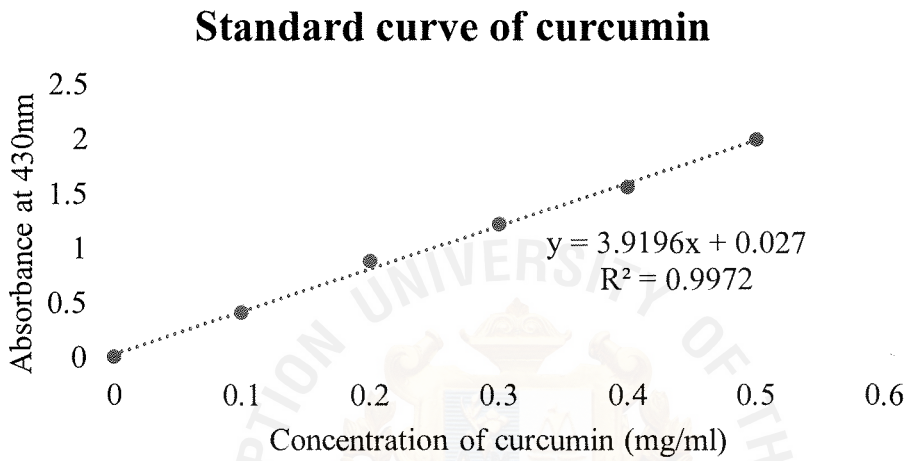



*Figure 26: Standard curve plotted between absorbance at 430nm and concentration of curcumin (mg/ml)*

*Table 19: Absorbance of curcumin solution in ethanol at 10% and 50% ethanol concentration.*

| **Treatment** | **Abs at 430nm** | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| Cur 50% EtOH (1:1 Dilution) | 1.997 | 1.957 | 1.922 | 1.984 |
| Cur 10% EtOH | 0.695 | 0.711 | 0.646 | 0.573 |

| **Treatment** | **Abs at 430nm** | | | | **Average±SD** |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | |
| Cur 50% EtOH | 3.994 | 3.914 | 3.844 | 3.968 | 3.930±0.0574 |
| Cur 10% EtOH | 0.695 | 0.711 | 0.646 | 0.573 | 0.6562±0.0536 |

*Table 20: Absorbance of free curcumin in supernatant from C-CS-NPs using A, B, and C treatment, from initial curcumin concentration of 0.5467mg/ml in 50% ethanol*

| Treatment | Abs at 430nm of supernatant | | | | | Average | SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| A | 0.088 | 0.092 | 0.092 | 0.089 | 0.091 | 0.090 | 0.002 |
| B | 0.09 | 0.083 | 0.09 | 0.092 | 0.081 | 0.087 | 0.004 |
| C | 0.079 | 0.085 | 0.084 | 0.092 | 0.087 | 0.085 | 0.004 |

*Table 21: Raw data of percent encapsulation and loading efficiency of free curcumin and curcumin concentration in CS-NPs using A, B, and C treatment, from initial curcumin concentration of 0.5467mg/ml in 50% ethanol*

| Treatment | Abs 430nm | Final conc. Free curcumin | Final conc. Free curcumin x 1:1 Dilution | Cur conc. in NPs | %Encapsulation efficiency (%EE) | %Loading efficiency (%LE) |
|---|---|---|---|---|---|---|
| A | 0.088 | 0.01556281 | 0.03112563 | 0.51557437 | 94.30 | 4.40 |
| | 0.092 | 0.01658332 | 0.03316665 | 0.51353335 | 93.93 | 4.38 |
| | 0.092 | 0.01658332 | 0.03316665 | 0.51353335 | 93.93 | 4.38 |
| | 0.089 | 0.01581794 | 0.03163588 | 0.51506412 | 94.21 | 4.40 |
| | 0.091 | 0.0163282 | 0.03265639 | 0.51404361 | 94.02 | 4.39 |
| Average±SD | 0.090±0.002 | 0.0162±0.0004 | 0.0324±0.0008 | 0.5143±0.0008 | 94.08±0.1516 | 4.39±0.0071 |
| B | 0.09 | 0.01607307 | 0.03214614 | 0.51455386 | 94.1199676 | 3.79464499 |
| | 0.083 | 0.01428717 | 0.02857434 | 0.51812566 | 94.7733045 | 3.82098569 |
| | 0.09 | 0.01607307 | 0.03214614 | 0.51455386 | 94.1199676 | 3.79464499 |
| | 0.092 | 0.01658332 | 0.03316665 | 0.51353335 | 93.9332999 | 3.7871191 |
| | 0.081 | 0.01377692 | 0.02755383 | 0.51914617 | 94.9599722 | 3.82851158 |
| Average±SD | 0.087±0.004 | 0.0154±0.0011 | 0.0307±0.0022 | 0.5160±0.0022 | 94.38±0.4064 | 3.81±0.0164 |
| C | 0.079 | 0.01326666 | 0.02653332 | 0.52016668 | 95.1466399 | 4.99200269 |
| | 0.085 | 0.01479743 | 0.02959486 | 0.51710514 | 94.5866368 | 4.96262131 |
| | 0.084 | 0.0145423 | 0.0290846 | 0.5176154 | 94.6799706 | 4.96751823 |
| | 0.092 | 0.01658332 | 0.03316665 | 0.51353335 | 93.9332999 | 4.92834309 |
| | 0.087 | 0.01530768 | 0.03061537 | 0.51608463 | 94.3999691 | 4.95282754 |
| Average±SD | 0.085±0.004 | 0.0149±0.0010 | 0.0298±0.0021 | 0.5169±0.0021 | 94.55±0.3942 | 4.96±0.0207 |

$y = 3.9196x + 0.027$

**Find final conc. of free curcumin**

Ex1: $0.090 = 3.9196x + 0.027$

$$\Rightarrow x = \frac{0.090-0.027}{3.9196} = 0.0156 \text{ mg/ml}$$

**Final conc. Free curcumin x (1:1) Dilution**

x = 0.0162

Final conc. free curcumin x Dilution = 0.0161 x 2 = 0.0321 mg/ml

**Concentration of curcumin in NPs**

Conc. cur in NPs = Stock conc. – Final conc. free curcumin x Dilution
= 0.5467 mg/ml – 0.0321 mg/ml
= 0.5140 mg/ml

**% Encapsulation efficiency**

$$EE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{total amount of curcumin}} \times 100$$

$$EE\% = \frac{0.5467-0.0311}{0.5467} \times 100 = 94.11\%$$

$$LE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{Mass of C-CS-NPs after freeze dried}} \times 100$$

$$LE\% = \frac{0.5467-0.0162}{11.7} \times 100 = 4.41\%$$

*Table 22: Absorbance of free curcumin in supernatant from C-CS-NPs using 0.5% and 1% CS, from initial curcumin concentration of 0.5467mg/ml in 50% ethanol*

| Treatment | Abs at 430nm of supernatant | | | | | Average±SD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| C-CS-NPs (using CS 0.5%) | 0.146 | 0.141 | 0.147 | 0.143 | 0.145 | 0.1444±0.0022 |
| C-CS-NPs (using CS 1%) | 0.143 | 0.132 | 0.140 | 0.140 | 0.154 | 0.1418±0.0071 |

*Table 23: Raw data of percent encapsulation efficiency (%EE) and loading efficiency (%LE) of free curcumin and curcumin concentration in CS-NPs using 0.5% and 1% CS, from initial curcumin concentration of 0.5467mg/ml in 50% ethanol*

| Replicate | Abs 430nm | Final conc. Free curcumin | Final conc. Free curcumin x 1:1 Dilution | Cur conc. in NPs | %EE | %LE |
|---|---|---|---|---|---|---|
| C-CS-NPs (using CS 0.5%) | 0.146 | 0.0304 | 0.0607 | 0.486 | 88.89 | 1.36 |
| | 0.141 | 0.0291 | 0.0582 | 0.4885 | 89.36 | 1.37 |
| | 0.147 | 0.0306 | 0.0612 | 0.4855 | 88.8 | 1.36 |
| | 0.143 | 0.0296 | 0.0592 | 0.4875 | 89.17 | 1.36 |
| | 0.145 | 0.0301 | 0.0602 | 0.4865 | 88.99 | 1.36 |
| Average±SD | 0.1444±0.0022 | 0.0300±0.0005 | 0.0599±0.0011 | 0.4868±0.0011 | 89.04±0.20 | 1.37±0.030 |
| C-CS-NPs (using CS 1% | 0.143 | 0.02959486 | 0.05918971 | 0.48751029 | 89.17 | 1.63 |
| | 0.132 | 0.02678845 | 0.0535769 | 0.4931231 | 90.19 | 1.65 |
| | 0.14 | 0.02882947 | 0.05765894 | 0.48904106 | 89.45 | 1.64 |
| | 0.14 | 0.02882947 | 0.05765894 | 0.48904106 | 89.45 | 1.64 |
| | 0.154 | 0.03240127 | 0.06480253 | 0.48189747 | 88.14 | 1.62 |
| Average±SD | 0.1418±0.0071 | 0.0293±0.0018 | 0.0586±0.0036 | 0.4881±0.0036 | 89.29±0.66 | 1.64±0.0122 |

$y = 3.9196x + 0.027$

**Find final conc. of free curcumin**

Ex1: $0.1444 = 3.9196x + 0.027$

$\Rightarrow x = \frac{0.1444-0.027}{3.9196} = 0.03035$ mg/ml

**Final conc. Free curcumin x (1:1) Dilution**

$x = 0.0304$

Final conc. free curcumin x Dilution = 0.03035 x 2 = 0.0607 mg/ml

**Concentration of curcumin in NPs**

Conc. cur in NPs = Stock conc. – Final conc. free curcumin x Dilution

= 0.5467 mg/ml – 0.0607 mg/ml

= 0.4861 mg/ml

**% Encapsulation efficiency**

$$EE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{total amount of curcumin}} \times 100$$

$$EE\% = \frac{0.5467-0.0607}{0.5467} \times 100 = 88.89\%$$

**% Loading efficiency**

$$LE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{Mass of C-CS-NPs after freeze dried}} \times 100$$

$$LE\% = \frac{0.5467 - 0.0607}{35.64} \times 100 = 1.36\%$$

*Table 24: Raw data of percent encapsulation and loading efficiency of free curcumin and curcumin concentration in FCS-NPs using 0.5% CS, treatment C, from initial curcumin concentration of 0.5467mg/ml in 50% ethanol*

| Replicate | Abs 430nm | Final conc. Free curcumin | Final conc. Free curcumin x 1:1 Dilution | Cur conc. in NPs | %Encapsulation efficiency (%EE) | %Loading efficiency (%LE) |
|---|---|---|---|---|---|---|
| 1 | 0.075 | 0.012246 | 0.0245 | 0.5222 | 95.52 | 5.87 |
| 2 | 0.054 | 0.006888 | 0.0138 | 0.5329 | 97.48 | 5.99 |
| 3 | 0.052 | 0.006378 | 0.0128 | 0.5339 | 97.67 | 6.00 |
| 4 | 0.044 | 0.004337 | 0.0087 | 0.538 | 98.41 | 6.04 |
| 5 | 0.054 | 0.006888 | 0.0138 | 0.5329 | 97.48 | 5.99 |
| 6 | 0.055 | 0.007144 | 0.0143 | 0.5324 | 97.39 | 5.98 |
| 7 | 0.056 | 0.007399 | 0.0148 | 0.5319 | 97.29 | 5.98 |
| 8 | 0.06 | 0.008419 | 0.0168 | 0.5299 | 96.92 | 5.95 |
| 9 | 0.054 | 0.006888 | 0.0138 | 0.5329 | 97.48 | 5.99 |
| 10 | 0.056 | 0.007399 | 0.0148 | 0.5319 | 97.29 | 5.98 |
| Average ±SD | 0.056± 0.0074 | 0.0074±0.0019 | 0.0148±0.0038 | 0.5319±0.0038 | 97.29±0.6922 | 5.98±0.0424 |

$y = 3.9196x + 0.027$

**Find final conc. of free curcumin**

Ex1: $0.075 = 3.9196x + 0.027$

$$\Rightarrow x = \frac{0.075 - 0.027}{3.9196} = 0.012246 \text{ mg/ml}$$

**Final conc. Free curcumin x (1:1) Dilution**

$x = 0.012246$

Final conc. free curcumin x Dilution $= 0.012246 \times 2 = 0.0245$ mg/ml

**Concentration of curcumin in NPs**

Conc. cur in NPs = Stock conc. – Final conc. free curcumin x Dilution

= 0.5467 mg/ml – 0.0245 mg/ml

= 0.5222 mg/ml

**% Encapsulation efficiency**

$$EE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{total amount of curcumin}} \times 100$$

$$EE\% = \frac{0.5467 - 0.0245}{0.5467} \times 100 = 95.52\%$$

**%Loading efficiency**

$$LE\% = \frac{\text{Total amount of curcumin} - \text{Amount of free curcumin}}{\text{Mass of C-FCS-NPs after freeze dried}} \times 100$$

$$LE\% = \frac{0.5467-0.0245}{8.9} \times 100 = 5.87\%$$

*Table 25: Result of DPPH as absorbance value of sample at 517nm*

| | Abs 517nm | | |
|---|---|---|---|
| | **1** | **2** | **3** |
| Blank (methanol) | 0 | 0 | 0 |
| Control (acetic acid) | 0.456 | 0.438 | 0.448 |
| CS-NPs | 0.406 | 0.429 | 0.372 |
| C-CS-NPs | 0.426 | 0.423 | 0.417 |
| C-FCS-NPs | 0.325 | 0.297 | 0.318 |

DPPH scavenging effect (inhibition) = $(\frac{Ao-A1}{Ao})$x 100

$A_0$ = the absorbance of the control reaction

$A_1$ = absorbance in presence of sample

% Radical scarvenging = $(\frac{0.456-0.406}{0.456})$x 100 = 10.96

*Table 26: Result of DPPH as % radical scavenging*

| | % Radical scavenging | | | Average±SD |
|---|---|---|---|---|
| Blank (methanol) | 0 | 0 | 0 | 0 |
| Control (acetic acid) | - | - | - | - |
| CS-NPs | 10.96 | 6.62 | 12.54 | 10.03±2.49 |
| C-CS-NPs | 6.58 | 3.42 | 6.92 | 5.64±1.57 |
| C-FCS-NPs | 28.73 | 32.19 | 29.02 | 29.98±1.57 |

*Table 27: Stock preparation and dilution sequence for preparation of gallic acid standard curve*

| | Gallic acid concentration (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **6.25** | **12.5** | **25** | **50** | **100** |
| **Abs 765nm** | 0 | 0.114 | 0.251 | 0.5 | 0.957 | 1.706 |
| | 0 | 0.126 | 0.254 | 0.503 | 0.96 | 1.698 |
| | 0 | 0.117 | 0.241 | 0.503 | 0.964 | 1.742 |
| **Mean±SD** | 0 | 0.119±0.0051 | 0.249±0.0056 | 0.502±0.0014 | 0.960±0.0029 | 1.715±0.0191 |

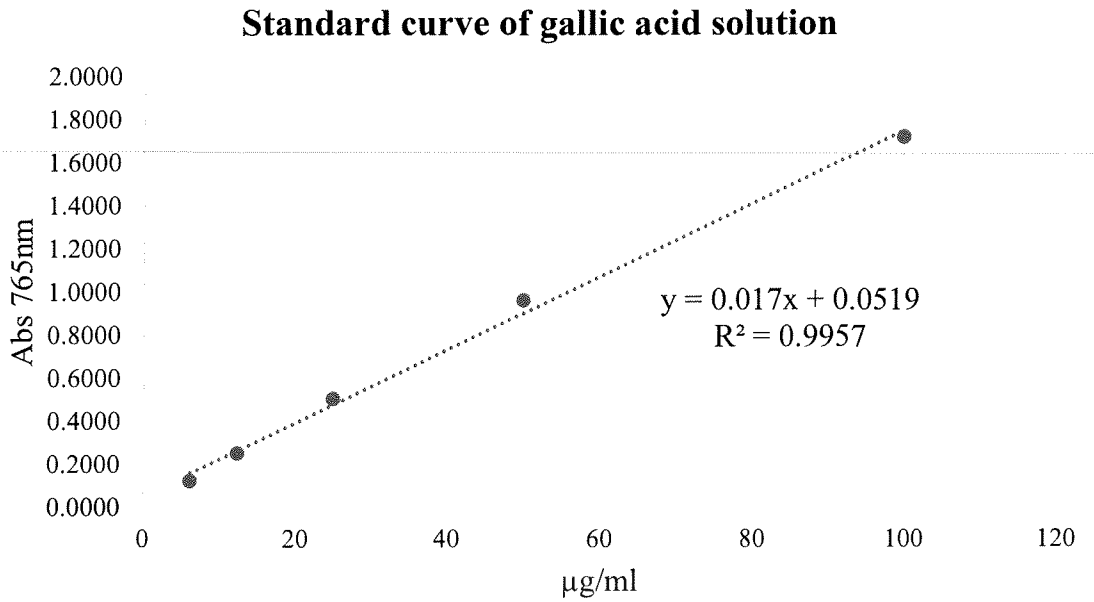



*Figure 27: Standard curve of gallic acid for evaluation of total phenolic content*

*Table 28: Result of total phenolic content as absorbance value at 765nm*

| | Abs 765nm | | |
|---|---|---|---|
| | **1** | **2** | **3** |
| Blank (water) | 0 | 0 | 0 |
| CS-NPs | 0.051 | 0.033 | 0.048 |
| C-CS-NPs | 0.098 | 0.087 | 0.109 |
| C-FCS-NPs | 0.124 | 0.115 | 0.135 |

*Table 29: Result of total phenolic content as mg GAE/ml sample*

| | mg GAE/ml | | | Average±SD |
|---|---|---|---|---|
| Blank (water) | - | - | - | - |
| CS-NPs | 0 | 0 | 0 | 0 |
| C-CS-NPs | 2.712 | 2.065 | 3.359 | 2.712±0.528 |
| C-FCS-NPs | 4.241 | 3.712 | 4.888 | 4.28±0.481 |

$y = 0.017x + 0.0519$

**Find total phenolic content (mg GAE/ml):**

Ex1: $0.051 = 0.017x + 0.0519$

$\Rightarrow x = \frac{0.051 - 0.0519}{0.017} = 0$ mg GAE/ml

Ex2: $0.098 = 0.017x + 0.0519$

$\Rightarrow x = \frac{0.098 - 0.0519}{0.017} = 2.712$ mg GAE/ml

*Table 30: Result of chelating ability of sample as absorbance at 562nm*

| Sample Formulation | Abs 562nm | | | | | Average±SD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Blank | - | - | - | - | - | - |
| Control | 0.834 | 0.841 | 0.842 | 0.842 | 0.843 | 0.8404±0.0033 |
| CS-NPs | 0.036 | 0.029 | 0.025 | 0.035 | 0.023 | 0.0296±0.0052 |
| C-CS-NPs | 0.007 | 0.015 | 0.014 | 0.010 | 0.011 | 0.0114±0.0029 |
| C-FCS-NPs | 0.007 | 0.006 | 0.006 | 0.007 | 0.018 | 0.0088±0.0046 |

$$Chelating\ ability\ (\%) = (\frac{Acontrol - Asample}{Acontrol}) \times 100$$

$$Chelating\ ability\ (\%) = (\frac{0.834 - 0.036}{0.836}) \times 100 = 95.68\%$$

*Table 31: Result of chelating ability of samples as %chelating ability*

| Sample Formulation | Chelating ability (%) | | | | | Average±SD |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Blank | - | - | - | - | - | - |
| Control | - | - | - | - | - | - |
| CS-NPs | 95.68 | 96.55 | 97.03 | 95.84 | 97.27 | 96.48±0.63 |
| C-CS-NPs | 99.16 | 98.22 | 98.34 | 98.81 | 98.69 | 98.64±0.34 |
| C-FCS-NPs | 99.16 | 99.29 | 99.29 | 99.17 | 97.86 | 98.95±0.55 |

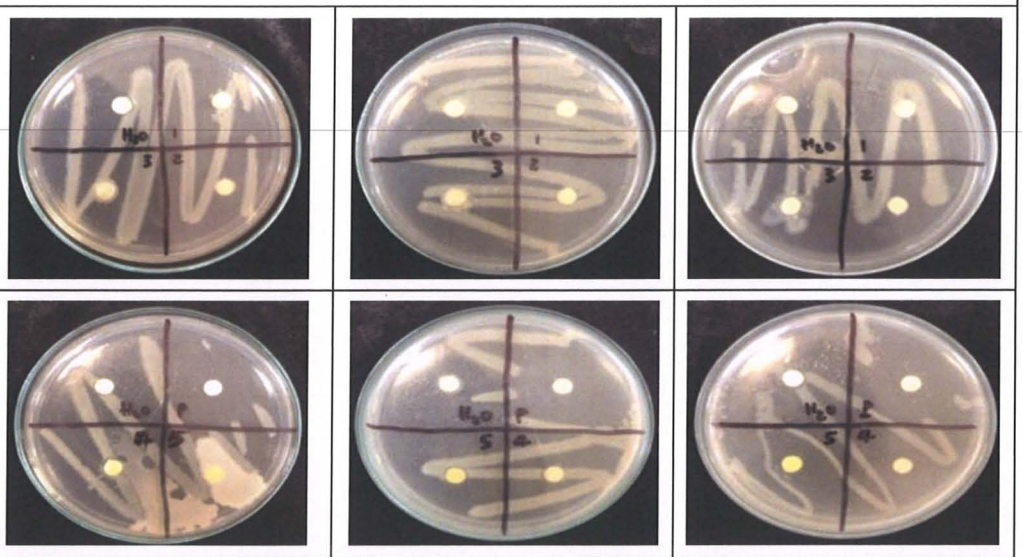
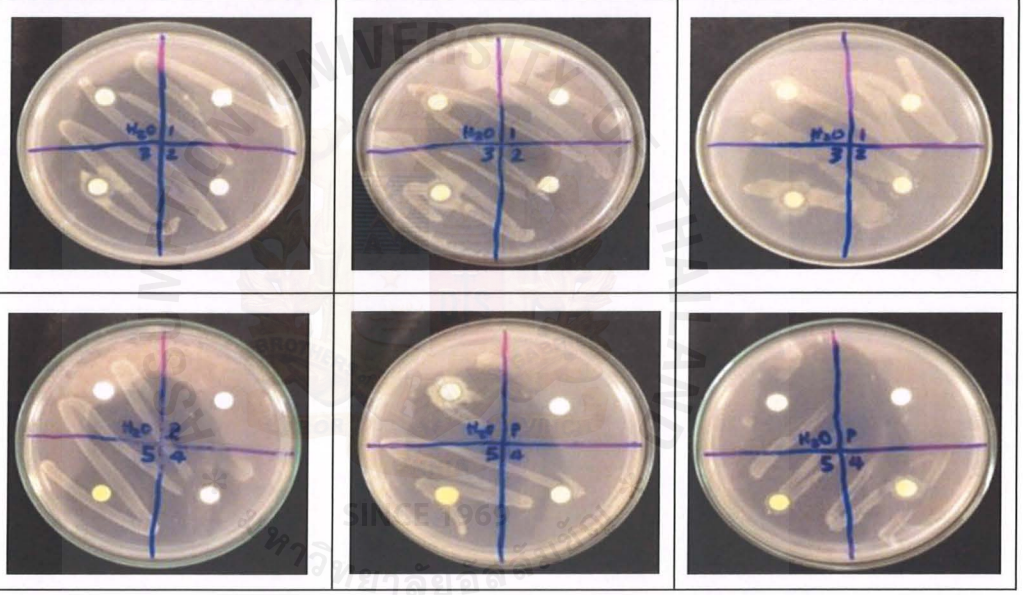
| Pathogen | Pictures of disk diffusion |
|---|---|
| *E. coli* |  |
| *S. aureus* |  |

*Figure 28: Result of antimicrobial activity of E. coli and S. aureus of (1:CS-NPs, 2: C-CS-NPs, 3: FCS-NPs, 4: C-FCS-NPs, 5: Pure curcumin) using disk diffusion method*

*Table 32: Result of antimicrobial activity of E. coli and S. aureus as diameter of inhibition zone of (1:CS-NPs, 2: C-CS-NPs, 3: FCS-NPs, 4: C-FCS-NPs, 5: Pure curcumin) using disk diffusion method*

| | **Antimicrobial activity of 5 sample formulations** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Water ($H_2O$) | Ampicillin (P) | CS-NPs (1) | C-CS-NPs (2) | FCS-NPs (3) | C-FCS-NPs (4) | Pure curcumin (5) |
| | Diameter of inhibition zone (mm) | | | | | | |
| *E. coli* | 6 | 28 | 6 | 6 | 6 | 8 | 6 |
| | 6 | 28 | 6 | 6 | 6 | 8 | 8 |
| | 6 | 28 | 6 | 7 | 6 | 10 | 7 |
| Average± SD | 6 | 28 | 6 | 6.33±0.47 | 6 | 8.67±0.94 | 7±0.82 |
| *S. aureus* | 6 | 36 | 6 | 7 | 8 | 16 | 7 |
| | 6 | 32 | 6 | 6 | 8 | 10 | 8 |
| | 6 | 40 | 6 | 10 | 8 | 8 | 9 |
| Average± SD | 6 | 36±3.26 | 6 | 7.67±1.69 | 8 | 11.33±3.39 | 8±0.82 |

## Appendix B: R-program Code and Output

## Code:

Independent t-test

Statistics >> means >> Independent sample t-test >> Assume equal variances? "Yes"

| Group | Response variable | |
|---|---|---|
| -trt | -y | |
| Alternative hypothesis | Confidence Level | Assume equal variance |
| -Two-sided | -0.95 | -Yes |

One way ANOVA for RCBD

```
attach(Dataset)
Dataset
RCBD<-aov(y~trt+rep,data=Dataset)
summary(RCBD)
```

Comparison in Duncan

```
library(agricolae)
attach(Dataset)
Dataset
model<-aov(y~trt, data=Dataset)
comparison<-duncan.test(model,"trt",main="y dealt with different trt")
duncan.test(model,"trt",alpha=0.05,console=TRUE)
```

## Output:

**Curcumin solutions:**

| **Two Sample t-test** | |
|---|---|
| | |
| data: y by trt | |
| t = 72.1195, df = 6, **p-value = 4.783e-10** | |
| alternative hypothesis: true difference in means is not equal to 0 | |
| 95 percent confidence interval: | |
| 3.162676 | 3.384824 |
| sample estimates: | |
| mean in group 1 | mean in group 2 |
| 3.9300* | 0.65625 |

*p-value < 0.05; significantly different

**Optimization of encapsulation method:**

**Treatments:**

A (Curcumin added last after Chitosan and TPP)

B (Curcumin mix with TPP before encapsulate into chitosan)

C (Curcumin mix with chitosan then TPP added last)

***** % Encapsulation Efficiency**

| **Source** | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr > F** |
|---|---|---|---|---|---|
| **Treatment** | 2 | 0.5705 | 0.2852 | 1.9941 | 0.1787 |
| **Residuals** | 12 | 1.7167 | 0.1430 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

Pr>F < 0.05; significantly different

| **Level of Treatment** | **N** | **% Encapsulation efficiency** | |
|---|---|---|---|
| | | **Mean** | **Std. err** |
| **A** | 5 | 94.078 | 0.0754586 |
| **B** | 5 | 94.380 | 0.2032486 |
| **C** | 5 | 94.550 | 0.1981666 |

Mean Square Error: 0.14379, alpha: 0.05; Df Error: 12

| **Means with the same letter are not significantly different.** | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | C | 94.55 |
| a | B | 94.38 |
| a | A | 94.08 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

*** % Loading Efficiency

| Source | Df | Sum Sq | Mean Sq | F value | Pr > F |
|---|---|---|---|---|---|
| **Treatment** | 2 | 3.330 | 1.6648 | 5257 | <2e-16 *** |
| **Residuals** | 12 | 0.004 | 0.0003 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| Level of Treatment | N | % Loading efficiency | |
|---|---|---|---|
| | | Mean | Std. err |
| **A** | 5 | 4.390 | 0.004472136 |
| **B** | 5 | 3.804 | 0.008717798 |
| **C** | 5 | 4.958 | 0.009695360 |

Mean Square Error: 0.0003166667, alpha: 0.05 ; Df Error: 12

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | C | 4.958 |
| b | A | 4.39 |
| c | B | 3.804 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

**Two different chitosan concentrations in the encapsulation of curcumin into chitosan nanoparticles (C-CS-NPs):**

*** % Encapsulation Efficiency

| Two Sample t-test | |
|---|---|
| | |
| data: y by trt | |
| t = -0.6871, df = 8, **p-value = 0.5114** | |
| alternative hypothesis: true difference in means is not equal to 0 | |
| 95 percent confidence interval: | |
| -1.0367367 | 0.5607367 |
| sample estimates: | |
| mean in group 1 | mean in group 2 |
| 89.042 | 89.280 |

***p-value < 0.05; significantly difference

*** % Loading Efficiency

| Two Sample t-test |
|---|
| |
| data: y by trt |
| t = -50.0253, df = 8, **p-value = 2.823e-11** |
| alternative hypothesis: true difference in means is not equal to 0 |
| 95 percent confidence interval: |

| | |
|---|---|
| -0.2866305 | -0.2613695 |
| sample estimates: | |
| mean in group 1 | mean in group 2 |
| 1.362 | 1.636* |

***p-value < 0.05; significant difference

Therefore, there is significant difference between 1%CS and 0.5%CS.

**DPPH method of antioxidant assay:**

| No | Treatments |
|---|---|
| (1) | CS-NPs |
| (2) | C-CS-NPs |
| (3) | C-FCS-NPs |

| Source | Df | Sum Sq | Mean Sq | F value | Pr > F |
|---|---|---|---|---|---|
| **Treatment** | 2 | 1009.2 | 504.6 | 90.14 | 3.34e-05 *** |
| **Residuals** | 6 | 33.6 | 5.6 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| Level of Treatment | N | % Scavenging | |
|---|---|---|---|
| | | Mean | Std. err |
| **1** (CS-NPs) | 3 | 10.041 | 1.769 |
| **2** (C-CS-NPs) | 3 | 5.641 | 1.113 |
| **3** (C-FCS-NPs) | 3 | 29.979 | 1.109 |

Mean Square Error: 5.597888, alpha: 0.05; Df Error: 6

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | 3 (C-FCS-NPs) | 29.98 |
| b | 1 (CS-NPs) | 10.04 |
| b | 2 (C-CS-NPs) | 5.641 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

**Total Phenolic Content Assay:**

| Source | Df | Sum Sq | Mean Sq | F value | Pr > F |
|---|---|---|---|---|---|
| **Treatment** | 2 | 28.136 | 14.068 | 55.13 | 0.000137 *** |
| **Residuals** | 6 | 1.531 | 0.255 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| Level of Treatment | N | % Scavenging | |
|---|---|---|---|
| | | Mean | Std. err |
| **1** (CS-NPs) | 3 | 0.000 | 0.000 |
| **2** (C-CS-NPs) | 3 | 2.712 | 0.373 |
| **3** (C-FCS-NPs) | 3 | 4.280 | 0.341 |

Mean Square Error: 0.2551711, alpha: 0.05; Df Error: 6

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | 3 (C-FCS-NPs) | 4.28 |
| b | 2 (C-CS-NPs) | 2.712 |
| c | 1 (CS-NPs) | 0 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

**Chelating ability:**

| **Source** | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr > F** |
|---|---|---|---|---|---|
| **Treatment** | 2 | 18.259 | 9.129 | 26.98 | 0.000036 *** |
| **Residuals** | 12 | 4.061 | 0.338 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| **Level of Treatment** | **N** | **% Scavenging** | |
|---|---|---|---|
| | | **Mean** | **Std. err** |
| **1** (CS-NPs) | 5 | 96.474 | 0.315 |
| **2** (C-CS-NPs) | 5 | 98.644 | 0.169 |
| **3** (C-FCS-NPs) | 5 | 98.954 | 0.275 |

Mean Square Error: 0.3383967, alpha: 0.05; Df Error: 12

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | 3 (C-FCS-NPs) | 98.95 |
| a | 2 (C-CS-NPs) | 98.64 |
| b | 1 (CS-NPs) | 96.47 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

**Antimicrobial activity:**

| **No** | **Treatments** |
|---|---|
| (1) | CS-NPs |
| (2) | C-CS-NPs |
| (3) | FCS-NPs |
| (4) | C-FCS-NPs |
| (5) | Pure curcumin |

*** ***Escherichia coli***

| **Source** | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr > F** |
|---|---|---|---|---|---|
| **Treatment** | 4 | 15.067 | 3.767 | 7.062 | 0.00573 ** |
| **Residuals** | 10 | 5.333 | 0.533 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| Level of Treatment | N | Inhibition Diameter (mm) | |
|---|---|---|---|
| | | Mean | Std. err |
| **1** (CS-NPs) | 3 | 6.00 | 0.000 |
| **2** (C-CS-NPs) | 3 | 6.33 | 0.333 |
| **3** (FCS-NPs) | 3 | 6.00 | 0.000 |
| **4** (C-FCS-NPs) | 3 | 8.67 | 0.667 |
| **5** (Pure curcumin) | 3 | 7.00 | 0.577 |

Mean Square Error: 0.5333333, alpha: 0.05 ; Df Error: 10

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | 4 (C-FCS-NPs) | 8.667 |
| b | 5 (Pure curcumin) | 7 |
| b | 2 (C-CS-NPs) | 6.333 |
| b | 1 (CS-NPs) | 6 |
| b | 3 (FCS-NPs) | 6 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |

*** ***Staphylococcus aureus***

| Source | Df | Sum Sq | Mean Sq | F value | Pr > F |
|---|---|---|---|---|---|
| **Treatment** | 4 | 45.07 | 11.267 | 2.485 | 0.111 |
| **Residuals** | 10 | 45.33 | 4.533 | | |
| --- | | | | | |
| Signif. codes: | 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | |

| Level of Treatment | N | Inhibition Diameter (mm) | |
|---|---|---|---|
| | | Mean | Std. err |
| **1** (CS-NPs) | 3 | 6.00 | 0.000 |
| **2** (C-CS-NPs) | 3 | 7.67 | 1.202 |
| **3** (FCS-NPs) | 3 | 8.00 | 0.000 |
| **4** (C-FCS-NPs) | 3 | 11.33 | 2.404 |
| **5** (Pure curcumin) | 3 | 8.00 | 0.577 |

Mean Square Error: 4.533333, alpha: 0.05 ; Df Error: 10

| Means with the same letter are not significantly different. | | |
|---|---|---|
| **Duncan Grouping** | **Treatments** | **Mean** |
| a | 4 (C-FCS-NPs) | 11.33 |
| ab | 3 (FCS-NPs) | 8 |
| ab | 5 (Pure curcumin) | 8 |
| ab | 2 (C-CS-NPs) | 7.667 |
| b | 1 (CS-NPs) | 6 |
| > | | |
| duncan.test(model,"trt",alpha=0.05,console=TRUE) | | |