Centella asiatica Extract Loaded BSA Nanoparticles Using the Organic and Conventional *C. asiatica* to Improve Bioavailability Activity and Drug Delivery System.

Ms. Patteera Chanapongpisan

ID: 591-9702

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Master of Science in Food Biotechnology

Department of Food Technology

Assumption University

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Advisor: Assist. Prof. Dr. Patchanee Yasurin

Level of study: Master of Science

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(Assist. Prof. Dr. Patchanee Yasurin)

Thesis Advisor

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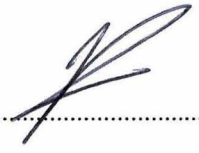
The manuscript has been read and in satisfaction of the thesis requirements for the Master of Science in Food Biotechnology



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Last but not least, the grateful for my mother and my family whose supports and encourages me in any depressed moment. They made my battery life full feel all the time even though when I felt no more power to move on but they are my goal to succeed. Thank you all.

Ms. Patteera Chanapongpisan

September, 2018

ABSTRACT

Centella asiatica Extract Loaded BSA Nanoparticles Using the Organic and Conventional _C. asiatica_ to Improve Bioavailability Activity and Drug Delivery System.

Patteera Chanapongpisan

Biotechnology Faculty, Assumption University, Bangkok, 10240, Thailand

Centella asiatica is Thai traditional herb which is very famous and also one of top five of Thailand Champion Herbal Products by the Department for Development of Thai Transitional and Alternative Medicine, Ministry of Public Health. *C. asiatica* crude extracts showed excellent potential *in-vitro* but the problem was occurred *in-vivo* due to their poor lipid solubility or improper molecular size, resulting in poor bioavailability. The nanoparticles were prepare by desolvation method using three different ratio *C. asiatica* crude chloroform extracts: BSA (1:2, 1:3, and 1:4). Then, it was tested with the well agar diffusion method was used for evaluating antibacterial activity with different concentration (100, 200, and 300 µg/ml) against seven food borne pathogens, the antioxidant activity with two different assay as FRAP assay and DPPH assay to evaluate antioxidant activity. The entrapment efficiency, loading efficiency and solubility also use to test the efficiency of the nanoparticles. Next, it was test for release kinetic *in Vitro* during the whole period of 6 hours in both artificial gastric and intestinal juice. For the result can be seen that the different ratio of the concentation of *C. asiatica* to BSA (1:2, 1:3, and 1:4) and conventional and organic not signigicant effect to the bioavailability of *C. asiatica* extract -loaded BSA nanoparticles. ($p<0.05$) while the used of solvent extraction as Ethanol, Chloroform and hexane was significant different for the bioavailability of *C. asiatica* extract -loaded BSA nanoparticles, the highest was ethanol extraction solvent. ($p>0.05$) So, the most effective of economic and less consumption is the *C. aisatica* loaded BSA nanoparticles with 1:2 ratio and extract with ethanol because it used less of BSA to prepare and get more biological activity than chloroform and hexane extract.

Keyword; *Centella asiatica* , BSA-nano particles, Bioavailability, Organic

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INTRODUCTION

Centella asiatica is top five of Thailand Champion Herbal Product (TCHP) needed in the world market and consisted of high efficiency which can be used to produce pharmacy, make up and in food industry. It has been already investigated for its bioavailability activities, antimicrobial activity, antioxidant activity, anti-inflammatory activity, wound healing activity and anticancer activity. (Yasurin P et al., 2016) the major bioactive compounds of *Centella* extract are triterpene glycosides (saponins), such as asiaticoside and madecassoside, and their respective aglycones (sapogenins), including asiatic acid and madecassic acid. (Jacinda T. J. and Ian A., 2009)

Although *C. asiatica* showed their extra ordinary potential *in-vitro* but less or no *in-vivo* activity due to their poor lipid solubility and improper molecular size, resulting in poor absorption, slow delivery, poor dosing and poor bioavailability. It is not easy for water soluble biological active compounds to enter through cell membrane of both human and pathogenic microorganism which has hydrophobic characteristic. (Kesonbuakao K. And Yasurin P., 2016) these affect to the biological absorption activity and loading system of both in human and in microbial.

The nanotechnology is one of the best ways to solve this problem by improve drug delivery system of crude extract and increase the efficiency of drugs. This technology also can reduce cost of biological extraction and the process of obtaining the pure biological active compound. In United State, organic food production is a $16 billion a year industry, according to the Organic Trade Association (OTA) and it's rising up precipitously. The organic product also growing at a sizzling rate of 17-20% per year as compared to a glacial rate of 2-3% for conventional products (OTA, All thing organic conference, 2015)

The aim of this study was to improve the bioavailability and drug delivery system of *C. asiatica* by BSA-nanoparticles and to compare the organic and conventional to increase product's value.

LITERATURE REVIEW

Centella asiatica

C. asiatica is one of five Thailand Champion Herbal Product (TCHP) needed in the world market from Strategic Plan Development Herbs: Thai Herbs - World Product (2013-2017) by the Department for Development of Thai Transitional and Alternative Medicine, Ministry of Public Health. *C. asiatica* is belonging to the family *Umbellifere(Apiceae)* commonly found in most tropical and subtropical countries (Kashmira J. G. et al., 2010). It has small fan shaped green leaves, thin, alternate with long petioles (Llkay E. O., 2012). It has been already investigated for its bioavailability activities, antimicrobial activity, antioxidant activity, anti-inflammatory activity, wound healing activity and anticancer activity. (Yasurin P et al., 2016) it can be used as traditional drug to decrease blood pressure, cure the fresh wound, heal bruised and diuretic (Ullah et al., 2009). Normally the whole part of *C. asiatica* including steam, leaves, and aerial part areused. In Ayurveda, an Indian system of medicine, *C. asiatica* is used in the management of central nervous system, skin and gastrointestinal disorder (Subathra et al., 2005). The major biologically active compounds of *C. asiatica* extract are monoterpenes, sesquiterpene, triterpenoids (Rattanakom, 2015).

Figure 1: the picture of Centella asiatica

(http://www.khawchawbannews.com/board/index.php?topic=25.0)

Biological activities

The major bioactive compounds of *C. asiatica* extract are triterpene glycosides (saponins), such as asiaticoside and madecassoside, and their respective aglycones (sapogenins), including asiatic acid and madecassic acid. (Jacinda T. J. and Ian A., 2009) Asiaticoside appears to be poorly soluble in both ethanol and water and more soluble in chloroform, ethyl acetate, and methanol. (Verma R.K. et al., 1999) The triterpene saponins and their sapogenins are mainly responsible for the wound healing and vascular effects by inhibiting the production of collagen at the wound site. (Kashmira J. G. et al., 2010)

Table 1 Main active compounds group of C. asiatica

Main groups	Active compound	References
Terpenoids	Triterpenes, asiaticoside, centelloside, madecassoside,brahmoside, brahminoside (saponin glycosides), asiaticentoic acid, centellic acid,centoic acid, madecassicacid, terminolic acid and betulic acid.	Barnes et al.,2007; Jamil et al., 2007
	Various terpenoids: β-caryophyllene, trans β-farneseneand germacrene D (sesquiterpenes), α-pinene and β-pinene.	Barnes et al.,2007; Jamil et al., 2007
Phenols	Flavonoids: Kaempferol, kaempferol-3-o-dglucuronide,castilliferol, quercetin, quercetin-3-o-β-dglucuronide,castillicetin, apigenin, rutin, luteolin,naringin	Bhandari etal.,2007; Zheng and Qin,2007; Chong NJ and Aziz, 2011

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	Phenylpropanoids: Rosmarinic acid, chlorogenic acid,3,4-di-o-caffeoyl quinic acid, 1,5-di-o-caffeoyl quinic acid, 3,5-di-o-caffeoyl quinic acid, 4,5-di-o-caffeoylquinic acid, isochlorogenic acid	Chong NJ and Aziz, 2011
	Tannin: Tannin, phlobatannin	Chong NJ and Aziz, 2011

C. asiatica 95% Ethanol extracted has the ability in antimicrobial activity to restrain the growth of *Bacillus cereus* and *Listeria monocytogenes* at normal, osmotic stress and high acidic conditions states. (Pitinidhipat N. and Yasurin P., 2012; Utami V.C. et al., 2012) also can inhibit the growth of pathogenic bacteria in intestines (Mamtha B. et al.,2004) and the activity to inhibit the growth of Gram-positive and Gram-negative bacteria (Rattanakom S. and Yasurin P., 2015) The 1000 µg/disc of hot methanolic *C. asiatica* crude extracts were moderately effective on *Staphylococcus aureus* and *Methicillin Resistant S. aureus* (Wild Type) (Zaidan et al., 2005). *Micobacterium tuberculosis* and *M. leprae* were reported to be more sensitive to liposomal asiatocoside particles than free asiatocoside of *C. asiatica* (Fugh- Berman, 2003).

The study of antioxidant activity from *C. asiatica* extracted by 3 different solvents; water, ethanol and petroleum ether was shown that *C. asiatica* extracted by ethanol has the highest antioxidant activity while *C. asiatica* extracted by petroleum ether has no effect on antioxidant activity. (Hamid A.A. et al., 2002). Antioxidant activity of *C. asiatica* may have a variety of mechanisms such as the ability to inhibit the creation of oxidation (Hatano, et al., 1989), the ability to inhibit the formation of free radicals and stop the formation of free radicals as p-coumaric acids. (Laranjinha J. et al., 1995), and *C. asiatica* extracted by ethanol showed higher FRAP value than chloroform and hexane extracted. (Rattanakom S. and Yasurin P., 2015)

Table 2 Bioactive compound of C. asiatica and its biological property

Bioactive compound	Biological property	References
Asiatic acid	Aids in generation of neuroglia; promotes wound healing, promotes cuticle cornification; stimulates granulation; induces gene expression changes, enhancing learning and memory properties, antinociceptive activity, anti-inflammation activity, acetylcholinesterase inhibitory activity, anti-apoptotic activity	Huang et al., 2011; Nasir et al., 2011,2012; Zhou et al., 2011; Song et al., 2012; Zhang et al., 2012
Asiaticoside	Anti- inflammatory; antioxidant induces gene expression changes, wound healing, reduces scar formation, neuroprotective activity, improve collagen biosynthesis	Tang et al., 2011; Zhou et al., 2011; Lee et al., 2012; Nowwarote et al.,2012; Paolino et al., 2012; Xu et al., 2012
Madecassoside	Induces gene expression changes, protection of endothelial cells from oxidative injury	Zhou et al., 2011; Bian et al., 2012
Quercetin	Anti- HIV- 1, antiasthmatic, antibacterial, antihepatotoxin, antihypertensive, anti-inflammatory, antitussive, antiviral, coronary vasodilator, ntihypercholesterolemic, 5-HT inhibitor, smooth muscle relaxant, platelet aggregation inhibitor, 3',5'-cAMP-phosphodiesterase inhibitor, fatty acid synthetase inhibitor, aldose reductase inhibitor (eye lens) , protein kinase C inhibitor; antihypertensive, reduces blood capillary brittleness, antioxidant	Chong NJ and Aziz,2013; Zhou et al., 2011

Bioactive compound	Biological property	References
Quercitrin	Antibacterial, antineoplastic, antihepatotoxin, anti-inflammatory, antimutagenic, antiviral, diuretic, Hemostatic, aldose reductase inhibitor, antioxidant, insect antifeedant (Bombyx mor) , insect phagostimulant (Gastrophysa atriocyaea) , hepatoprotective	Bhandari et al., 2007; Zhou et al., 2011
Kaempferol	Anti- HIV- 1, antibacterial; anti- inflammatory, antitussive to cure trachitis, antioxidant, Δ5-lipoxygenase inhibitor; iodinate thyronine deiodinase inhibitor; aldose reductase inhibitor	Chong NJ and Aziz,2011; Zhou et al., 2011
Apigenin	Antibacterial, antiulcerative, antispasmodic (smooth muscle) , diuretic, aldose reductaseinhibitor, antihypertensive, anti- inflammatory, antioxidant, nodulation signal for metabiosis of pea and *Rhizobium leguminosarum*	Bhandari et al., 2007; Zhou et al., 2011
Luteolin	Antiallergic, antibacterial, antifungal, cytotoxic, anti inflammatory, antispasmodic, antitussive, antiviral, enhances arterial tension and lowers intravenous tension, enhances blood capillary permeability, immunoenhancer, increases coronary flow; dihydrocoenzyme I (NADH) oxidase inhibitor, iodine- induced thyronine deiodinase inhibitor, aldose reductase inhibitor, anti-inflammatory, anti-HIV activity	Bhandari et al., 2007; Zhou et al., 2011

Bioactive compound	Biological property	References
Naringin	Antibacterial, anti- inflammatory, antiviral, aldose reductase inhibitor, passive cutaneous anaphylaxis inhibitor	Zheng and Qin, 2007; Zhou et al., 2011
Betulic acid	Antineoplastic, cytotoxic, antitubercular, antibacterial	Jamil et al., 2007; Zhou et al., 2011
α-Pinene	Antifungal, antitussive, irritant	Barnes et al., 2007; Zhou et al., 2011
β-Pinene	Antifungal, anti-inflammatory, antitussive	Barnes et al., 2007; Zhou et al., 2011
Ascorbic acid	Antioxidant, antibacterial, anti-infective, antidote, antihypercholesterolemic, inhibits production of Carcinogen, induces tissue to produce collagen, hematopoietic activity	Chong NJ and Aziz,2011; Zhou et al., 2011
Chlorogenic acid	Antioxidant, antineoplastic, cytotoxic, antimutagenic, antiviral, choleretic, hemostatic, leukopoietic, antimalarial.	Chong NJ and Aziz,2011; Zhou et al., 2011
Irbic acid	Strong radical scavenging, collagenase inhibitory activity	Antognoni et al., 2011

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Nanotechnology and nanoparticles.

Nanotechnology can be used in many field but much more introduced into several aspects of the food science, including encapsulations and delivery systems. Nanoparticles can protect and deliver functional food ingredient, bioactive ingredients such as nutrients, phytochemicals, nutraceuticals, and drugs may be incorporated into nanoparticles to maximize delivery efficiency and increase desirable benefits (Rhaese et al., 2003)

Nanoparticles are the particles that less than 100 nanometers which are suitable for drug delivery. Presently, nanotechnology was applied with plant extracted to improve drug delivery system, bioactive compound released and increase the efficiency of compound. These technological discoveries have revolutionized drug delivery. The new drug delivery systems have the ability not only to increase the effectiveness of active components, but also to reintroduce other components that were discarded because they were not useful in formulation (Bonifácio et al., 2014).

The principle of BSA nanoparticles formation

Bovine Serum Albumin (BSA) is one of protein nanocarriers which can soluble in water and diluted salt solution freely. The albumin protein can be stable in the pH range from 4 to 9 and can be heated at 60°C up to 10 hours without any deleterious effects. (Felix Kratz, 2008) The high solubility of this protein can up to about 40% (w/v) and at pH 7.4 makes it an attractive macromolecular carrier capable of accommodating a wide variety of drugs. (Lohcharoenkal et al., 2014)

BSA is the capable of binding reversibly with many exogenous and endogenous drugs. Various drugs with albumin nanoparticles have been synthesized by the desolvation method; BSA endows the drug with high dispersibility and biocompatibility, and it also has a multifunctional surface for conjugation with other targeting molecules. (Singh et al., 2017) The yield of the nanoparticles was nearly 93% (w/w) and encapsulation efficiency of 82% (w/w). (Verma et al., 2018)

There are many methods to prepare protein nanoparticles based on two things which are attractive and repulsive force in the protein. Subsequent thermal or chemical crosslinking leads to the formation of cross-linked nanoparticles with entrapped drug molecules. The commonly methods use to prepare nanoparticles is desolvation or coacervation method. (Lohcharoenkal et al., 2014)

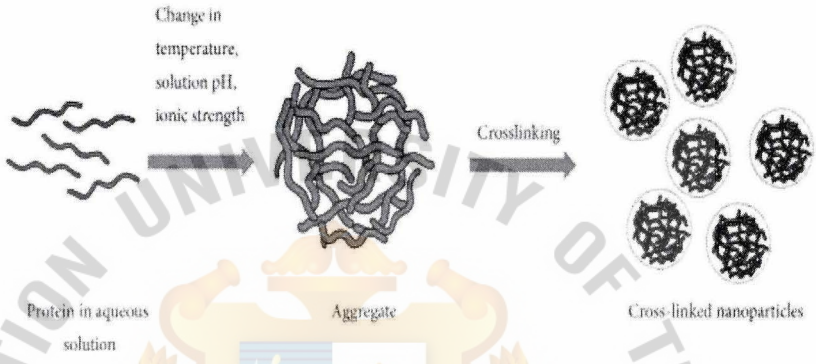

Figure 2: Preparation of protein nanoparticles by desolvation methods (Lohcharoenkul et al., 2014)

Desolvation method is usually based on the different solubility of protein in solvents as a function of solvent polarity, ionic strength, pH and the presence of electrolytes. When the protein structure changes it will coacervation or get precipitation from the addition of desolvation agent such as acetone and ethanol. After nanoparticles are formed, they are cross-linked by agents such as glutaraldehyde and glyoxal (Langer et al., 2003)

Crosslinking can stabilizes the protein nanoparticles and reduces enzymatic degradation and drug release from the nanoparticles. It also affect the stability of drugs, particularly protein drugs in the nanoparticles. Surface coating can be used to stabilize nanoparticles instead of crosslinking. (Wang et al., 2008) Drugs or bioactive compounds can be loaded into particles by surface adsorption or by entrapping the drugs in the particles during preparation process. (Figure 3)

However, the loading efficiency depends on drug properties as well as other factors such as the ratio of drug or polymer. (Lohcharoenkal et al., 2014)

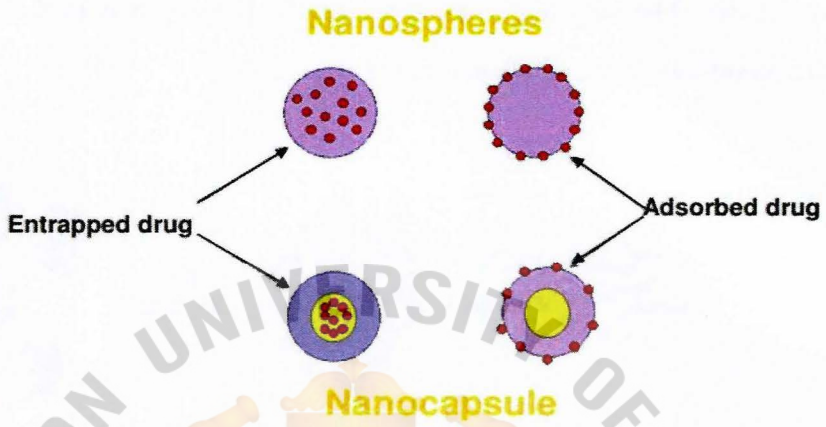

Figure 3: the diagram of entrapping or adsorption of the bioactive compounds or drug into nanoparticles in drug delivery system (Lohcharoenkal et al., 2014)

Entrapment and Loading efficiencies

The entrapment efficiency is defined as the amount of sample that has been entrapped into the carrier as percentage comparing with the total quantity of initial sample. The ratio of weight of entrapped sample to the weight of total carrier system is showing loading capacity. The loading capacity is varied depending on the methods for producing the particles.The loading capacity of sample is depending on the concentration of sample that is presented into the system that means higher concentration of initial sample possibly increases loading capacity of sample as revealed in various research works. (Dora et al., 2010). The relationship between loading capacity and concentration of crude extract could be explained by the interaction of hydrophobic effect and hydrogen bonding under synergistic. That means only specific bioactive compounds that are available in crude extract and compatible with functional groups on structure can be attached to the matrix of nanoparticles. (Bennick et al., 2002)

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Drug release kinetic

For drug delivery system, drug release has been an important topic. The two basic properties for drug delivery system are the delivery of drugs according to the need of the body over period of the treatment time as the first property and the second properties is the targeted delivery to site of the action. (U. Shah et al., 2012) Drug release system depend on the carrier of drugs which can be effect to the drug controlled release that maintain the drug levels to target cells

Apllication of kinetic model

Invitro kinetic release the result will plot into model for easy to analyze the mechanism of drug release kinetics as zero order, first order, Higuchi ,etc. (Gokhale A.,2014)

Application

Zero order

This relationship can be used to describe the drug dissolution of several types of modified release dosage forms as in the case of transdermal systems and matrix tablets with low solubility of drugs, coated forms, osmotic systems etc Pharmaceutical dosage forms following this profile release the same amount of drug by unit of time and it is ideal method of drug release in order to achieve a pharmacological prolonged action. (Bhowmik et al., 2012)

First order

Application: This relationship can be used to describe the drug dissolution in pharmaceutical dosage forms such as those containing water-soluble drugs in porous matrices

Higuchi model

Application: This relationship can be used to describe the drug dissolution from several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems and matrix tablets with water soluble drugs.

The controlled release system is to deliver a constant supply of the active ingredient, usually at a zero-order rate, by continuously releasing, for a certain period of time, an amount of the eliminated by the body. An ideal Controlled drug delivery system is the one, which delivers the drugs at a predetermined rate, locally or systematically, for a specific period of time. (S. Ummadi et al., 2013) the best fit model base on the (r) value in various medel.

Organic farming

There are some research report comparing quantity and quality of nutrient and bioactive compound between organic and conventional product. The result found that juice from organic tomatoes has higher β-carotene, chlorogenic acid, phenolic acid, garlic acid, flavonoids and quercetin than conventional (Hallmann E. et al., 2013) The organic marionberries has significant higher total phenolic than conventional. (Asami D.K. et al., 2003). Organic kiwi ethanol extracted has significant higher flavonoids than conventional kiwi ethanol extracted however water extra ction of both organic and conventional kiwi was no significant different. (Park YS. Et al., 2013)

Organic product growing at a sizzling rate of 17-20% per year as compared to a glacial rate of 2-3% for conventional products (OTA, All thing organic conference, 2015). The global organic for foods and beverages product market size reached to USD 91 billion in 2015. This because of the awareness about health benefits associated with consumption of organic products is expected to drive the demand over the forecast period, the demand for organic also grown over and over the past few years. (OTA, 2016)

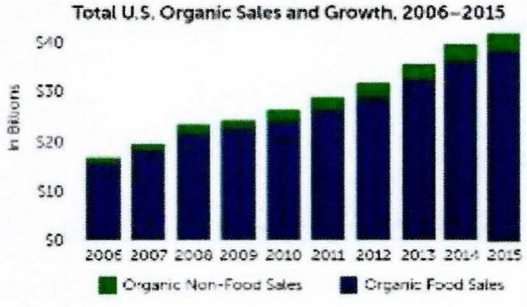

Figure 4: the growing up graph of organic product in United State (OTA., 2016)

OBJECTIVE

1. To increase bio efficiency, absorption and drug delivery system of *C. asiatica* drude extracted.

2. To compare the bio efficiency and pharmacological activity of Bovine Serum Albumin (BSA) nanoparticles of organic and conventional.

3. To study the result of the solvents that use to extract and the concentration of *C. asiatica* crude extracted from organic and conventional in BSA nanoparticle.

4. To compare the physical activity of *C. asiatica* BSA nanoparticles from organic and conventional.

5. To compare the bio activity of *C. asiatica* BSA nanoparticles from organic and conventional.

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MATERIALS AND METHODS

1. Preparation of sample

C. asiatica was purchased from local markets in Bangkok, Thailand. The aerial part of *C. asiatica* was used. Fresh *C. asiatica* were washed with tap water and cut into small pieces. Then it was air dried in oven (Memmert UM500) at 45°C. The dried samples was finely ground into powder. The powder were kept at 4°C before used. (Rattanakom and Yasurin, 2015)

2. Preparation of C. asiatica crude extract

C. asiatica from commercial and organic production are extracted with the extraction solvent as 95% Ethanol, Chloroform and Hexane using 1:10 ratio (g/mL). The mixtures are macerated at room temperature, 120 rpm, for 48 hours and then are filtered using Whatman filter paper No. 4. The crude extracts are concentrated using rotary evaporators at 50°C are kept at -20°C before use. The *C. asiatica* crude extracts are further used for preparation of *C. asiatica*- BSA nanoparticles (Rattanakorn and Yasurin, 2015)

3. Preparation of C. asiatica extract-loaded BSA nanoparticles

C. asiatica extract-loaded BSA nanoparticles was prepared by the desolvation method (Yu et al., 2014). The 100 mg of BSA were dissolved in 1 ml of sodium chloride solution (10 mM). Then, 8.0 ml of ethanol was added dropwise into the BSA solution under magnetic stirring (400 rpm) at room temperature. Subsequently, the as-prepared BSA nanoparticles were cross-linked with 0.2% glutaraldehyde (GA). Then, *C. asiatica* crude extract was added into the solution for 24 hours at different ratio of *C. asiatica* crude extracted to BSA (1:2, 1:3, and 1:4) in the preparation of nanoparticles. The particles were centrifuged and washed with distilled water. The centrifuged particles were resuspended and disperse in 2% mannitol, then freeze-dried at -40°C for 24 hours. The dried nanopowder were kept at room temperature before use.

4. Entrapment efficiency and loading efficiency

C. asiatica crude extract was run absorbance spectrum to find the best the wavelength (λmax) at which the absorbance is the greatest by UV-vis spectrophotometer. The 2 mg *C. asiatica* extract-loaded BSA nanoparticles were dissolved in 1 ml methanol and gently shaken for 24 hours at 37 °C to completely extract *C. asiatica* crude chloroform extract to methanol. Then the solutions were centrifuged at 12000 rpm for 10 min, and the supernatant was kept and measured optical density (OD) by a UV-vis spectrophotometer at λmax. The amount of *C. asiatica* crude extract entrapped and loaded in is express as entrapment efficiency and loading efficiency calculated as follows (Xie et al., 2011):

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

All measurements were done in triplicate and three replications independently.

5. Solubility and stability

To compare the solubility of *C. asiatica* crude extract before and following the encapsulation process, saturation solubility is determined (Xie et al., 2011). Excessive samples (*C. asiatica* crude extract and *C. asiatica* extract-loaded BSA nanoparticles) were dispersed into 1 mL water and at 200 rpm, 37 °C. After 24 hours, samples were taken out and filtered through a 0. 22 μm Millipore membrane. Filtrate was diluted appropriately, and the optical density (OD) were measured by a UV- vis spectrophotometer at λmax

The 1 mg/mL of *C. asiatica* extract-loaded BSA nanoparticles in phosphate buffer solution (0.01M, pH7.4) were incubated at 200 rpm, 37 °C, for 24 hours. At designated time points (0, 0.5, 1, 2, 3, 4, 5, 6 hours), the mixture was sampled and the optical density (OD) was measured by UV-vis spectrophotometer at λmax (Xie et al., 2011). The stability of CBNP is calculated as follows:

$$\text{Percentage reduction of } C.asiatica = 100\left(\frac{C_0 - C_t}{C_0}\right)$$

Where C_0 is the initial absorbance

C_t is the absorbance of the sample at time point

All measurements were done in triplicate and three replications independently.

6. Antimicrobial activity

The modified agar well diffusion method (Rattanakom, 2015) is used. The 100 µl of bacteria (approximately 1.5×10^8 CFU/ml) is swab on Mueller-Hinton agar (MHA) plate. The 50 µl of *C. asiatica* crude extract and *C. asiatica*- BSA nanoparticles at concentration 100, 200, and 300 µg/ml diluted with distilled water were used to test antibacterial activity against seven human pathogens. The 20 µl of 50 mg/ml penicillin G was used as positive control. The inhibition zones were measured to determine the effectiveness of the *C. asiatica* crude extract and *C. asiatica*- BSA nanoparticles against each bacterium. The experiment was done in duplicate and three replication independently.

7. Antioxidant activity by Ferric reducing antioxidant potential assay (FRAP)

The modified ferric reducing antioxidant potential assay (Benzie and Strain, 1999) was used to determine FRAP value of *C. asiatica* crude extract and *C. asiatica*-BSA nanoparticles. The FRAP reagent was prepared using 300 mmol sodium acetate buffer at pH 3.6, 20 mmol iron chloride and 10 mmol 2,4,6-tripyridyl-s-triazine dissolved in 40 mmol hydrochloric acid at a ratio of 10:1:1 (v:v:v). The reagent was incubated at 37°C for 10 minutes before use. The 20µl of 1 mg/ml the extract and *C. asiatica*- BSA nanoparticles was added, followed by adding 1000µl of FRAP reagent vigorously and kept in the dark for 30 minutes. The optical density (OD) of this mixture was measured at 593 nm. FRAP values were expressed as mmol Fe^{2+}/mg of sample. All measurements were done in triplicate and three replications independently.

8. Antioxidant activity by DPPH radical scavenging activity

The modified DPPH radical scavenging activity (Brand-Williams et al., 1995) was used for percentage DPPH radical scavenging determination. The 100 µl of 1 mg/ml *C. asiatica* crude extract and *C. asiatica*- BSA nanoparticles were mixed with 3.9 ml DPPH reagent (50 µM). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 minutes. The optical density (OD) was measured at 517 nm. The results were expressed as percentage reduction of DPPH (Molyneux, 2004).

$$\text{Percentage reduction of DPPH} = 100\left(\frac{A_0 - A_c}{A_0}\right)$$

Where A_0 is the initial absorbance

A_c is the value for added sample concentration

All measurements were done in triplicate and three replications independently.

9. Release kinetic *in Vitro*

Release kinetic (Xie et al., 2011) methodology was modified. The release of *C. asiatica* crude extract from *C. asiatica*- BSA nanoparticles was done by dissolving 20 mg of *C. asiatica*-BSA nanoparticles in 15 ml artificial gastric juice (0.01 M PBS pH 2.0) and intestinal juice without enzymes (0.01 M PBS pH 7.4). The mixture is incubated at 37 °C at 200 rpm. At designated time points (0, 1, 2, 3, 4, 5, 6 hours), mixture is sampled and centrifuged at 3000 rpm for 10 min. The pellet is resuspended in 100 µL of methanol to determine the amount of *C. asiatica* crude extract released by measuring optical density (OD) by UV-vis spectrophotometer at λmax. . All measurements were done in triplicate and three replications independently then the results will express to drug release models (D. Bhowmik et al., 2012)

Zero order model.

This is when drug dissolution from pharmaceutical dosage which is slowly release drug can be presented by the following equation

Where

$$Q_0 = Q_t + K_0\, t$$

Q_t = amount of drug released in time t

Q_0 = initial amount of drug in solution

K_0 =zero order release constant

t = time in hours

First order model.

The application of this model was proposed by Gibaldi and Feldman (1967) and Wangner (1969) which is the logarithm model of amount of remained VS time can be expressed by the following equation

$$\text{Log } Q_t = \log Q_e + (K_{it} \div 2.303)$$

Where

Q_t = amount of drug released in time t

Q_e = initial amount of drug in solution

K_t = first order release constant

Higuchi model.

The developed of Higuchi (1961, 1963), the release of water soluble and low soluble drugs incorporated in solid matrices. This model describes the drug release characteristics as diffusion process based on fick's law related with square root of time dependent and it expressed by using the formula.

$$Q_t = K_H \sqrt{t}$$

Where

Q_t = amount of drug released in time t

K_H = Higuchi constant

$\sqrt{t}$ = dependent square root of time

10. In vitro silulated gastointestional conditions

The determination of bioactivity of *C. asiatica*-gelatin nanoparticles is modified from Xie, et al., 2011 and Verruck, et al., 2015 by dissolving 20 mg of *C. asiatica*-gelatin nanoparticles in mastication step after pH adjusted to 6.9 with addition of 1 mol/L $NaHCO_3$ and *C. asiatica*-gelatin nanoparticles are treated with 100 U/mL of α-amylase and 1 mmol/L $CaCl_2$ as saliva solution at the rate of 0.6 mL/min for 2 minutes, 200 rpm. (Choi, et al., 2007) The treated sample is continuously added with 1 mol/mL HCl until pH reached 2.0 to create the oesophagus-stomach condition in addition 0.05 ml/g of sample pepsin solution is added and the sample is stirred at 130 rpm for 90 minutes. The sample is added with 0.25 ml/g of sample pancreatin-bovine bile salts solution and the condition of sample is changed to pH 5 by adding of 1 mol/L $NaHCO_3$. Sample is stirred at 45 rpm for 20 minutes as the duodenum section. Lastly, pH of sample increases to 6.5 by adding 1 mol/L $NaHCO_3$ and stirred at 45 rpm for 90 minutes. The mixture in all sections are incubated at 37 °C constantly and taken out and centrifuged at 3000 rpm for 10 minutes. The pellet is collected and determine antioxidant acitivities. Pellets from each gastrointestinal section are collected according to the table 2 to determine antioxidant and antimicrobial acitivities.

Table3 Processing conditions used in each step of simulated gastrointestinal conditions (Adapted from Verruck et al., 2015)

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

11. Statistical analysis and Experimental design

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with Duncan's multiple range tests ($p < 0.05$) by SAS software version 9.4.

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RESULT AND DISCUSSION

Entrapment efficiency

Table 4: Entrapment efficientcy of C. asiatica crude extract loaded BSA Nanoparticles

Sample		Entrapment efficientcy (%)	
Solvent Extraction	Ratio of Crude : BSA	Conventional	Organic
Ethanol	1:2	46.26 ± 0.97^{E}	43.23 ± 0.37^{E}
	1:3	70.91 ± 2.31^{B}	66.40 ± 1.12^{BC}
	1:4	93.12 ± 0.96^{A}	90.60 ± 0.31^{A}
Chloroform	1:2	43.22 ± 3.29^{E}	43.18 ± 0.36^{E}
	1:3	56.21 ± 2.48^{D}	64.72 ± 0.68^{BC}
	1:4	94.00 ± 4.51^{A}	89.10 ± 0.46^{A}
Hexane	1:2	42.99 ± 1.99^{E}	39.19 ± 2.04^{E}
	1:3	67.86 ± 1.94^{BC}	63.26 ± 4.44^{BC}
	1:4	87.82 ± 3.31^{A}	89.23 ± 2.74^{A}

Note: Different superscript within a column showed significant different at $p<0.05$

The entrapment efficiency were calculated as the entrapment efficiency is the amount of crude in nanoparticles to the total amount of crude added, the result was show in the table 4. The results were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version9.4 *C. asiatica* extract-loaded BSA nanoparticles with three extracted solvent and two different farming production which were conventional and organic. The result showed that nanoparticles preparation in the concentration of crude exract to BSA nanoparticles in ratio 1:4 got significant higher in the percentage entrapment efficiency than 1:2 and 1:3 ratio. ($p<0.05$) When focus on BSA used for the preparation of *C. asiatica* crude loaded BSA nanoparticle, the higher result in entrapment is the used of the amount of BSA to prepare *C. asiatica* crude loaded BSA nanoparticles.

Loading efficiency

Table 5 : Loading efficiency of C. asiatica crude extract loaded BSA Nanoparticles

Sample		Loading efficiency (%)	
Solvent Extraction	Ratio of Crude : BSA	Conventional	Organic
Ethanol	1:2	39.32 ± 0.82^{A}	36.74 ± 0.31^{ABC}
	1:3	39.00 ± 1.27^{AB}	36.52 ± 0.62^{ABC}
	1:4	37.34 ± 0.38^{ABC}	$36.24 \pm .013^{ABC}$
Chloroform	1:2	36.74 ± 2.80^{ABC}	36.71 ± 0.31^{ABC}
	1:3	30.92 ± 1.37^{D}	35.60 ± 0.37^{ABCD}
	1:4	37.60 ± 1.80^{ABC}	$35.64 \pm .018^{ABCD}$
Hexane	1:2	36.54 ± 1.69^{ABC}	33.31 ± 1.74^{CD}
	1:3	37.32 ± 1.07^{ABC}	34.79 ± 2.44^{CD}
	1:4	35.13 ± 1.32^{ABCD}	35.69 ± 1.10^{ABCD}

Note: Different superscript within a column showed significant different at $p<0.05$

Loading efficiency is the amount *C. asiatica* extract-loaded BSA nanoparticles to the total of BSA added. The result shown that there were no significant different for loading efficiency in all ratio of *C. asiatica* extract-loaded BSA nanoparticles ($p>0.05$) which are not different drude extract from both organic and conventional nanoparticles product. The result show that the trand of the loading effiecincy is about 40%. This can be say that the maximum amout of drug or bioactive compound can be load in BSA nanoparticles as 40% maximum. Solvent extraction and the concentration raito of *C. asiatica* extract-loaded into BSA nanoparticles also nosignifiant different. ($p>0.05$)

Entrapment and loading efficiencies are parameters used to measure the ability for the bioactive compounds to be trapped into the carrier system and the quantity of bioactive compounds loaded into carrier, respectively. (Kittithat Y.,2017)

Solubility

Table 6 : Solubility of C. asiatica crude extract loaded BSA Nanoparticles and C. asiatica crude extract

Sample		*Solubility*	
Solvent Extraction	Ratio of Crude : BSA	Conventional	Organic
Ethanol	1:2	202.62 ± 7.17^{EF}	209.30 ± 5.39^{DE}
	1:3	176.08 ± 2.69^{FGH}	196.42 ± 7.84^{EF}
	1:4	159.37 ± 4.23^{H}	200.71 ± 6.92^{EF}
Chloroform	1:2	220.46 ± 2.35^{CD}	224.66 ± 2.21^{CD}
	1:3	169.18 ± 6.22^{FGH}	211.55 ± 2.69^{CDE}
	1:4	192.43 ± 6.49^{EFG}	194.06 ± 2.64^{EFG}
Hexane	1:2	167.75 ± 6.42^{GH}	183.88 ± 8.38^{FG}
	1:3	173.37 ± 2.80^{FGH}	166.34 ± 5.16^{H}
	1:4	167.75 ± 5.64^{H}	173.07 ± 8.39^{FGH}
C. asiatica Crude Extracts			
Ethanol		374.21 ± 4.49^{A}	298.98 ± 5.53^{B}
Chloroform		245.40 ± 9.10^{C}	234.68 ± 1.38^{C}
Hexane		254.13 ± 5.28^{C}	234.08 ± 4.73^{C}

Note: Different superscript within a column showed significant different at $p<0.05$

The evaluation of solubility was performed under controlled condition in distilled water at neutral pH and temperature of 37 °C for 24 hours. The result of solubility showed that BSA nanoparticles were barely dissolved in water comparing to *C. asiatica* crude extract. Therefore, BSA nanoparticles from desolvation methods can be use to protect from undesired condition and deliver hydrophilic bioactive compounds to attach and penetrate cell membranes of human and pathogenic bacteria with slow release rate in which, their cell membranes allow only hydrophobic compounds to access. (Kommareddy, et al., 2005)

Stability

To determine the solubility of *C. asiatica* crude extracts before and following the encapsulation process, *C. asiatica* crude extracts and *C. asiatica* extract-loaded BSA nanoparticles were dissolved in water. The results were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.4. The result was about 40 to 60 % stables as can indegate that nanoparticles process made the stability of *C. asiatica* crude extracts by protecting it from hydrolysis and biotransformation. The nanoparticles also can provided stability of the *C. asiatica* crude extract so, improving of hydrophobic capacity of crude extract make it can be better in entering through the cell because cell membrane of both human and pathogenic microorganism, are hydrophobic characteristics. The stability of BSA nanoparticles protein can up to about 40% (w/v) and at pH 7.4 makes it an attractive macromolecular carrier capable of accommodating a wide variety of drugs. (Lohcharoenkal et al., 2014)

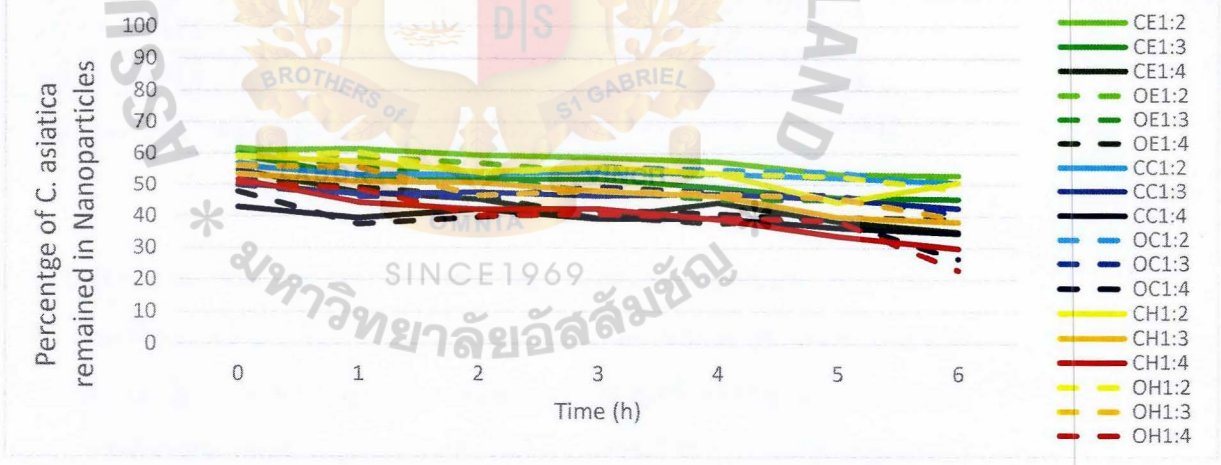

Figure 5: Stability of C. asiatica loaded-BSA nanoparticles *in PBS (pH7.4) at 37°C over period of 6 hours.*

Note: CE = Conventional C. asiatica Ethanol crude extract loaded BSA
CC = Conventional C. asiatica Chloroform crude extract loaded BSA
CH = Conventional C. asiatica Hexane crude extract loaded BSA
CE = Organic C. asiatica Ethanol crude extract loaded BSA
CC = Organic C. asiatica Chloroform crude extract loaded BSA
CH = Organic C. asiatica Hexane crude extract loaded BSA
1:2, 1:3 and 1:4 = ratio used of crude : BSA nanoparticles

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Release kinetic in vitro at pH 2.0

For release kinetic in *vitro*, *C. asiatica* extract-loaded BSA nanoparticles with 3 different extraction solvents and two different farmimg production were tested with artificial gastric juice (0.01 M PBS pH 2.0) as to imitate the environment in stomach. The release rate of *C. asiatica* extract-loaded BSA nanoparticles with using same ratio in the gastric juice was tend to higher than intestinal juice. At pH 2.0 the BSA protein was unfolded and release *C. asiatica* out from the particles as showed in figure 6. The release kinetic of *C. asiatica* extract-loaded BSA nanoparticles at this pH, the result was show slightly increase of *C. asiatica*. However, the acidic condition in gastric juice at pH 2.0 could denature the protein structure which caused the structure to unfold and bioactive compounds were released at higher rate comparing to the release rate of *C. asiatica* extract-loaded BSA nanoparticles in PBS pH 7.4. (Figure 10) The denaturation of protein could be explained by when the pH was out of isoelectric point. (Lin, et al., 2002) Then the result was countinue calculated for the release mechanism model of the drug release or the bioactive compound release.

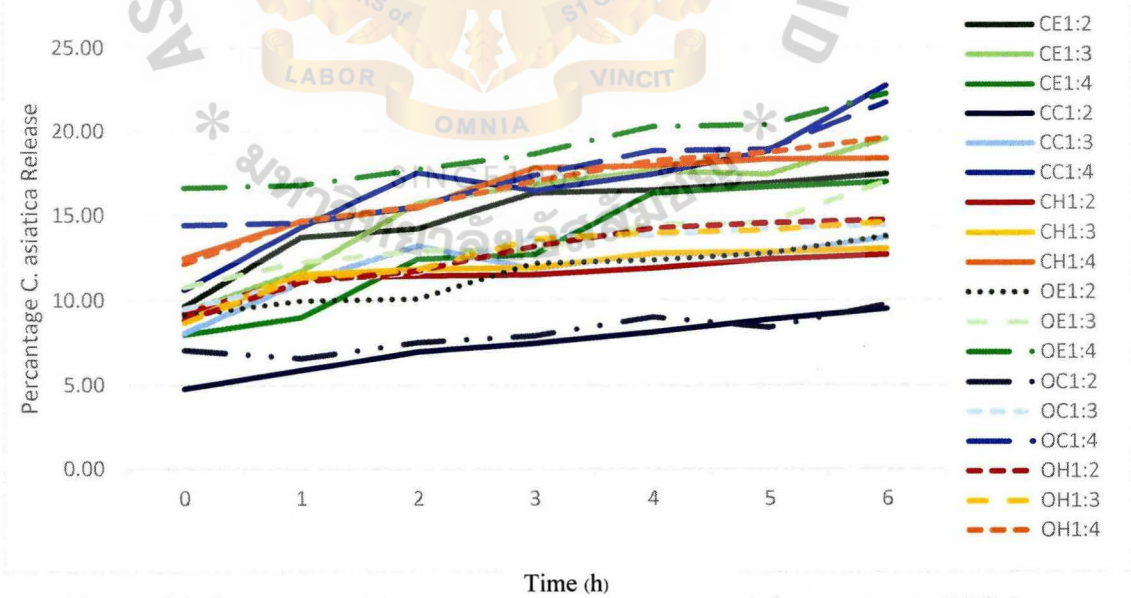

Figure 6: Release rate of C. asiatica from BSA-nanoparticles in vitro in PBS (in artificial gastric juice at pH 2.0) at 37°C over period of 6 hours

There are 3 mechanism model use to anlyze the result which are zero order model, first order model and Higuchi model. Then, the result of all model was countinue compare the R^2 of each value together. The best fits model is the best R^2 or the highest R^2 from C. asiatica releases out of each model. So, the trand of the release model was zero order model. As the higher R^2 from 2 different model as shown in table 7.

For zero model is the ideal Controlled drug delivery system which delivers the drugs or bioactive compounds at a predetermined rate, locally or systematically, for a specific period of time. (S. Ummadi et al., 2013) In the case of transdermal systems and matrix tablets with low solubility of drugs, coated forms, osmotic systems etc Pharmaceutical dosage forms following this profile release the same amount of drug by unit of time . (Bhowmik et al., 2012)

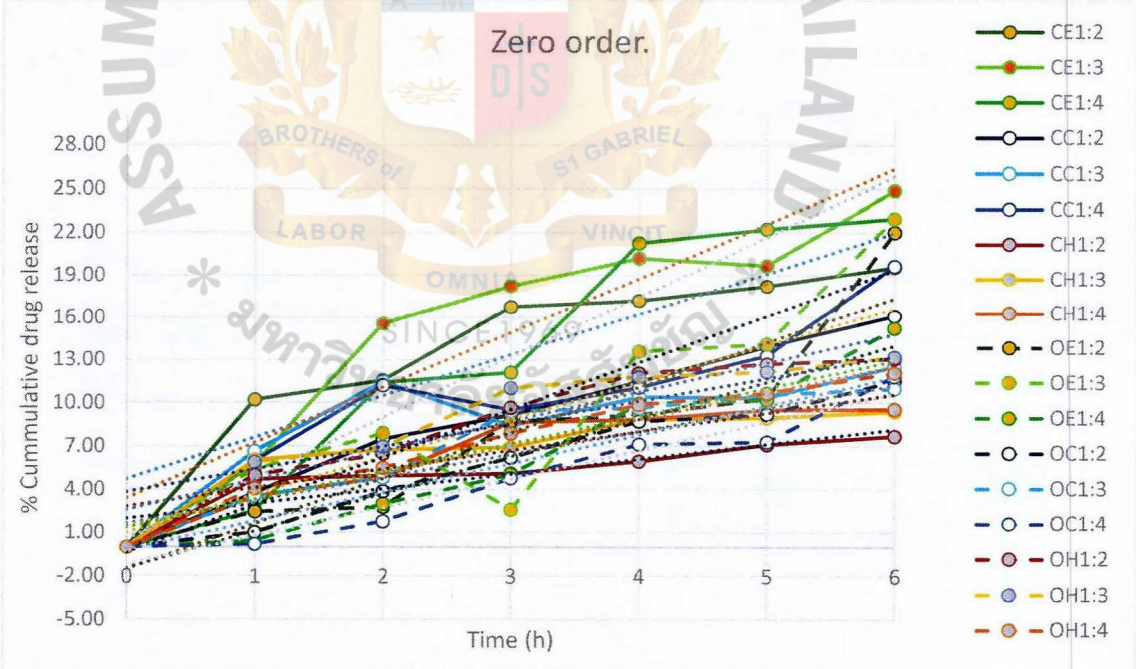

Figure 7: Release rate of C. asiatica from BSA-nanoparticles Zero order model.

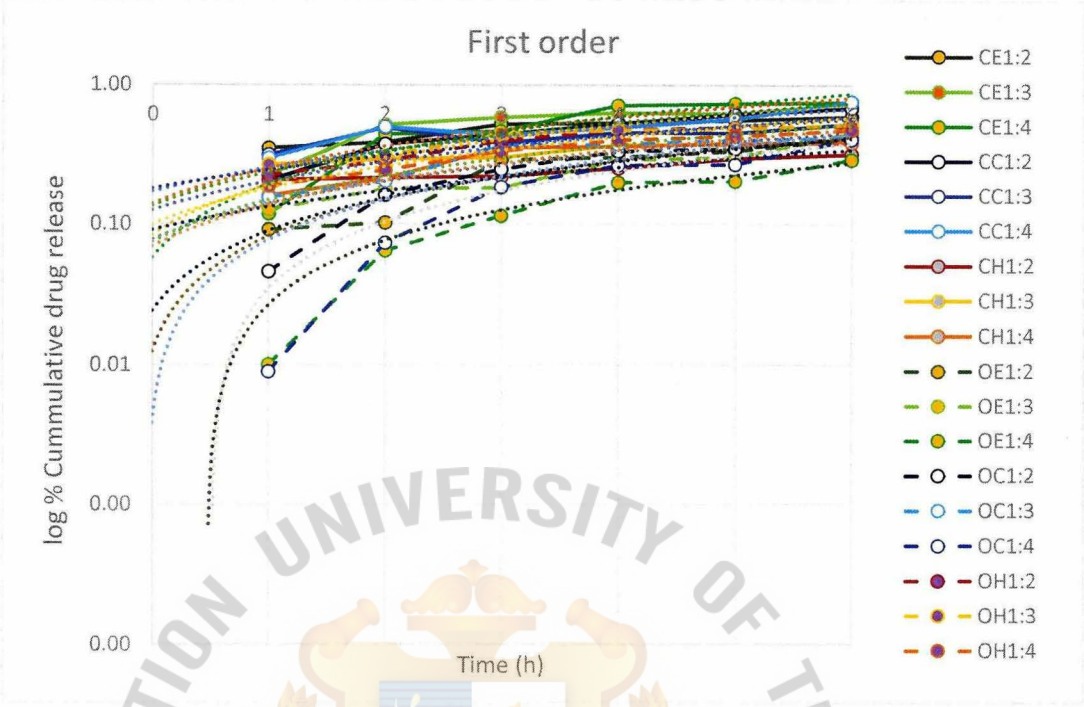

Figure 8: Release rate of C. asiatica from BSA-nanoparticles First order model.

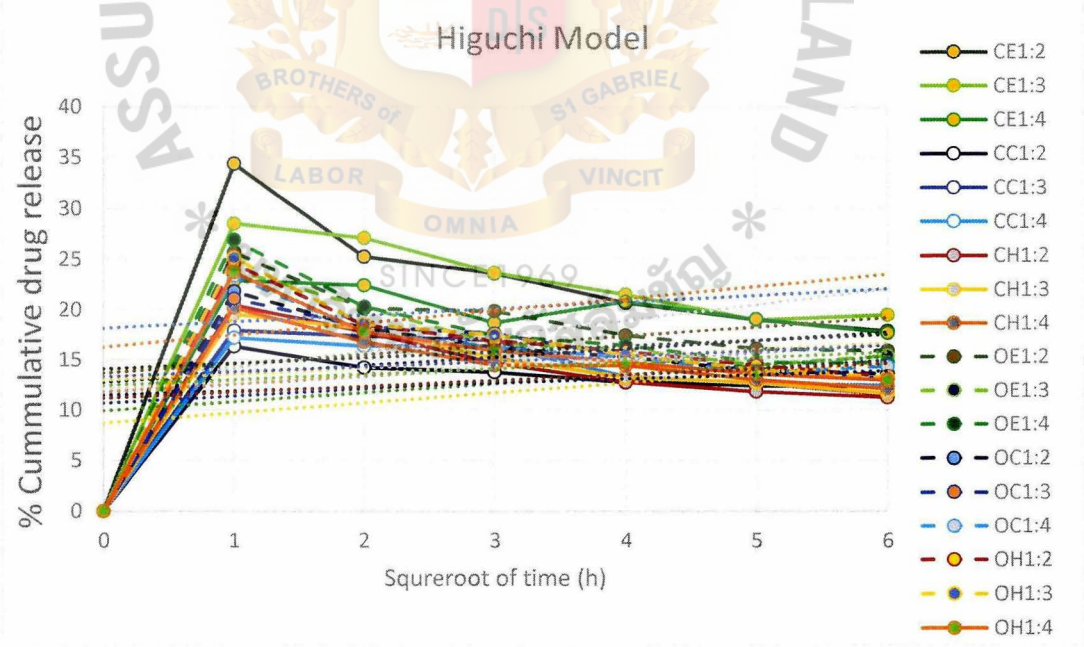

Figure 9: Release rate of C. asiatica from BSA-nanoparticles Higuchi model

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Table 7: The R^2 from zero order, firs order and Higuchi model of C. asiatica crude extract - loaded BSA nanoparticles from conventional and organic crude extract.

BSA- nonaparticles	Ratio of Crude to BSA	Zero order (R^2)	First order (R^2)	Higuchi model (R^2)
conventional				
Ethanol extraction solvent	1:02	0.8747	0.7653	0.0181
	1:03	0.8769	0.8313	0.0753
	1:04	0.9334	0.9125	0.1818
Chloroform extraction solvent	1:02	0.9842	0.951	0.1612
	1:03	0.6406	0.6138	0.0614
	1:04	0.9842	0.8363	0.1081
Hexane extraction solvent	1:02	0.7904	0.7541	0.0183
	1:03	0.749	0.9272	0.0039
	1:04	0.8654	0.8449	0.0276
Organic				
Ethanol extraction solvent	1:02	0.8525	0.9405	0.0619
	1:03	0.8029	0.9379	0.0563
	1:04	0.9548	0.9674	0.0229
Chloroform extraction solvent	1:02	0.9818	0.9739	0.0617
	1:03	0.8946	0.8752	0.0642
	1:04	0.9458	0.9579	0.053
Hexane extraction solvent	1:02	0.7904	0.8828	0.0311
	1:03	0.8663	0.8231	0.0214
	1:04	0.9583	0.9272	0.0276

Release kinetic in vitro at pH 7.4

For release kinetic in *vitro*, *C. asiatica* extract-loaded BSA nanoparticles with 3 different extraction solvents and two different farmimg production were tested intestinal juice without enzymes (0.01 M PBS pH 7.4) as to imitate the environment in intestine. In pH 7.4 this is the nuture pH which can made *C. asiatica* extract-loaded BSA nanoparticles to stable all the whole 6 hr as in figure 10. The albumin protein can be stable in the pH range from 4 to 9 and can be heated at 60°C up to 10 hours without any deleterious effects. (Felix Kratz, 2008). So, there are less of release shown in figure 10.

This can be because the albumin protein can be stable in the pH range from 4 to 9 and can heated at 60°C up to 10 hours without any deleterious effects. (Felix Kratz, 2008)

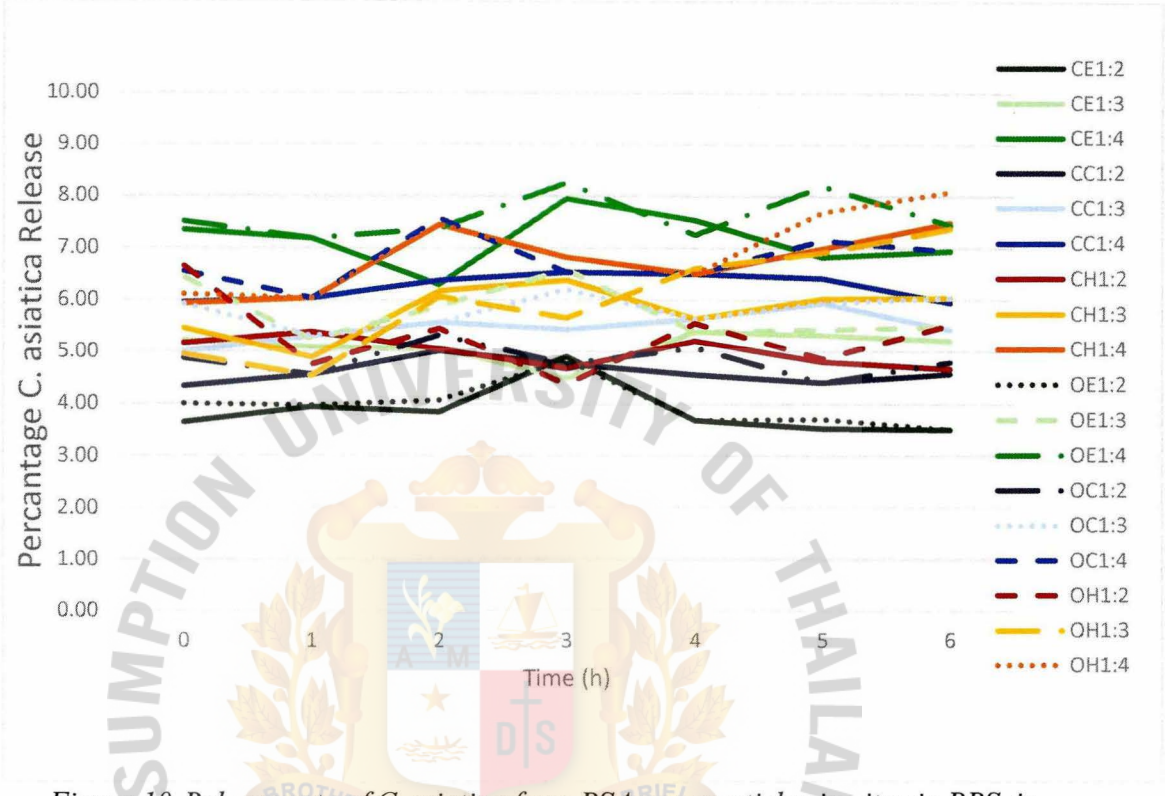

Figure 10. Release rate of C. asiatica from BSA-nanoparticles in vitro in PBS (in artificial intestinal juice at pH 7.4) at 37°C over period of 6 hours

Antioxidant activity (FRAP)

The antioxidant testing which is the measured by the ability of the compound that can catch free radicals compounds by scavenging or trapping methods (Huang et al., 2005). There are two assay to measure the antioxidant activity of *C. asiatica* crude extracts and *C. asiatica* extract-loaded BSA nanoparticles.

The FRAP assay, there are no free radicals involved. The assay determine ability of *C. asiatica* extract-loaded BSA nanoparticles or *C. asiatica* crude extracts to reduce of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The result show that crude extract was significant higher in FRAP reaction more than nanoparticles. ($p<0.05$)

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Table 8: Ferric reducing antioxidant potential of NPs and crude extract

Sample		FRAP (mmol Fe^{2+}/mg dried weight)	
Solvent	Ratio	Conventional	Organic
Ethanol	1:2	0.85 ± 0.08^{C}	0.86 ± 0.05^{C}
	1:3	0.72 ± 0.03^{CD}	0.75 ± 0.08^{CD}
	1:4	0.67 ± 0.09^{DE}	0.62 ± 0.07^{DE}
Chloroform	1:2	0.57 ± 0.08^{EFG}	0.54 ± 0.06^{EFGH}
	1:3	0.47 ± 0.06^{FGH}	0.52 ± 0.03^{FGH}
	1:4	$0.45 \pm .010^{GH}$	0.50 ± 0.05^{FGH}
Hexane	1:2	0.47 ± 0.07^{FGH}	0.44 ± 0.11^{GH}
	1:3	0.42 ± 0.05^{GH}	0.45 ± 0.07^{GH}
	1:4	0.41 ± 0.03^{H}	0.42 ± 0.04^{GH}
C. asiatica Crude Extracts			
Ethanol		2.12 ± 0.16^{A}	2.04 ± 0.10^{A}
Chloroform		1.13 ± 0.09^{B}	1.12 ± 0.13^{B}
Hexane		1.09 ± 0.10^{B}	1.15 ± 0.05^{B}

Note: Different superscript within a column showed significant different at $p<0.05$

Antioxidant activity (DPPH)

DPPH radical scavenging assay measures the reducing ability of antioxidants toward DPPH. The antioxidant effect is proportional to the disappearance of DPPH in a methanolic solution when added with *C. asiatica* extract-loaded BSA nanoparticles. Or *C. asiatica* crude extracts. The result from Table 7 showed that *C. asiatica* crude extract was significant highest in antioxidant activity than all *C. asiatica* extract-loaded BSA nanoparticles. ($p<0.05$).

For the result of antioxidant activity from both DPPH and FRAP can be because of the bioactive compound was bind with BSA already which can made NPs to be inactive form or its take time for the chemical reaction when *C. asiatica* crude extract was already loaded in BSA Nanoparticles. According to previous study on *C. asiatica*

Extract-Loaded BSA nanoparticles, the nanoparticles has slow release rate up to 6 hours and its antioxidant activity showed less or equal compare to crude extract. (Kesornbuakoa and Yasurin., 2016).

Table 9: DPPH radical scavenging of NPs and crude extract

Sample		DPPH (% DPPH radical scavenging)	
Solvent	Ratio	Conventional	Organic
Ethanol	1:2	18.18 ± 4.01^{C}	17.63 ± 2.09^{C}
	1:3	11.43 ± 2.09^{DEFGH}	13.55 ± 2.08^{CDE}
	1:4	8.31 ± 2.54^{EFGH}	7.36 ± 2.14^{FGH}
Chloroform	1:2	14.87 ± 3.96^{CD}	13.23 ± 3.05^{CDEF}
	1:3	8.43 ± 1.38^{EFGH}	10.91 ± 2.79^{DEFGH}
	1:4	7.75 ± 2.48^{EFGH}	8.35 ± 0.78^{EFGH}
Hexane	1:2	13.18 ± 1.47^{CDEF}	12.92 ± 2.51^{CDEF}
	1:3	10.76 ± 2.45^{DEFGH}	8.01 ± 2.33^{EFGH}
	1:4	7.03 ± 1.39^{GH}	6.75 ± 1.97^{H}
C. asiatica Crude Extracts			
Ethanol		31.94 ± 4.34^{A}	29.91 ± 5.71^{AB}
Chloroform		26.42 ± 2.99^{AB}	25.55 ± 2.40^{B}
Hexane		27.22 ± 4.77^{AB}	24.23 ± 5.33^{B}

Note: Different superscript within a column showed significant different at $p<0.05$

Antibacterial Activity

For antibacterial activity, *C. asiatica* extract and *C. asiatica* extract -loaded BSA nanoparticles was used for evaluating antibacterial activity with different concentration (100, 200, and 300 µg/ml) against seven food borne pathogens (*Escherichia coli*, *Bacillus cereus*, *Bacillus sereus*, *stephylocuccus aureus*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), *S. enterica* 4,5,12:i:- (human) US clone.

The results showed in table5. It was found that antibacterial activities trend of *C. asiatica* extract -loaded BSA nanoparticles increased from *C. asiatica* crude extracts significantly ($p<0.05$). Most of the inhibition zone were higher in Gram positive bacteria than Gram negative bacteria because of the sensitivity. Gram negative bacteria has more protective layer which are outer membrane and lipopolysaccharide while Gram positive bacteria got only peptidoglycan that why they can be inhibit easier. Bovine serum albumin (BSA) of is the protein can attract macromolecular and carry variety of molecule of active compound (Kratz, 2008). It also can readily bind and release small molecule (Kratz, 2008). All of these reasons made it can increase an efficiency of absorption to the cells.

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Table 10: The inhibition zone of NPs and crude extract against 7 different microorganisms in the unit of cm.

Sample / Solvent	Ratio	Conc. (µg/ml)	Inhibition Zone of 7 microorganisms (cm)						
			E. coli	*B. cereus*	*B. subtilis*	*S. aureus*	ST	SE	SUS
Conventional Ethanol	1:2	100	0.65 ± 0.05^{CDEFbc}	0.75 ± 0.05^{BCDa}	0.80 ± 0.01^{DEFa}	0.77 ± 0.03^{DEFa}	0.72 ± 0.13^{BCDab}	0.60 ± 0.01^{DEFc}	0.63 ± 0.03^{CDEFbc}
		200	0.65 ± 0.05^{CDEFb}	0.65 ± 0.05^{EFb}	0.80 ± 0.05^{DEFa}	0.80 ± 0.05^{DEFa}	0.67 ± 0.03^{BCDEb}	0.62 ± 0.03^{CDEFb}	0.67 ± 0.12^{BCDEb}
		300	0.77 ± 0.06^{Ba}	0.63 ± 0.03^{EFGa}	0.83 ± 0.10^{DEFbc}	0.73 ± 0.06^{EFGab}	0.63 ± 0.03^{CDEbc}	0.62 ± 0.03^{CDEFbc}	0.63 ± 0.06^{CDEFbc}
	1:3	100	0.75 ± 0.09^{BCb}	0.62 ± 0.03^{EFGb}	0.90 ± 0.10^{CDa}	0.97 ± 0.13^{BCa}	0.68 ± 0.03^{BCDEb}	0.62 ± 0.03^{CDEFb}	0.63 ± 0.08^{CDEFb}
		200	0.65 ± 0.05^{CDEFb}	0.67 ± 0.06^{EFb}	0.87 ± 0.08^{CDa}	0.83 ± 0.10^{CDEa}	0.65 ± 0.01^{BCDEb}	0.63 ± 0.03^{CDEFb}	0.70 ± 0.08^{BCb}
		300	0.75 ± 0.15^{BCbc}	0.73 ± 0.10^{CDc}	0.93 ± 0.03^{Ca}	0.88 ± 0.06^{CDab}	0.67 ± 0.08^{BCDEc}	0.65 ± 0.05^{CDEc}	0.63 ± 0.03^{CDEFc}
	1:4	100	0.73 ± 0.12^{BCDa}	0.77 ± 0.08^{BCDa}	0.95 ± 0.05^{Ca}	0.85 ± 0.13^{CDEa}	0.75 ± 0.13^{BCa}	0.75 ± 0.13^{Ba}	0.75 ± 0.10^{Ba}
		200	0.68 ± 0.14^{CDEFb}	0.68 ± 0.03^{DEFb}	0.88 ± 0.03^{CDa}	0.75 ± 0.09^{DEFb}	0.63 ± 0.03^{CDEb}	0.63 ± 0.06^{CDEFb}	0.65 ± 0.01^{BCDEb}
		300	0.70 ± 0.05^{BCDEbc}	0.82 ± 0.10^{Bab}	1.15 ± 0.13^{Ba}	0.80 ± 0.15^{DEFab}	0.63 ± 0.06^{CDEc}	0.62 ± 0.03^{CDEFc}	0.68 ± 0.03^{BCDbc}
Conventional Chloroform	1:2	100	0.60 ± 0.01^{DEFGa}	0.68 ± 0.01^{DEFa}	0.72 ± 0.06^{EFGHa}	0.77 ± 0.15^{DEFa}	0.62 ± 0.10^{CDEFa}	0.63 ± 0.06^{CDEFa}	0.65 ± 0.05^{BCDEa}
		200	0.65 ± 0.01^{CDEFa}	0.70 ± 0.09^{CDEa}	0.77 ± 0.06^{DEFa}	0.72 ± 0.08^{EFGa}	0.72 ± 0.12^{BCDa}	0.75 ± 0.10^{Ba}	0.68 ± 0.10^{BCDa}
		300	0.62 ± 0.03^{DEFGab}	0.68 ± 0.08^{DEFab}	0.80 ± 0.15^{DEFa}	0.73 ± 0.06^{EFGab}	0.67 ± 0.03^{BCDEab}	0.65 ± 0.01^{CDEab}	0.70 ± 0.15^{BCab}
	1:3	100	0.67 ± 0.08^{CDEFb}	0.60 ± 0.01^{FGb}	0.85 ± 0.01^{CDa}	0.73 ± 0.03^{EFGab}	0.63 ± 0.08^{CDEb}	0.67 ± 0.16^{BCDb}	0.63 ± 0.03^{CDEFb}
		200	0.58 ± 0.03^{EFGc}	0.78 ± 0.08^{BCa}	0.73 ± 0.06^{EFGHab}	0.77 ± 0.06^{DEFa}	0.75 ± 0.05^{BCc}	0.60 ± 0.05^{DEFc}	0.60 ± 0.01^{CDEFc}
		300	0.65 ± 0.05^{CDEFab}	0.68 ± 0.06^{DEFab}	0.75 ± 0.05^{DEFGab}	0.77 ± 0.08^{DEFa}	0.70 ± 0.09^{BCDab}	0.63 ± 0.06^{CDEFb}	0.63 ± 0.08^{CDEFb}
	1:4	100	0.58 ± 0.03^{EFGb}	0.65 ± 0.05^{EFab}	0.75 ± 0.09^{DEFGa}	0.70 ± 0.01^{FGHab}	0.63 ± 0.03^{CDEab}	0.63 ± 0.10^{CDEFab}	0.75 ± 0.09^{Ba}
		200	0.60 ± 0.01^{DEFGc}	0.78 ± 0.15^{BCab}	0.83 ± 0.06^{CDa}	0.77 ± 0.06^{DEFabc}	0.63 ± 0.06^{CDEbc}	0.70 ± 0.10^{BCabc}	0.63 ± 0.03^{CDEFbc}
		300	0.65 ± 0.01^{CDEFc}	0.73 ± 0.15^{CDbc}	0.91 ± 0.03^{CDa}	0.85 ± 0.01^{CDab}	0.63 ± 0.03^{CDEc}	0.62 ± 0.05^{CDEFc}	0.68 ± 0.03^{BCDc}
Conventional Hexane	1:2	100	0.60 ± 0.01^{DEFGa}	0.63 ± 0.03^{EFa}	0.68 ± 0.14^{EFGHa}	0.68 ± 0.10^{FGHa}	0.60 ± 0.05^{CDEFa}	0.62 ± 0.03^{CDEFa}	0.65 ± 0.01^{BCDEa}
		200	0.65 ± 0.01^{CDEFab}	0.63 ± 0.03^{EFab}	0.67 ± 0.08^{EFGHa}	0.63 ± 0.03^{HIab}	0.63 ± 0.03^{CDEab}	0.57 ± 0.06^{EFGb}	0.60 ± 0.05^{CDEFab}
		300	0.63 ± 0.03^{DEFGa}	0.72 ± 0.18^{CDa}	0.67 ± 0.03^{EFGHa}	0.68 ± 0.08^{FGHa}	0.62 ± 0.12^{CDEFa}	0.60 ± 0.09^{DEFa}	0.65 ± 0.09^{BCDEa}

Sample / Solven	Ratio	Conc. (µg/ml)	Inhibition Zone of 7 microorganisms (cm)						
			E. coli	*B. cereus*	*B. subtilis*	*S. aureus*	ST	SE	SUS
Conventional Hexane	1:3	100	$0.57 \pm 0.03^{EFG/c}$	$0.62 \pm 0.03^{EFG/bc}$	$0.68 \pm 0.13^{EFGH/ab}$	$0.72 \pm 0.03^{EFG/a}$	$0.58 \pm 0.06^{DEF/bc}$	$0.58 \pm 0.03^{DEF/bc}$	$0.57 \pm 0.03^{EFG/c}$
		200	$0.62 \pm 0.03^{DEFG/ab}$	$0.62 \pm 0.08^{EFG/ab}$	$0.68 \pm 0.06^{EFGH/a}$	$0.58 \pm 0.06^{HIJ/b}$	$0.63 \pm 0.03^{CDE/ab}$	$0.57 \pm 0.03^{EFG/b}$	$0.62 \pm 0.03^{CDEF/ab}$
		300	$0.58 \pm 0.03^{EFG/ab}$	$0.60 \pm 0.05^{EFG/ab}$	$0.65 \pm 0.05^{FGH/ab}$	$0.68 \pm 0.06^{FGH/a}$	$0.63 \pm 0.08^{CDE/ab}$	$0.57 \pm 0.06^{EFG/b}$	$0.60 \pm 0.05^{CDEF/ab}$
	1:4	100	$0.57 \pm 0.03^{EFG/a}$	$0.60 \pm 0.01^{EFG/a}$	$0.68 \pm 0.20^{EFGH/a}$	$0.67 \pm 0.06^{FGH/a}$	$0.63 \pm 0.06^{CDE/a}$	$0.63 \pm 0.10^{CDEF/a}$	$0.60 \pm 0.01^{CDEF/a}$
		200	$0.60 \pm 0.01^{DEFG/a}$	$0.65 \pm 0.05^{EF/a}$	$0.66 \pm 0.06^{FGH/a}$	$0.62 \pm 0.03^{HI/a}$	$0.57 \pm 0.08^{DEF/a}$	$0.60 \pm 0.10^{DEF/a}$	$0.62 \pm 0.16^{CDEF/a}$
		300	$0.60 \pm 0.05^{DEFG/b}$	$0.58 \pm 0.03^{FGH/b}$	$0.68 \pm 0.03^{DEFGH/ab}$	$0.78 \pm 0.06^{DEF/a}$	$0.72 \pm 0.08^{BCD/b}$	$0.65 \pm 0.09^{CDE/b}$	$0.62 \pm 0.06^{CDEF/b}$
Organic Ethanol	1:2	100	$0.75 \pm 0.03^{BC/c}$	$0.75 \pm 0.05^{BCD/c}$	$0.85 \pm 0.10^{FGH/b}$	$0.97 \pm 0.03^{BC/a}$	$0.67 \pm 0.03^{BCDE/cd}$	$0.65 \pm 0.05^{CDE/d}$	$0.63 \pm 0.03^{CDEF/d}$
		200	$0.75 \pm 0.01^{BCD/bc}$	$0.78 \pm 0.03^{BC/b}$	$0.83 \pm 0.08^{DEF/b}$	$0.95 \pm 0.05^{BC/a}$	$0.67 \pm 0.06^{BCDE/c}$	$0.67 \pm 0.06^{BCD/c}$	$0.63 \pm 0.03^{CDEF/c}$
		300	$0.73 \pm 0.06^{BCD/bc}$	$0.82 \pm 0.03^{B/b}$	$0.82 \pm 0.03^{DEF/b}$	$0.93 \pm 0.06^{BCD/a}$	$0.78 \pm 0.06^{B/b}$	$0.67 \pm 0.03^{BCD/c}$	$0.67 \pm 0.06^{BCDE/c}$
	1:3	100	$0.72 \pm 0.03^{BCD/b}$	$0.73 \pm 0.03^{CD/b}$	$0.73 \pm 0.03^{EFGH/b}$	$1.00 \pm 0.09^{BC/a}$	$0.68 \pm 0.03^{BCDE/b}$	$0.63 \pm 0.06^{CDEF/b}$	$0.65 \pm 0.05^{BCDE/b}$
		200	$0.72 \pm 0.03^{BCD/bc}$	$0.73 \pm 0.06^{CD/bc}$	$0.82 \pm 0.08^{DEF/a}$	$0.80 \pm 0.05^{DEF/ab}$	$0.72 \pm 0.03^{BCD/bc}$	$0.63 \pm 0.03^{CDEF/d}$	$0.65 \pm 0.01^{BCDE/cd}$
		300	$0.73 \pm 0.03^{BCD/cd}$	$0.78 \pm 0.03^{BC/bc}$	$0.85 \pm 0.09^{DEF/ab}$	$0.88 \pm 0.10^{CD/a}$	$0.72 \pm 0.03^{BCD/cd}$	$0.68 \pm 0.03^{BCD/cd}$	$0.66 \pm 0.03^{BCDE/d}$
	1:4	100	$0.70 \pm 0.01^{BCDE/bc}$	$0.77 \pm 0.03^{BC/b}$	$0.77 \pm 0.06^{DEFG/b}$	$1.08 \pm 0.10^{B/a}$	$0.63 \pm 0.03^{CDE/c}$	$0.65 \pm 0.05^{CDE/c}$	$0.70 \pm 0.05^{BC/bc}$
		200	$0.67 \pm 0.06^{CDEF/b}$	$0.73 \pm 0.06^{CD/b}$	$0.77 \pm 0.03^{DEFG/b}$	$0.97 \pm 0.03^{BC/a}$	$0.68 \pm 0.06^{BCDE/b}$	$0.68 \pm 0.08^{BCD/b}$	$0.70 \pm 0.09^{BC/b}$
		300	$0.70 \pm 0.01^{BCDE/b}$	$0.72 \pm 0.03^{CD/b}$	$0.72 \pm 0.03^{FG/b}$	$0.87 \pm 0.03^{CD/a}$	$0.72 \pm 0.03^{BCD/b}$	$0.72 \pm 0.03^{BC/b}$	$0.70 \pm 0.05^{BC/b}$
Organic Chloroform	1:2	100	$0.63 \pm 0.03^{DEFG/a}$	$0.65 \pm 0.05^{DEF/a}$	$0.67 \pm 0.08^{EFGH/a}$	$0.65 \pm 0.05^{GHI/a}$	$0.60 \pm 0.01^{CDEF/a}$	$0.62 \pm 0.03^{CDEF/a}$	$0.63 \pm 0.03^{CDEF/a}$
		200	$0.65 \pm 0.05^{CDEF/a}$	$0.63 \pm 0.08^{EFG/a}$	$0.77 \pm 0.16^{DEFG/a}$	$0.63 \pm 0.06^{GHI/a}$	$0.62 \pm 0.03^{CDE/a}$	$0.63 \pm 0.08^{CDEF/a}$	$0.62 \pm 0.03^{CDEF/a}$
		300	$0.67 \pm 0.03^{CDEF/a}$	$0.63 \pm 0.03^{EFG/a}$	$0.65 \pm 0.05^{FGH/a}$	$0.67 \pm 0.03^{FGH/a}$	$0.65 \pm 0.05^{BCDE/a}$	$0.67 \pm 0.03^{BCD/a}$	$0.63 \pm 0.06^{CDEF/a}$
	1:3	100	$0.65 \pm 0.05^{CDEF/b}$	$0.63 \pm 0.03^{EFG/b}$	$0.88 \pm 0.06^{CD/a}$	$0.65 \pm 0.09^{FGH/b}$	$0.62 \pm 0.03^{CDE/b}$	$0.60 \pm 0.09^{DEF/b}$	$0.65 \pm 0.05^{BCDE/b}$
		200	$0.58 \pm 0.06^{EFG/b}$	$0.61 \pm 0.03^{FG/ab}$	$0.70 \pm 0.09^{EFGH/a}$	$0.63 \pm 0.03^{HI/ab}$	$0.60 \pm 0.01^{CDEF/b}$	$0.60 \pm 0.05^{DEF/b}$	$0.63 \pm 0.03^{CDEF/b}$
		300	$0.62 \pm 0.08^{DEFG/b}$	$0.68 \pm 0.03^{DEF/ab}$	$0.73 \pm 0.08^{FGH/a}$	$0.65 \pm 0.01^{FGH/ab}$	$0.63 \pm 0.03^{CDE/b}$	$0.63 \pm 0.03^{CDEF/b}$	$0.63 \pm 0.03^{CDEF/b}$
	1:4	100	$0.58 \pm 0.03^{EFG/c}$	$0.70 \pm 0.05^{CDE/b}$	$0.83 \pm 0.06^{CD/a}$	$0.63 \pm 0.03^{HI/bc}$	$0.62 \pm 0.03^{CDE/c}$	$0.63 \pm 0.03^{CDEF/bc}$	$0.57 \pm 0.03^{EFG/c}$
		200	$0.58 \pm 0.03^{EFG/b}$	$0.67 \pm 0.03^{DEF/ab}$	$0.77 \pm 0.06^{DEFG/a}$	$0.67 \pm 0.12^{FGH/ab}$	$0.63 \pm 0.03^{CDE/ab}$	$0.67 \pm 0.13^{BCD/ab}$	$0.60 \pm 0.10^{CDEF/b}$
		300	$0.62 \pm 0.03^{DEFG/c}$	$0.70 \pm 0.05^{CDE/b}$	$0.78 \pm 0.06^{DEF/a}$	$0.65 \pm 0.05^{FGH/bc}$	$0.62 \pm 0.03^{CDE/c}$	$0.65 \pm 0.01^{CDE/bc}$	$0.63 \pm 0.03^{CDEF/c}$

Sample / Solvent	Ratio	Conc. (µg/ml)	Inhibition Zone of 7 microorganisms (cm)						
			E. coli	*B. cereus*	*B. subtilis*	*S. aureus*	ST	SE	SUS
Organic Hexane	1:2	100	0.60 ± 0.05 ^{DEFGa}	0.70 ± 0.17 ^{CDEa}	0.75 ± 0.02 ^{DEFGa}	0.72 ± 0.13 ^{EFGa}	0.58 ± 0.03 ^{DEFa}	0.63 ± 0.19 ^{CDEFa}	0.58 ± 0.03 ^{DEFGa}
		200	0.62 ± 0.03 ^{DEFGa}	0.63 ± 0.03 ^{EFGa}	0.75 ± 0.09 ^{DEFGa}	0.75 ± 0.17 ^{DEFa}	0.60 ± 0.05 ^{CDEFa}	0.62 ± 0.03 ^{CDEFa}	0.67 ± 0.16 ^{BCDEa}
		300	0.60 ± 0.05 ^{DEFGa}	0.80 ± 0.26 ^{BCa}	0.68± 0.03 ^{EFGHa}	0.65± 0.01 ^{FGHa}	0.63± 0.06 ^{CDEa}	0.62± 0.03 ^{CDEFa}	0.63± 0.06 ^{CDEFa}
	1:3	100	0.58 ± 0.03 ^{EFGa}	0.57 ± 0.03 ^{FGHa}	0.55 ± 0.05^{HIJa}	0.53 ± 0.03 ^{IJa}	0.55 ± 0.05 ^{EFGa}	0.58 ± 0.03 ^{DEFa}	0.55 ± 0.05 ^{EFGa}
		200	0.62 ± 0.06 ^{DEFGa}	0.62 ± 0.03 ^{EFGa}	0.67 ± 0.06 ^{FGHa}	0.65 ± 0.01 ^{GHIa}	0.65 ± 0.05 ^{BCDEa}	0.60 ± 0.05 ^{DEFa}	0.63 ± 0.03 ^{CDEFa}
		300	0.65 ± 0.01 ^{CDEFa}	0.65 ± 0.05 ^{DEFa}	0.65 ± 0.05 ^{FGHa}	0.67 ± 0.03 ^{FGHa}	0.65 ± 0.05 ^{BCDEa}	0.63 ± 0.03 ^{CDEFa}	0.62 ± 0.03 ^{CDEFa}
	1:4	100	0.55 ± 0.05 ^{FGHa}	0.58 ± 0.03 ^{FGHa}	0.58 ± 0.03 ^{FGHa}	0.60 ± 0.01 ^{HIa}	0.57 ± 0.03 ^{DEFa}	0.53 ± 0.03 ^{FGa}	0.55 ± 0.05 ^{EFGa}
		200	0.60 ± 0.01 ^{DEFGa}	0.61 ± 0.03 ^{EFGa}	0.60 ± 0.01 ^{FGHa}	0.60 ± 0.01 ^{HIa}	0.62 ± 0.03 ^{CDEa}	0.62 ± 0.03 ^{CDEFa}	0.63 ± 0.03 ^{CDEFa}
		300	0.58 ± 0.01 ^{EFGa}	0.62 ± 0.01 ^{EFGa}	0.62 ± 0.03 ^{FGHa}	0.63 ± 0.08 ^{GHIa}	0.63 ± 0.03 ^{CDEa}	0.62 ± 0.08 ^{CDEFa}	0.62 ± 0.06 ^{CDEFa}
Conventional Crude									
Ethanol		100	0.53 ± 0.03 ^{FGHa}	0.53 ± 0.03 ^{GHa}	0.52 ± 0.03 ^{HIJa}	0.55 ± 0.05 ^{IJa}	0.55 ± 0.05 ^{EFGa}	0.55 ± 0.05 ^{EFGa}	0.55 ± 0.01 ^{EFGa}
		200	0.55 ± 0.01 ^{FGHa}	0.58 ± 0.03 ^{FGHa}	0.58 ± 0.03 ^{FGHa}	0.58 ± 0.03 ^{HIJa}	0.57 ± 0.03 ^{DEFa}	0.53 ± 0.03 ^{FGa}	0.56 ± 0.03 ^{EFGa}
		300	0.52 ± 0.03 ^{FGHb}	0.55 ± 0.03 ^{GHab}	0.55 ± 0.05 ^{HIJa}	0.53 ± 0.03 ^{IJa}	0.58 ± 0.03 ^{DEFa}	0.57 ± 0.03 ^{EFGab}	0.58 ± 0.03 ^{DEFGa}
Chloroform		100	0.50 ± 0.01 ^{GHa}	0.52 ± 0.03 ^{GHa}	0.48 ± 0.06 ^{Ja}	0.47 ± 0.03 ^{Ja}	0.50 ± 0.05 ^{Ha}	0.48 ± 0.03 ^{Ga}	0.50 ± 0.05 ^{FGHa}
		200	0.48 ± 0.03 ^{Ha}	0.53 ± 0.08 ^{GHa}	0.55 ± 0.05 ^{HIJa}	0.52 ± 0.03 ^{IJa}	0.52 ± 0.03 ^{GHa}	0.55 ± 0.05 ^{EFGa}	0.53 ± 0.03 ^{FGa}
		300	0.50 ± 0.01 ^{GHa}	0.52 ± 0.03 ^{GHa}	0.55 ± 0.05 ^{HIJa}	0.52 ± 0.03 ^{IJa}	0.53 ± 0.03 ^{GHa}	0.52 ± 0.06 ^{FGa}	0.53 ± 0.03 ^{FGa}
Hexane		100	0.50 ± 0.01 ^{GHa}	0.50 ± 0.09 ^{Ha}	0.48 ± 0.06 ^{Ja}	0.52 ± 0.10 ^{IJa}	0.52 ± 0.03 ^{GHa}	0.55 ± 0.05 ^{EFGa}	0.48 ± 0.06 ^{Ha}
		200	0.53 ± 0.06 ^{FGHa}	0.53 ± 0.06 ^{GHa}	0.55 ± 0.05 ^{HIJa}	0.55 ± 0.09 ^{HIJa}	0.52 ± 0.08 ^{GHa}	0.52 ± 0.03 ^{FGa}	0.52 ± 0.08 ^{FGHa}
		300	0.52 ± 0.03 ^{FGHa}	0.48 ± 0.03 ^{Ha}	0.52 ± 0.06 ^{IJa}	0.53 ± 0.08 ^{IJa}	0.50 ± 0.01 ^{Ha}	0.55 ± 0.09 ^{EFGa}	0.50 ± 0.05 ^{FGHa}
Organic Crude									
Ethanol		100	0.60± 0.01 ^{DEFGa}	0.60 ± 0.05 ^{FGa}	0.53 ± 0.06 ^{IJa}	0.55 ± 0.05 ^{HIJa}	0.55 ± 0.05 ^{EFGa}	0.55 ± 0.05 ^{EFGa}	0.52 ± 0.03 ^{FGHa}
		200	0.55 ± 0.01 ^{FGHa}	0.53 ± 0.03 ^{GHa}	0.57 ± 0.06 ^{FGHa}	0.53 ± 0.03 ^{IJa}	0.55 ± 0.05 ^{EFGa}	0.53 ± 0.03 ^{FGa}	0.58 ± 0.03 ^{DEFGa}
		300	0.57 ± 0.03 ^{EFGa}	0.57 ± 0.06 ^{FGHa}	0.57 ± 0.03 ^{FGHa}	0.57 ± 0.03 ^{HIJa}	0.61 ± 0.06 ^{CDEFa}	0.57 ± 0.03 ^{DEFa}	0.60 ± 0.05 ^{CDEFa}

Sample / Solvent	Ratio	Conc. (µg/ml)	Inhibition Zone of 7 microorganisms (cm)						
			E. coli	*B. cereus*	*B. subtilis*	*S. aureus*	ST	SE	SUS
Organic Crude									
Chloroform		100	$0.55 \pm 0.05^{FGH/a}$	$0.55 \pm 0.05^{GH/a}$	$0.57 \pm 0.06^{FGH/a}$	$0.53 \pm 0.03^{IJ/a}$	$0.57 \pm 0.03^{DEF/a}$	$0.48 \pm 0.03^{G/a}$	$0.55 \pm 0.05^{EFG/a}$
		200	$0.62 \pm 0.03^{DEFG/a}$	$0.60 \pm 0.05^{FG/ab}$	$0.53 \pm 0.03^{IJ/b}$	$0.57 \pm 0.03^{HIJ/ab}$	$0.55 \pm 0.05^{EFG/ab}$	$0.55 \pm 0.05^{EFG/ab}$	$0.58 \pm 0.03^{DEFG/ab}$
		300	$0.56 \pm 0.07^{EFG/a}$	$0.55 \pm 0.05^{BC/b/a}$	$0.57 \pm 0.06^{FGH/a}$	$0.60 \pm 0.05^{HI/a}$	$0.57 \pm 0.03^{DEF/a}$	$0.52 \pm 0.06^{FG/a}$	$0.62 \pm 0.03^{CDEF/a}$
Hexane		100	$0.57 \pm 0.03^{EFG/a}$	$0.58 \pm 0.06^{FGH/a}$	$0.57 \pm 0.06^{FGH/a}$	$0.57 \pm 0.03^{HIJ/a}$	$0.57 \pm 0.03^{DEF/a}$	$0.55 \pm 0.05^{EFG/a}$	$0.55 \pm 0.05^{EFG/a}$
		200	$0.62 \pm 0.03^{DEFG/a}$	$0.60 \pm 0.06^{FGH/ab}$	$0.60 \pm 0.01^{FGH/ab}$	$0.58 \pm 0.03^{HIJ/ab}$	$0.58 \pm 0.03^{DEF/ab}$	$0.52 \pm 0.03^{FG/b}$	$0.58 \pm 0.03^{DEFG/ab}$
		300	$0.58 \pm 0.08^{EFG/ab}$	$0.61 \pm 0.01^{FGH/ab}$	$0.60 \pm 0.05^{FGH/ab}$	$0.63 \pm 0.03^{HI/a}$	$0.60 \pm 0.01^{CDEF/ab}$	$0.55 \pm 0.09^{EFG/b}$	$0.60 \pm 0.01^{CDEF/ab}$
Penicillin G		300	$1.37 \pm 0.05^{A/d}$	$1.61 \pm 0.04^{A/c}$	$1.97 \pm 0.02^{A/b}$	$2.23 \pm 0.05^{A/a}$	$1.59 \pm 0.04^{A/c}$	$1.58 \pm 0.12^{A/c}$	$1.97 \pm 0.02^{A/d}$

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

ST stands for *S. enterica* Typhimurium
SE stands for *S. enterica* Enteritidis
SUS stands for *S. enterica* 4,5,12:i- (human) US clon

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Stimulus digestive

Hydrophilicity and drug regulated release are beneficial properties from the application of nanoparticles with bioactive hydrophobic compounds could enhance their effectiveness for the drug throughout *In-vitro* digestive system.(Ghosh et al.,2013) for antioxidant activity of stimulus digestive. The initiative antioxidant activity of crude extract in the mouth section was very low and prevention of oxidative stress showed the increasing trend after crude extract was exposed in the gastric juice in stomach section. The maximatum scavenging activity of crude extract was observed during the sample tested under pH 3.8. At low pH protein was unfold as lower it stable pH, the albumin protein can be stable in the pH range from 4 to 9 and can be heated at 60°C up to 10 hours without any deleterious effects. (Felix Kratz, 2008).

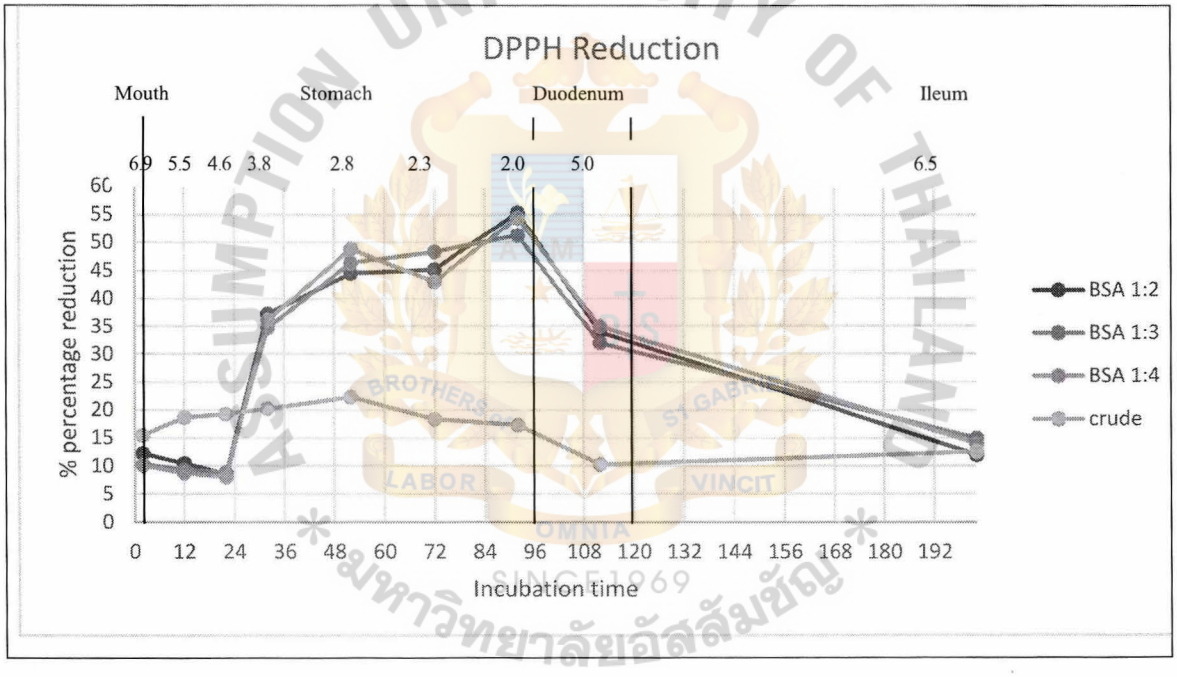

Figure 11: % DPPH radical scavenging to simulated gastrointestinal conditions throughout incubation time in minute. The upper left indicates simulated gastrointestinal system sections and pH values

Note: BSA 1:2 = Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles with 1:2 ratio
BSA 1:3 = Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles with 1:3 ratio
BSA 1:4 = Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles with 1:4 ratio
Crude = Organic C. asiatica Ethanol crude extract

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CONCLUSION

The nanoparticles was the technology that can be use to improving ability of *C. asaitica* crude extract in absorption to the cell. Higher hydrophobic capacity of the nanoparticles makes it can penetrate into cell membrane and enter to the cell. From BSA used for the preparation of *C. asiatica* crude loaded BSA nanoparticle, the higher result in entrapment is the used of the amount of BSA to prepare *C. asiatica* crude loaded BSA nanoparticles. The maximum amout of drug or bioactive compound can be load in BSA nanoparticles as 40% maximum. The Entrapment and loading efficiencies are parameters used to measure the ability for the bioactive compounds to be trapped into the carrier system and the quantity of bioactive compounds loaded into carrier, respectively. For solubility as the ability to soluble in water BSA nanoparticles from desolvation methods can be use to protect from undesired condition and deliver hydrophilic bioactive compounds. As BSA nanoparticles shown between 40 - 60%. BSA nanoparticles also high release at pH 2.0 but less release at pH 7.4 because the low pH can denature the structure of protein to unfold and bioactive compounds were released. Thus the albumin protein can be stable in the pH range from 4 to 9 and can be heated at 60°C up to 10 hours without any deleterious effects. (Felix Kratz, 2008) the model of kinetic release is zero order model which is the release the same amount of drug by unit of time . For the result of antioxidant activity from both FRAP and DPPH can be because of the bioactive compound was bind with BSA already which can made NPs to be inactive form as the high entrapment efficiency is the less antioxidant activity. Ane the time for the chemical reaction when *C. asiatica* crude extract was already loaded in BSA Nanoparticles should be more than 6 hrs as the result of stability. Most of the inhibition zone were higher in Gram positive bacteria than Gram negative bacteria because of the sensitivity. Gram negative bacteria has more protective layer which are outer membrane and lipopolysaccharide while Gram positive bacteria got only peptidoglycan that why they can be inhibit easier.

For the result can be seen that the different ratio of the concentation of *C. asiatica* to BSA (1:2, 1:3, and 1:4) and conventional and organic not signigicant effect to the bioavailability

of *C. asiatica* extract -loaded BSA nanoparticles.($p<0.05$) while the used of solvent extraction as Ethanol, Chloroform and hexae was significant different for the bioavailability of *C. asiatica* extract -loaded BSA nanoparticles, the highest was ethanol extraction solvent. ($p>0.05$)

So, the most effective of economic and less consumption is the *C. aisatica* loaded BSA nanoparticles with 1:2 ratio and extract with ethanol because it used less of BSA to prepare and get more biological activity than chloroform and hexane extract.

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APPENDIX A

Solution preparation

1. Garlic acid stock solution (conc. = 500 mg/L)

Composition per 100 ml:

Garlic acid	0.05 g
Ethanol	10 ml
Distilled water	Make volume to100 ml

Preparation: Dissolve 0.05 g of garlic acid in 10 ml of ethanol. Added distilled water, make volume to 100 ml using volumetric flask. This solution can be kept for 2 weeks.

2. Saturated sodium carbonate solution

Composition per 250 ml:

Distilled water	250 ml
Sodium carbonate	Added until it can't dissolve anymore

Preparation: Add 250 ml of distilled water into beaker and put on stirrer. Then constantly add sodium carbonate and keep stirring until it can't dissolve anymore.

3. DPPH (50 µM)

DPPH	0.0197 g
Distilled water	1 L

Preparation: Weight 0.0197 g of DPPH and add up distilled water to 1 L in volumetric flask

4. Ferrous sulphate (conc. = 1 mM)

Composition per 1L:

$FeSO_4 \cdot 7H_2O$	0.278 g
Distilled water	1 L

Preparation: Weight 0.278 g of $FeSO_4 \cdot 7H_2O$ and add 1 L of distilled water. Dissolve the solution completely.

5. **Acetate buffer (300 mM, pH 3.6)**

Composition per 100 ml:

Sodium acetate·$3H_2O$	0.31 g
Glacial acetic acid	1.6 ml
Distilled water	Add up to 100 ml

Preparation: Weight 0.31 g of sodium acetate·$3H_2O$ and add 50 ml of distilled water. Then add 1.6 ml of glacial acetic acid to the mixture and added distilled water up to 100 ml using volumetric flask. Check pH and store at 4°C.

6. **Diluted HCl (40 mM)**

Composition per 1L:

Conc. HCl (1M)	1.46 ml
Distilled water	Add up to1 L

Preparation: Added 1.46 ml of conc. HCl (1M) to 500 ml distilled water in volumetric flask. Then added distilled water up to 1 L. Store at room temperature

7. **TPTZ (10 mM)**

TPTZ	0.031 g
HCl (40 mM)	10 l

Preparation: Mix 0.031 g of TPTZ with 10 ml of 40 mM HCl. Dissolve at 50°C in water bath. Make fresh on day of assay in a new Corning tube.

8. **Phosphate buffer (1M)**

Na_2HPO_4	1.2 g
NaH_2PO_4	0.22 g
NaCl	8.5 g
Distilled water	Add up to 100 ml
HCl	Adjust pH

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Preparation: Weight Na_2HPO_4, NaH_2PO_4, and NaCl. Then dissolve with distilled water add up to 100 ml in volumetric flask and adjust pH with HCl to 2.0 and 7.4.

9. Ferric chloride (20 mM)

$FeCl_3 \cdot 6H_2O$	0.054 g
Distilled water	10 ml

Preparation: Mix 0.054 g of $FeCl_3 \cdot 6H_2O$ with 10 ml distilled water. Dissolve the solution completely. Make fresh on day of assay in a new Corning tube.

10. Phosphate buffer (1M)

Na_2HPO_4	1.2 g
NaH_2PO_4	0.22 g
NaCl	8.5 g
Distilled water	Add up to 100 ml
HCl	Adjust pH

Preparation: Weight Na_2HPO_4, NaH_2PO_4, and NaCl. Then dissolve with distilled water add up to 100 ml in volumetric flask and adjust pH with HCl to 2.0 and 7.4.

Media Composition and Preparation

1. Mueller Hinton Broth

Composition per liter:

Beef infusion form	300.0 g
Casien hydrolysate (acid)	17.5 g
Starch	1.5 g
Distilled water	1 L
pH 7.4±0.2 at 25°C	

Source: This medium is available as a premixed powder from Titan Biotech Ltd.

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Preparation: Dissolve 21 g in 1000 ml distilled water. Gently heat if required to dissolve medium completely. Distribute into tube or flask and sterilize by autoclaving at 15 psi (121 °C) for 15 minutes. Cool to room temperature prior to dispense.

2. Mueller Hinton Agar

Composition per liter:

Beef infusion form	300.0 g
Casien hydrolysate (acid)	17.5 g
Starch	1.5 g
Distilled water	1 L
Agar	15 g

pH 7.4±0.2 at 25°C

Source: This medium is available as a premixed powder from Titan Biotech Ltd as broth form. Added 15 grams of agar to make it turn to agar form.

Preparation: Dissolve 21 g in 1000 ml distilled water. Added 15 g of agar into previous mixed. Gently heat if required to dissolve medium completely. Distribute into tube or flask and sterilize by autoclaving at 15 psi (121 °C) for 15 minutes. Cool to room temperature prior to dispense.

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1. Entrapment efficiency and loading efficiency

- **Procedure**

C. asiatica crude extract was run absorbance spectrum to find the best the wavelength (λmax) at which the absorbance is the greatest by UV-vis spectrophotometer. The 2 mg C. *asiatica* loaded BSA nanoparticles were dissolved in 1 ml methanol and gently shaken for 24 hours at 37 °C to completely extract *C. asiatica* crude extract to methanol[11].Then the solutions were centrifuged at 12000 rpm for 10 min, and the supernatant was kept and measured optical density (OD) by a UV-vis spectrophotometer at λmax. The amount of *C. asiatica* crude extract entrapped and loaded in C. *asiatica* loaded BSA nanoparticles is express as entrapment efficiency and loading efficiency calculated as follows (Xie et al., 2011):

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

All measurements were done in triplicate and three replications independently.

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1. Conventional

1.1 Ethanol Extraction

- **Standard curve**

Table 11 Standard curve of Conventional C. asiatica Ethanol extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.115	0.123	0.119	0.125	0.114	0.120	0.102	0.129	0.116
0.0004 g/ml	40	0.204	0.213	0.209	0.226	0.188	0.207	0.227	0.214	0.221
0.0006 g/ml	60	0.342	0.317	0.330	0.329	0.336	0.333	0.321	0.332	0.327
0.0008 g/ml	80	0.487	0.472	0.480	0.497	0.485	0.491	0.488	0.480	0.484
0.0010 g/ml	100	0.601	0.589	0.595	0.611	0.598	0.605	0.622	0.612	0.617

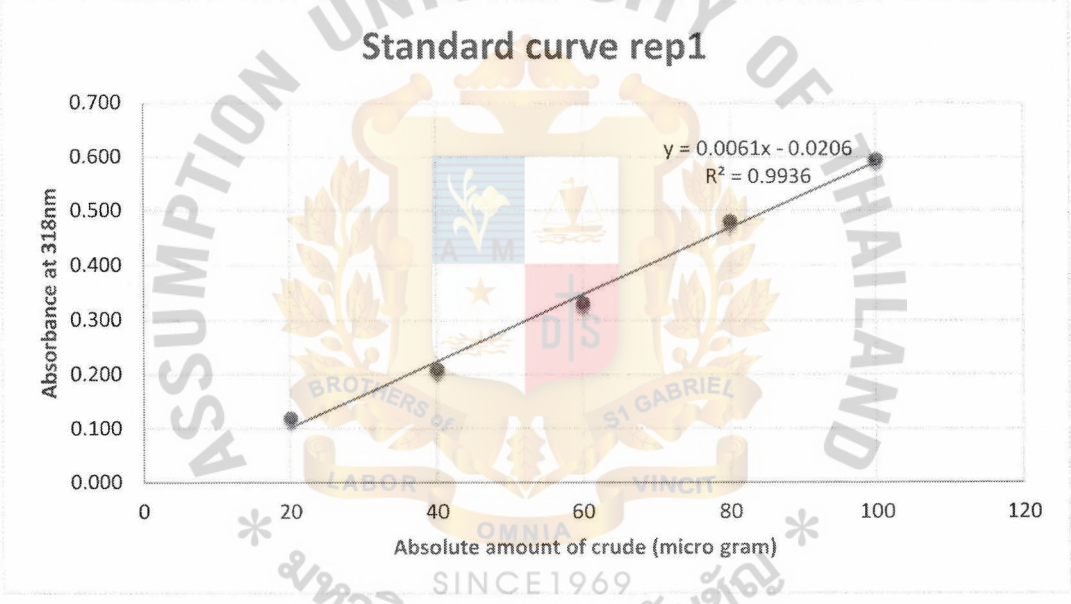

Figure 12 Standard curve plot between absorbance at 418 nm and absolute amount of crude

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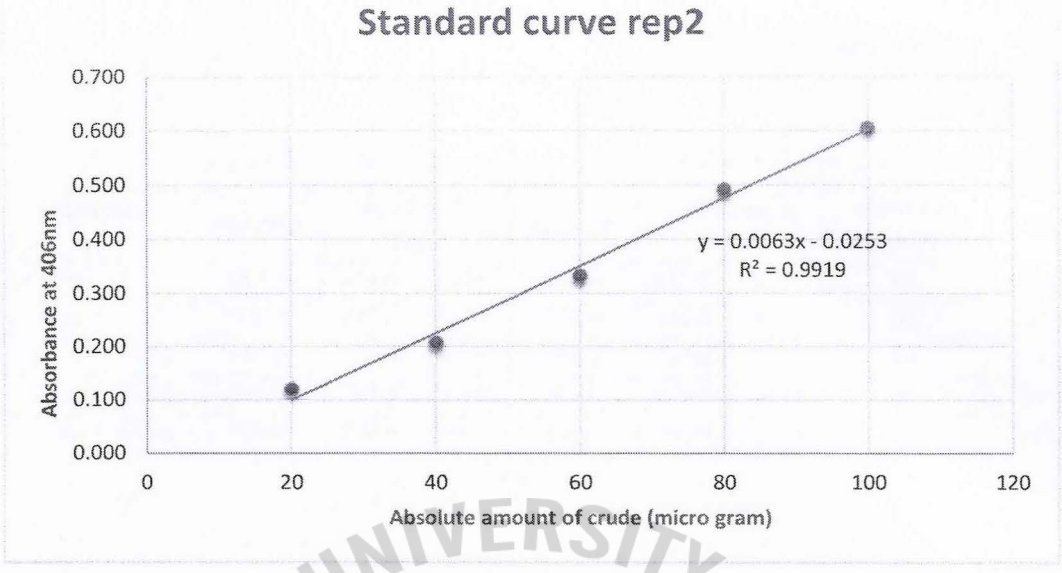

Figure 13 Standard curve plot between absorbance at 406 nm and absolute amount of crude

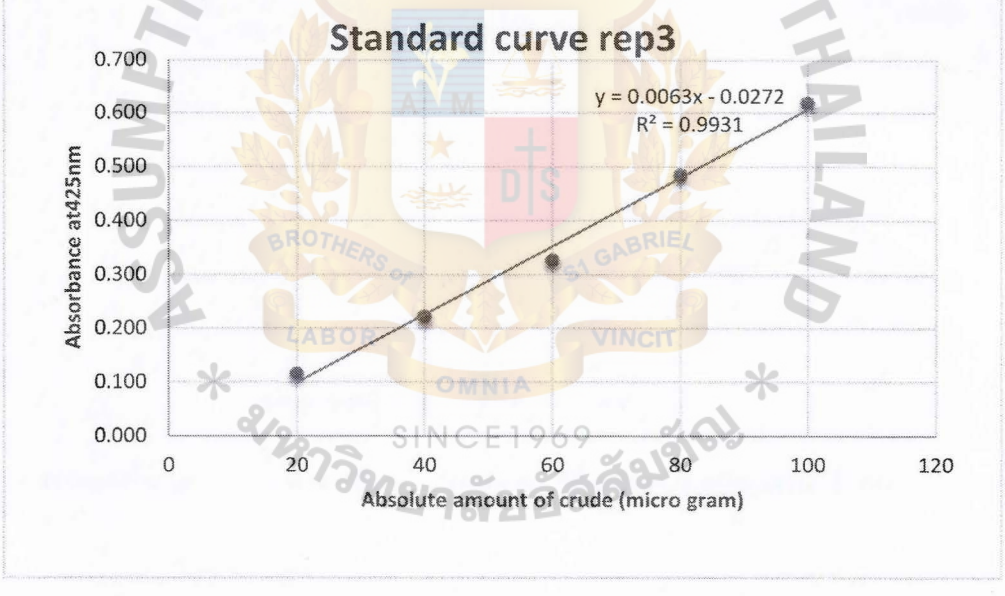

Figure 14 Standard curve plot between absorbance at 425 nm and absolute amount of crude

Result

From standard curve;

$y = 0.0061x - 0.0206$ for replication 1

$y = 0.0063x - 0.0253$ for replication 2

$y = 0.0063x - 0.0272$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Table 12 Result as absorbance value of conventional C. asiatica crude extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.454	0.458	0.477	0.467	0.464	0.459	0.416	0.458	0.437
2	0.471	0.465	0.439	0.423	0.455	0.465	0.456	0.437	0.426
3	0.499	0.454	0.475	0.462	0.473	0.488	0.454	0.449	0.437

Table 13 Result of calculated amount of crude extract in conventional C. asiatica crude extract loaded BSA nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	79.279	79.333	75.016
2	76.767	75.074	73.804
3	79.873	79.608	75.217

Table 14 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0004	0.0004	0.0004
2	0.0004	0.0004	0.0004
3	0.0004	0.0004	0.0004

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

Table 15 Entrapment efficiency of conventional C. asiatica crude extract loaded BSA nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	46.63	72.12	93.77
2	45.16	68.25	92.26
3	46.98	72.37	94.02

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}}\ x\ 100$$

Table 16 Loading efficiency of conventional C. asiatica crude extract loaded BSA nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	39.64	39.67	37.51
2	38.38	37.54	36.90
3	39.94	39.80	37.61

Table 17 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	46.26 ± 0.97^{E}	39.32 ± 0.82^{A}
1:3	70.91 ± 2.31^{B}	39.00 ± 1.27^{AB}
1:4	93.35 ± 0.96^{A}	37.34 ± 0.38^{ABC}

1.2 Chloroform Extraction

- **Standard curve**

Table 18 Standard curve of Conventional C. asiatica Choloroform extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.094	0.125	0.110	0.258	0.269	0.264	0.122	0.102	0.112
0.0004 g/ml	40	0.228	0.227	0.228	0.441	0.448	0.445	0.268	0.258	0.263
0.0006 g/ml	60	0.348	0.317	0.333	0.674	0.641	0.658	0.352	0.311	0.332
0.0008 g/ml	80	0.492	0.458	0.475	0.797	0.785	0.791	0.456	0.478	0.467
0.0010 g/ml	100	0.584	0.689	0.637	0.933	0.911	0.922	0.564	0.587	0.576

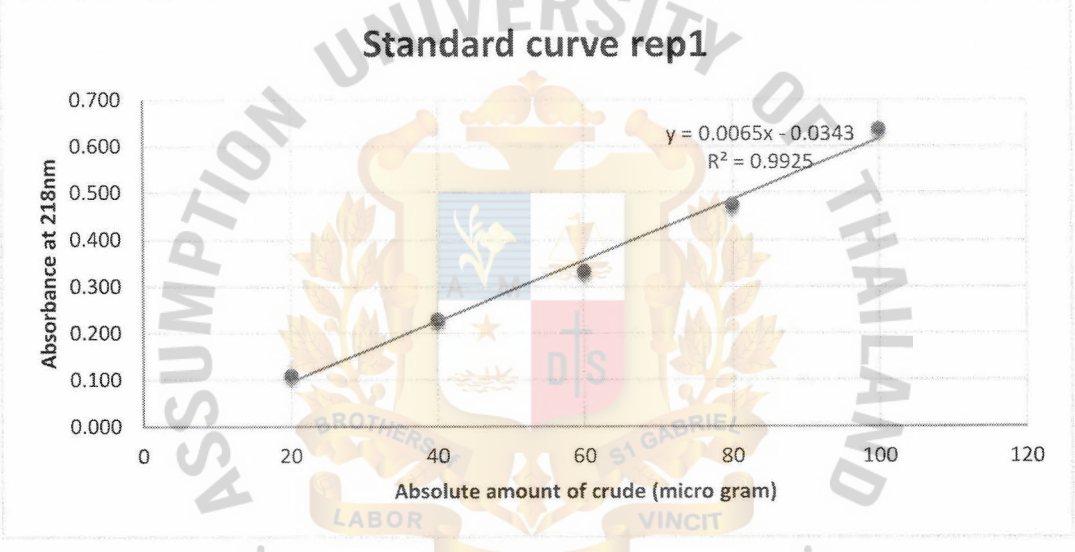

Figure 15 Standard curve plot between absorbance at 218 nm and absolute amount of Conventional C. asiatica Choloroform rep.1

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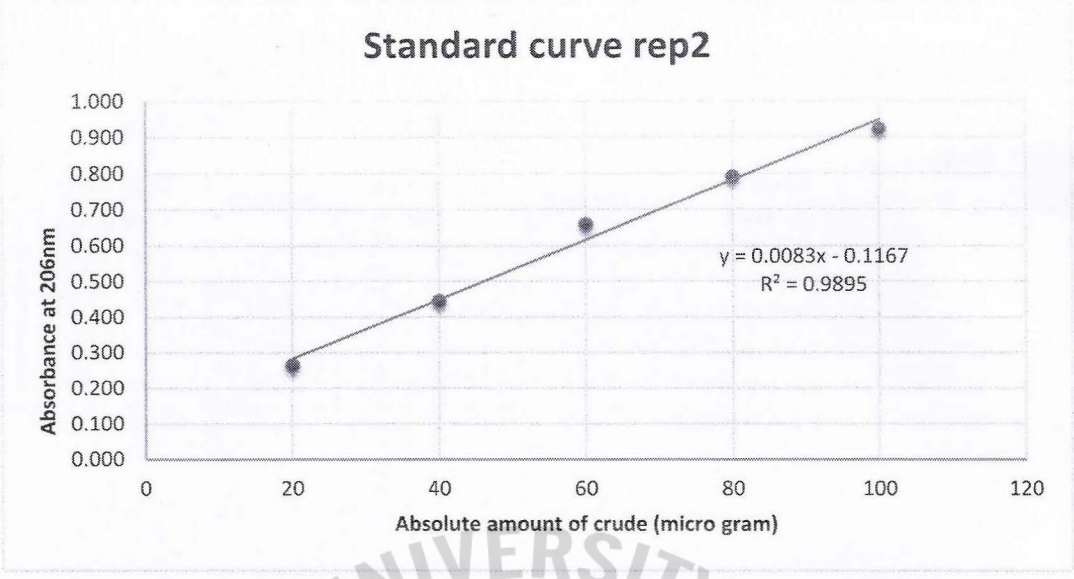

Figure 16 Standard curve plot between absorbance at 206 nm and absolute amount of Conventional C. asiatica Choloroform rep.2

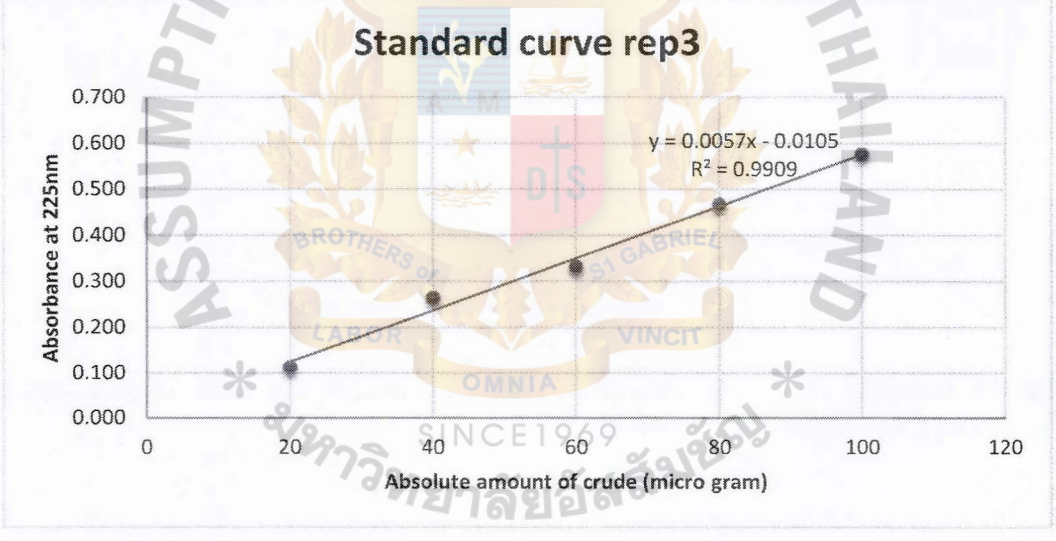

Figure 17 Standard curve plot between absorbance at 225 nm and absolute amount of Conventional C. asiatica Choloroform rep.3

Result

From standard curve;

$y = 0.0065x - 0.0343$ for replication 1

$y = 0.0083x - 0.1167$ for replication 2

$y = 0.0057x - 0.0105$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Table 19 Result as absorbance value of Conventional C. asiatica Choloroform loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.445	0.524	0.485	0.402	0.406	0.236	0.496	0.448	0.449
2	0.442	0.449	0.485	0.391	0.439	0.417	0.452	0.489	0.479
3	0.335	0.451	0.401	0.411	0.303	0.324	0.363	0.508	0.428

Table 20 Result of calculated amount of crude extract in Conventional C. asiatica Choloroform loaded BSA nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	79.841	58.815	76.713
2	69.321	64.141	71.088
3	71.257	62.544	77.807

Table 21 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0004	0.0003	0.0004
2	0.0003	0.0003	0.0004
3	0.0004	0.0003	0.0004

Entrapment efficiency (%)

$$= \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

Table 22 Entrapment efficiency of Conventional C. asiatica Choloroform loaded BSA nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	46.97	53.47	95.89
2	40.78	58.31	88.86
3	41.92	56.86	97.26

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

Table 23 Loading efficiency of Conventional C. asiatica Choloroform loaded BSA nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	39.92	29.41	38.36
2	34.66	32.07	35.54
3	35.63	31.27	38.90

Table 24 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	43.22 ± 3.29^{E}	36.74 ± 2.80^{ABC}
1:3	56.21 ± 2.48^{D}	30.92 ± 1.37^{D}
1:4	94.00 ± 4.51^{A}	37.60 ± 1.80^{ABC}

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1.3 Hexane Extraction

- **Standard curve**

Table 25 Standard curve of Conventional C. asiatica Hexane extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.094	0.089	0.092	0.125	0.096	0.111	0.102	0.113	0.108
0.0004 g/ml	40	0.218	0.227	0.223	0.256	0.208	0.232	0.216	0.238	0.227
0.0006 g/ml	60	0.335	0.327	0.331	0.335	0.349	0.342	0.356	0.321	0.339
0.0008 g/ml	80	0.512	0.478	0.495	0.497	0.485	0.491	0.495	0.512	0.504
0.0010 g/ml	100	0.595	0.589	0.592	0.603	0.611	0.607	0.596	0.617	0.607

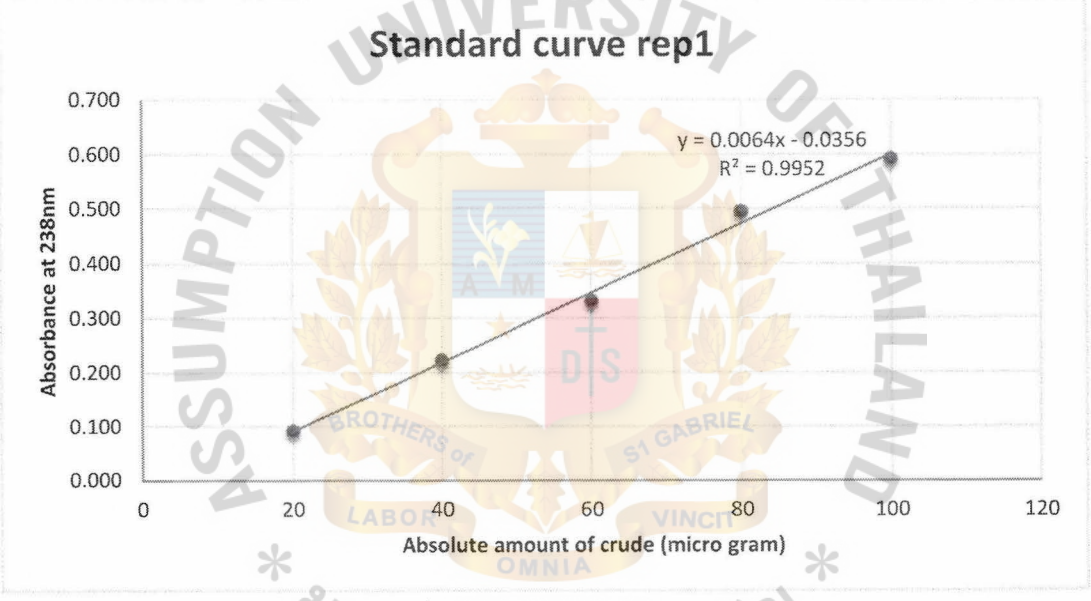

Figure 18 Standard curve plot between absorbance at 238 nm and absolute amount of Conventional C. asiatica Hexane crude rep.1

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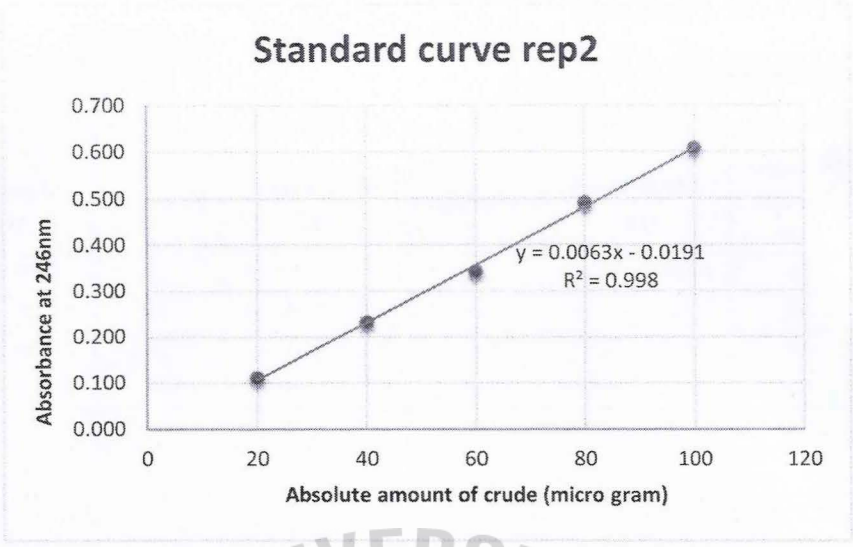

Figure 19 Standard curve plot between absorbance at 246 nm and absolute amount of Conventional C. asiatica Hexane crude extract crude rep.2

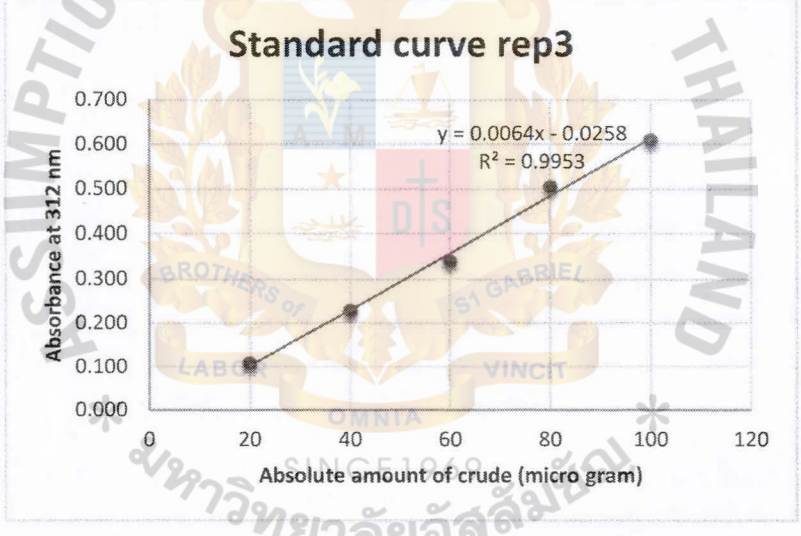

Figure 20 Standard curve plot between absorbance at 312 nm and absolute amount of Conventional C. asiatica Hexane crude extract crude rep.3

Result

From standard curve;

$y = 0.0065x - 0.0356$ for replication 1

$y = 0.0063x - 0.191$ for replication 2

$y = 0.0064x - 0.0258$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

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Table 26 Result as absorbance value of Conventional C. asiatica Hexane extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.465	0.475	0.423	0.442	0.496	0.435	0.382	0.452	0.457
2	0.451	0.385	0.426	0.424	0.455	0.445	0.421	0.425	0.428
3	0.457	0.394	0.471	0.472	0.443	0.424	0.432	0.422	0.365

Table 27 Result of calculated amount of crude extract in Conventional C. asiatica Hexane extract loaded BSA nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	76.552	77.073	72.802
2	69.804	73.085	70.439
3	72.885	73.771	67.521

Table 28 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0004	0.0004	0.0004
2	0.0003	0.0004	0.0004
3	0.0004	0.0004	0.0003

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

Table 29 Entrapment efficiency of Conventional C. asiatica Hexane extract loaded BSA nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	45.03	70.07	91.00
2	41.06	66.44	88.05
3	42.87	67.06	84.40

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

Table 30 Loading efficiency of Conventional C. asiatica Hexane extract loaded BSA nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	38.28	38.54	36.40
2	34.90	36.54	35.22
3	36.44	36.89	33.76

Table 31 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	42.99 ± 1.99^{E}	36.54 ± 1.69^{ABC}
1:3	67.86 ± 1.94^{BC}	37.32 ± 1.07^{ABC}
1:4	87.82 ± 3.31^{A}	35.13 ± 1.32^{ABCD}

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1. Organic farming

1.1 Ethanol Extraction

- **Standard curve**

Table 32 Standard curve of Organic C. asiatica Ethanol extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.120	0.125	0.123	0.112	0.104	0.108	0.115	0.132	0.124
0.0004 g/ml	40	0.212	0.222	0.217	0.206	0.211	0.209	0.201	0.222	0.212
0.0006 g/ml	60	0.354	0.336	0.345	0.339	0.346	0.343	0.332	0.345	0.339
0.0008 g/ml	80	0.465	0.454	0.460	0.475	0.455	0.465	0.445	0.482	0.464
0.0010 g/ml	100	0.589	0.602	0.596	0.601	0.588	0.595	0.585	0.596	0.591

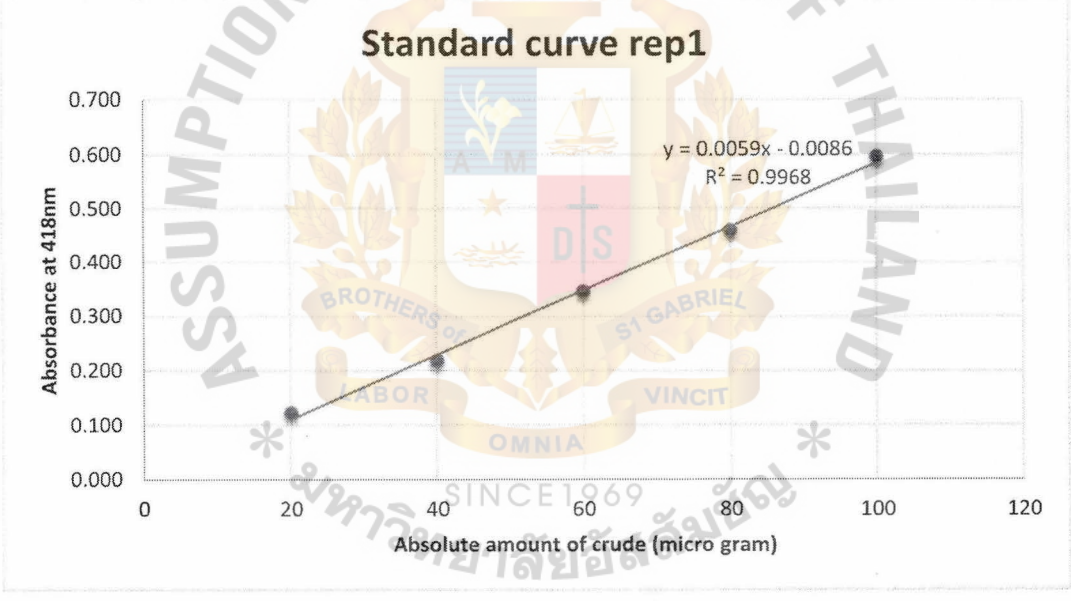

Figure 21 Standard curve plot between absorbance at 418 nm and absolute amount of Organic C. asiatica Ethanol crude rep.1

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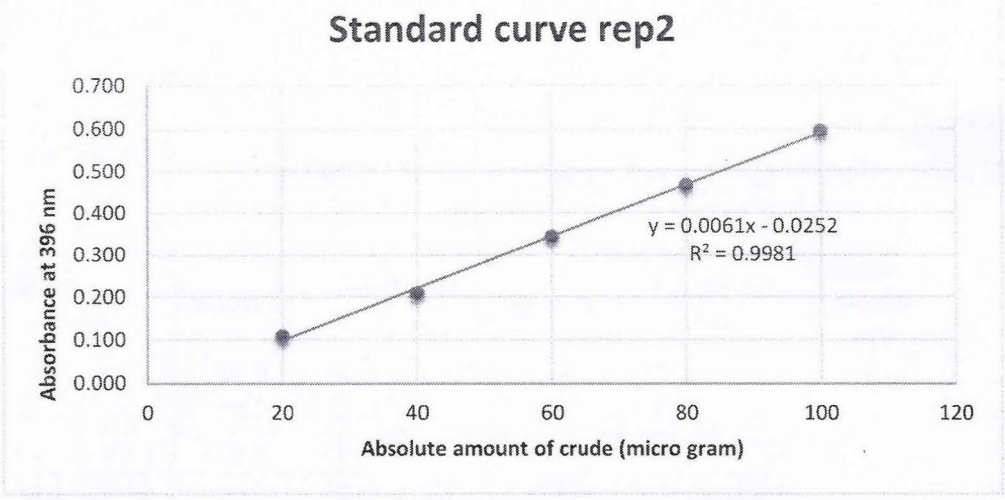

Figure 22 Standard curve plot between absorbance at 396 nm and absolute amount of Organic C. asiatica Ethanol crude rep.2

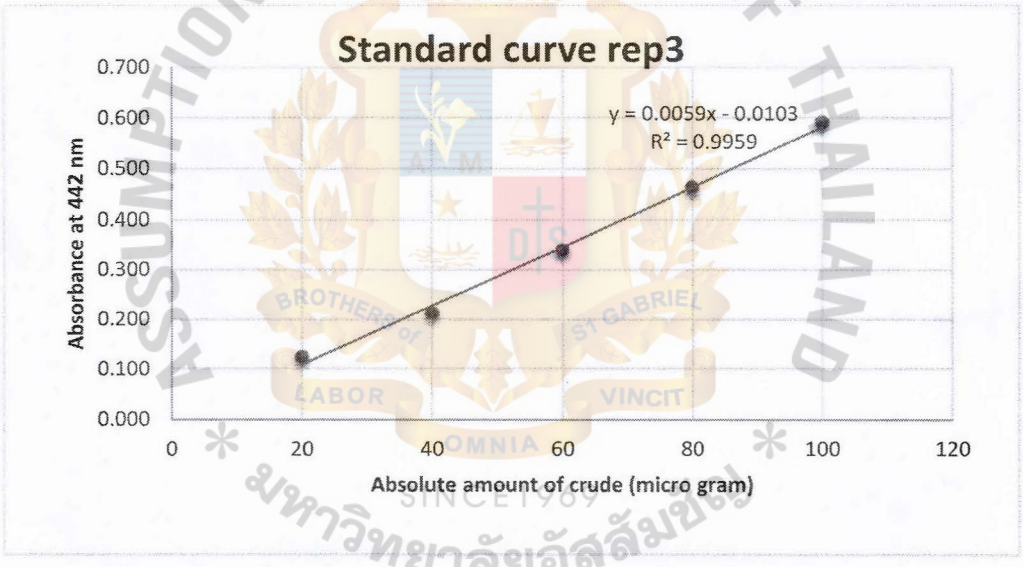

Figure 23 Standard curve plot between absorbance at 442 nm and absolute amount of Organic C. asiatica Ethanol crude rep.3

Result

From standard curve;

$y = 0.0059x - 0.0086$ for replication 1

$y = 0.0061x - 0.0252$ for replication 2

$y = 0.0059x - 0.0103$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

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Table 33 Result as absorbance value of Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.454	0.398	0.412	0.422	0.435	0.429	0.431	0.399	0.422
2	0.425	0.412	0.432	0.423	0.419	0.424	0.411	0.417	0.426
3	0.417	0.424	0.440	0.401	0.416	0.421	0.415	0.427	0.413

Table 34 Result of calculated amount of crude extract in Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	72.870	74.113	72.192
2	73.475	73.311	72.656
3	74.119	71.689	72.593

Table 35 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0004	0.0004	0.0004
2	0.0004	0.0004	0.0004
3	0.0004	0.0004	0.0004

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.asiatica \text{ crude extract}} \times 100$$

Table 36 Entrapment efficiency of Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	42.86	67.38	90.24
2	43.22	66.65	90.82
3	43.60	65.17	90.74

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\ asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

Table 37 Loading efficiency of Organic C. asiatica Ethanol crude extract loaded BSA nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	36.44	37.06	36.10
2	36.74	36.66	36.33
3	37.06	35.84	36.30

Table 38 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	43.23 ± 0.37^{E}	36.74 ± 0.31^{ABC}
1:3	66.40 ± 1.12^{BC}	36.52 ± 0.62^{ABC}
1:4	90.60 ± 0.31^{A}	36.24 ± 0.13^{ABC}

1.2 Chloroform Extraction

- **Standard curve**

Table 39 Standard curve of Organic C. asiatica Choloroform extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.135	0.102	0.119	0.126	0.117	0.122	0.112	0.120	0.116
0.0004 g/ml	40	0.224	0.203	0.214	0.216	0.201	0.209	0.231	0.216	0.224
0.0006 g/ml	60	0.334	0.327	0.331	0.312	0.354	0.333	0.311	0.332	0.322
0.0008 g/ml	80	0.485	0.477	0.481	0.499	0.458	0.479	0.498	0.475	0.487
0.0010 g/ml	100	0.598	0.589	0.594	0.607	0.603	0.605	0.598	0.603	0.601

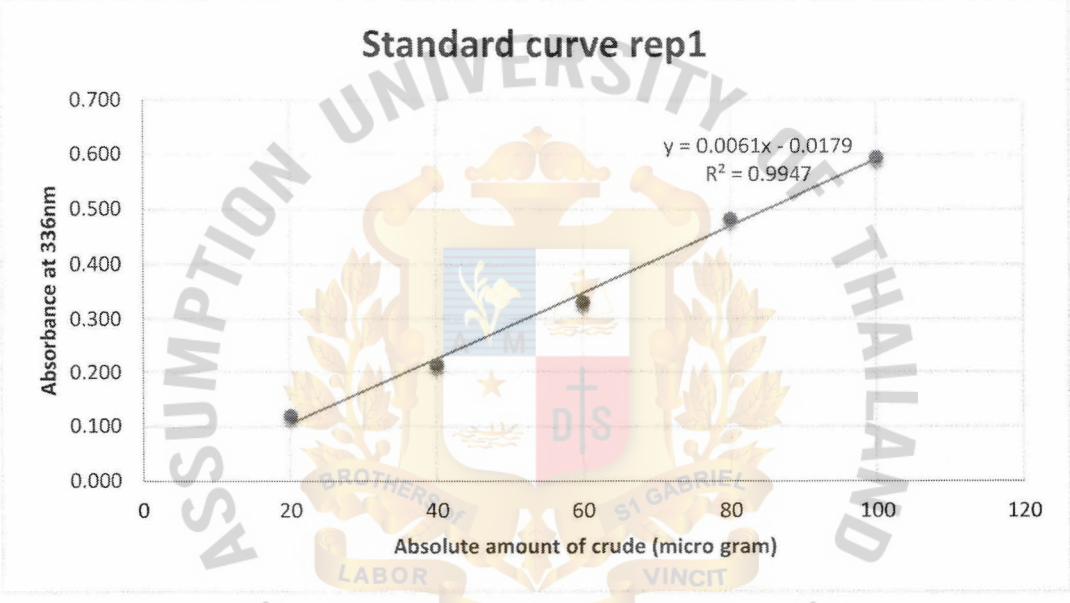

Figure 24 Standard curve plot between absorbance at 336 nm and absolute amount of Organic C. asiatica Choloroform crude rep.1

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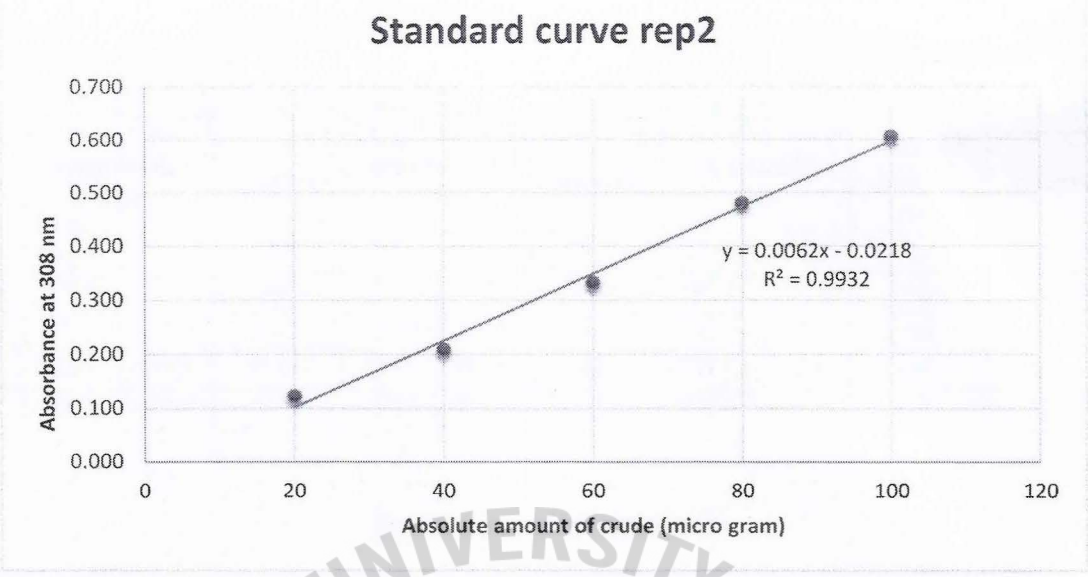

Figure 25 Standard curve plot between absorbance at 308 nm and absolute amount of Organic C. asiatica Choloroform crude rep.2

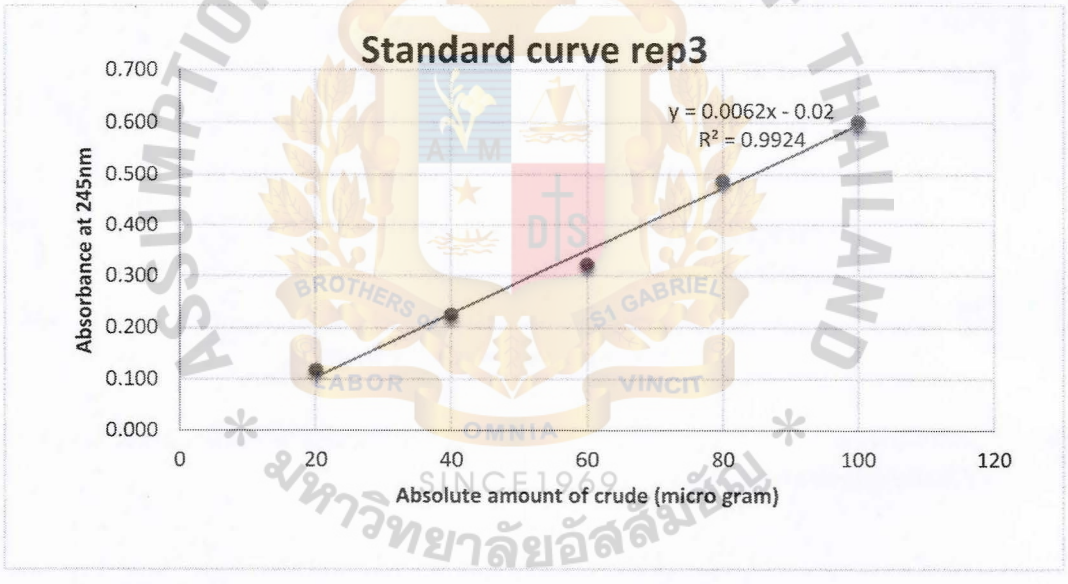

Figure 26 Standard curve plot between absorbance at 245 nm and absolute amount of Organic C. asiatica Choloroform crude rep.3

Result

From standard curve;

$y = 0.0061x - 0.0179$ for replication 1

$y = 0.0062x - 0.0218$ for replication 2

$y = 0.0062x - 0.0200$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Table 40 Result as absorbance value of Organic C. asiatica Choloroform crude extract loaded BSA nanoparticles at λmax

Replication	ODλmax								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.430	0.459	0.412	0.423	0.399	0.430	0.424	0.412	0.419
2	0.434	0.445	0.421	0.423	0.437	0.411	0.412	0.426	0.426
3	0.431	0.424	0.439	0.421	0.416	0.412	0.442	0.403	0.413

Table 41 Result of calculated amount of crude extract in Organic C. asiatica Choloroform crude extract loaded BSA nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	74.027	71.350	71.514
2	73.409	71.849	71.473
3	72.796	70.376	70.860

Table 42 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0004	0.0004	0.0004
2	0.0004	0.0004	0.0004
3	0.0004	0.0004	0.0004

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of total } C.\,asiatica \text{ crude extract}} \times 100$$

Table 43 Entrapment efficiency of Organic C. asiatica Choloroform crude extract loaded BSA nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	43.55	64.86	89.39
2	43.18	65.32	89.34
3	42.82	63.98	88.58

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

Table 44 Loading efficiency of Organic C. asiatica Choloroform crude extract loaded BSA nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	37.01	35.67	35.76
2	36.70	35.92	35.74
3	36.40	35.19	35.43

Table 45 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	43.18 ± 0.36^{E}	36.71 ± 0.31^{ABC}
1:3	64.72 ± 0.68^{BC}	35.60 ± 0.37^{ABCD}
1:4	89.10 ± 0.46^{A}	35.64 ± 0.18^{ABCD}

1.3 Hexane Extraction

- **Standard curve**

Table 46 Standard curve of Organic C. asiatica Hexane extract at λ_{max}

Concentration	Absolute amount (µg)	Absorbance rep1		Average	Absorbance rep2		Average	Absorbance rep3		Average
0.0002 g/ml	20	0.123	0.101	0.112	0.112	0.098	0.105	0.123	0.097	0.110
0.0004 g/ml	40	0.221	0.235	0.228	0.265	0.218	0.242	0.235	0.224	0.230
0.0006 g/ml	60	0.342	0.332	0.337	0.345	0.324	0.335	0.359	0.317	0.338
0.0008 g/ml	80	0.507	0.493	0.500	0.511	0.497	0.504	0.512	0.498	0.505
0.0010 g/ml	100	0.601	0.598	0.600	0.612	0.589	0.601	0.599	0.603	0.601

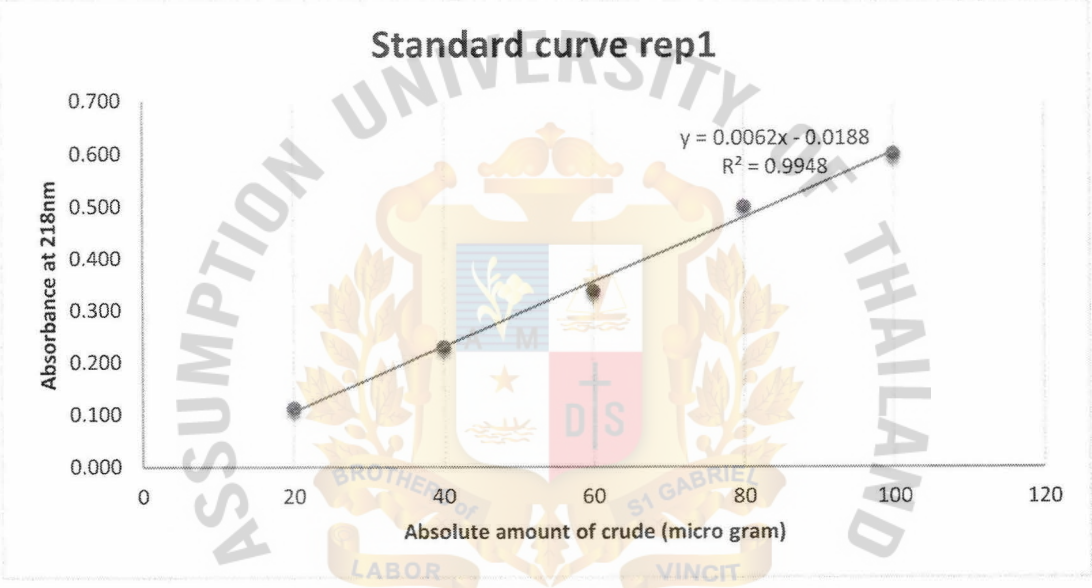

Figure 27 Standard curve plot between absorbance at 262 nm and absolute amount of Organic C. asiatica Hexane extract crude rep.1

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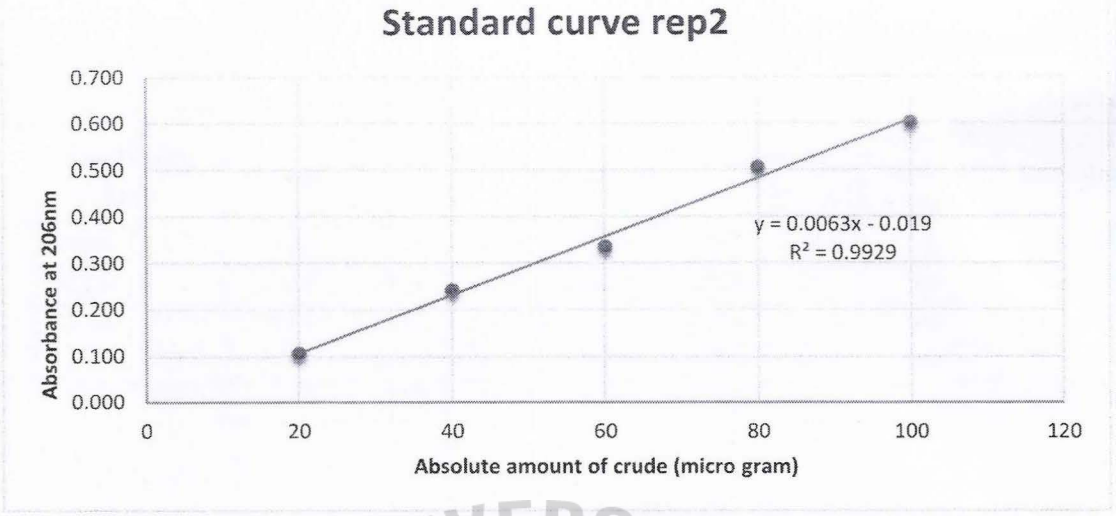

Figure 28 Standard curve plot between absorbance at 301 nm and absolute amount of of Organic C. asiatica Hexane extract crude rep.2

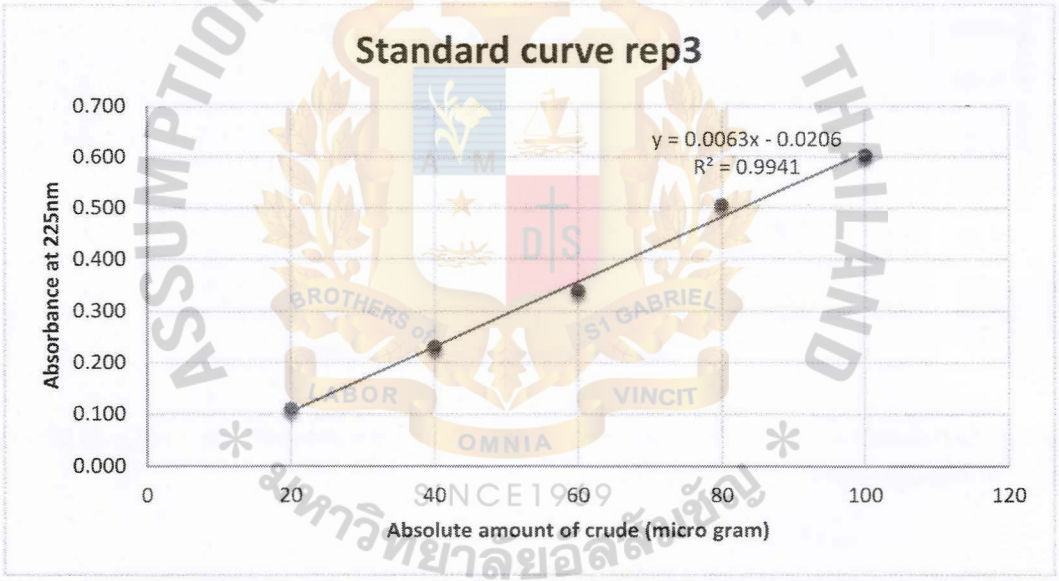

Figure 29 Standard curve plot between absorbance at 236 nm and absolute amount of Organic C. asiatica Hexane extract crude rep.3

Result

From standard curve;

$y = 0.0062x - 0.0188$ for replication 1

$y = 0.0063x - 0.019$ for replication 2

$y = 0.0063x - 0.0206$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Table 47 Result as absorbance value of Organic C. asiatica Hexane extract crude extract loased BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$								
	1:2			1:3			1:4		
	1	2	3	1	2	3	1	2	3
1	0.379	0.495	0.362	0.412	0.465	0.456	0.411	0.472	0.434
2	0.351	0.343	0.435	0.441	0.354	0.454	0.433	0.401	0.445
3	0.357	0.365	0.494	0.385	0.369	0.412	0.423	0.435	0.396

Table 48 Result of calculated amount of crude extract in Organic C. asiatica Hexane extract crude extract loased nanoparticles

Replication	microgram of crude extract in nps 100µl of 2mg/ml		
	1:2	1:3	1:4
1	69.484	74.699	73.839
2	62.751	69.101	70.688
3	67.608	64.963	69.619

Table 49 Result of calculated amount of crude extract in 1 mg nanoparticles

Replication	gram of crude extract in 1 mg nps		
	1:02	1:03	1:04
1	0.0003	0.0004	0.0004
2	0.0003	0.0003	0.0004
3	0.0003	0.0003	0.0003

$$\text{Entrapment efficiency (\%)} = \frac{\text{amount of □.□□□□□□□□ crude extract in nanopaticles}}{\text{amount of total □.□□□□□□□□ crude extract}} \times 100$$

Table 50 Entrapment efficiency of Organic C. asiatica Hexane extract crude extract loased nanoparticles

Replication	Entrapment Efficiency (%)		
	1:2	1:3	1:4
1	40.87	67.91	92.30
2	36.91	62.82	88.36
3	39.77	59.06	87.02

$$\text{Loading efficiency (\%)} = \frac{\text{amount of } C.\,asiatica \text{ crude extract in nanopaticles}}{\text{amount of nanoparticles}} \times 100$$

Table 51 Loading efficiency of Organic C. asiatica Hexane extract crude extract loased nanoparticles

Replication	Loading Efficiency (%)		
	1:2	1:3	1:4
1	34.74	37.35	36.92
2	31.38	34.55	35.34
3	33.80	32.48	34.81

Table 52 Summary of entrapment efficiency and loading efficiency in Mean±SD

CBNP with different ratio between Crude:BSA and crude	Entrapment efficiency (%)	Loading Efficiency (%)
1:2	39.19 ± 2.09^{E}	33.31 ± 1.74^{CD}
1:3	63.26 ± 4.44^{BC}	34.79 ± 2.44^{CD}
1:4	89.23 ± 2.74^{A}	35.69 ± 1.10^{ABCD}

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2. Solubility and stability

- Procedure

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

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

$$\text{Stability of } C.asiatica \text{ extract } (\%) = \frac{C_t}{C_o} \text{x } 100$$

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

1. Conventional farming

1.1 Ethanol Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

y = 0.0061x - 0.0206 for replication 1

y = 0.0063x - 0.0253 for replication 2

y = 0.0063x - 0.0272 for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

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Result

Table 53 The amount of Conventional C. asiatica Ethanol crudev extract presence in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	10.47	8.82	10.28
1	9.72	15.61	15.88
2	13.03	16.92	17.70
3	14.25	16.99	22.35
4	16.47	20.06	18.59
5	23.02	23.48	23.17
6	23.74	23.90	25.69

Table 54 The amount of crude presence in PBS at time point from 1 mg Conventional C. asiatica Ethanol extract crude

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	52.36	44.09	51.38
1	48.59	78.06	79.40
2	65.13	84.58	88.48
3	71.23	84.93	111.76
4	82.35	100.29	92.95
5	115.09	117.38	115.83
6	118.70	119.49	128.45

Table 55 Stability of Conventional C. asiatica Ethanol extract crude loaed BSAnanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	61	59	55
1	61	53	48
2	59	52	45
3	59	52	40
4	57	49	44
5	54	46	39
6	53	46	35

Table 56 Result as absorbance value of Conventional C. asiatica Ethanol extract crude loaed BSAnanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.111	0.098	0.088	0.089	0.077	0.098	0.063	0.085	0.075	0.191	0.222	0.211
2	0.107	0.121	0.099	0.079	0.092	0.088	0.083	0.076	0.075	0.187	0.211	0.198
3	0.107	0.103	0.114	0.098	0.078	0.092	0.084	0.081	0.073	0.209	0.214	0.226

Table 57 Solubility of Conventional C. asiatica Ethanol extract crude loaed BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	196.07	178.03	155.63	374.75
2	179.84	177.20	163.97	359.45
3	161.16	191.04	169.10	388.42

Table 58 Summary of solubility in Mean±SD of Conventional C. asiatica Ethanol extract crude loaed BSA nanoparticles

Sample	Solubility (µg/ml)
1:2	202.62 ± 7.17^{EF}
1:3	176.08 ± 2.69^{FGH}
1:4	210.62 ± 4.23^{H}
crude	374.21 ± 14.49^{A}

1.2 Chloroform Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

$y = 0.0065x - 0.0343$ for replication 1

$y = 0.0083x - 0.1167$ for replication 2

$y = 0.0057x - 0.0105$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Result

Table 59 The amount of conventional C. asiatica chloroform extract crude in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	18.68	18.61	19.76
1	23.53	22.36	22.50
2	23.15	21.71	20.25
3	22.02	23.04	22.72
4	23.09	22.37	22.64
5	25.43	24.29	24.92
6	28.04	27.29	26.32

Table 60 The amount of C. asiatica chloroform extract crude presence in PBS at time point from 1 mg nanoparticles

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	93	93	99
1	118	112	112
2	116	109	101
3	110	115	114
4	115	112	113
5	127	121	125
6	140	136	132

Table 61 Stability of conventional C. asiatica chloroform extract loaded BSA nanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	56	50	43
1	53	47	39
2	53	48	42
3	54	46	39
4	53	47	39
5	52	45	36
6	51	42	35

Table 62 Result as absorbance value of conventional C. asiatica chloroform extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.118	0.102	0.111	0.084	0.075	0.081	0.102	0.093	0.087	0.124	0.128	0.142
2	0.173	0.073	0.082	0.078	0.064	0.074	0.091	0.107	0.079	0.118	0.125	0.136
3	0.105	0.103	0.114	0.085	0.077	0.063	0.101	0.095	0.062	0.115	0.147	0.125

Table 63 Solubility of conventional C. asiatica chloroform extract loaded BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	222.513	175.846	197.385	254.821
2	220.974	163.538	194.821	236.654
3	217.897	168.154	185.077	244.737

Table 64 Summary of solubility in Mean±SD

Sample	Solubility (µg/ml)
1:2	220.46 ± 2.35^{CD}
1:3	169.18 ± 6.22^{FGH}
1:4	192.43 ± 6.49^{EFG}
crude	245.40 ± 9.10^{C}

1.3 Hexane Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

$y = 0.0065x - 0.0356$ for replication 1

$y = 0.0063x - 0.0191$ for replication 2

$y = 0.0064x - 0.0258$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Result

Table 65 The amount of Conventional C. saitica hexane crude extract presence in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	16.19	16.00	17.26
1	21.51	20.33	20.16
2	20.80	19.71	17.62
3	19.24	20.63	20.26
4	20.80	20.04	20.27
5	23.11	22.09	22.71
6	26.13	25.30	24.16

Table 66 The amount of Conventional C. saitica hexane crude presence in PBS at time point from 1 mg nanoparticles

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	81	80	86
1	108	102	101
2	104	99	88
3	96	103	101
4	104	100	101
5	116	110	114
6	131	126	121

Table 67 Stability of Conventional C. saitica hexane crude extract loaded BSA nanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	58	53	46
1	54	49	42
2	55	49	45
3	56	49	42
4	55	49	42
5	53	47	39
6	52	44	37

Table 68 Result as absorbance value of C. saitica hexane crude extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.118	0.102	0.111	0.084	0.075	0.081	0.102	0.093	0.087	0.124	0.128	0.142
2	0.173	0.073	0.082	0.078	0.064	0.074	0.091	0.107	0.079	0.118	0.125	0.136
3	0.105	0.103	0.114	0.085	0.077	0.063	0.101	0.095	0.062	0.115	0.147	0.125

Table 69 Solubility of C. saitica hexane crude extract loaded BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	222.513	175.846	197.385	254.821
2	220.974	163.538	194.821	236.654
3	217.897	168.154	185.077	244.737

Table 70 Summary of solubility in Mean±SD

Sample	Solubility (µg/ml)
1:2	167.75 ± 6.42^{GH}
1:3	173.37 ± 2.80^{FGH}
1:4	167.75 ± 5.64^{GH}
crude	254.13 ± 5.28^{C}

2. Organic farming

2.1 Ethanol Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

$y = 0.0059x - 0.0086$ for replication 1

$y = 0.0061x - 0.0252$ for replication 2

$y = 0.0059x - 0.0103$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

.Result

Table 71 the amount of Organic C. asiatica ethanol extract crude presence in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	12.03	11.06	11.55
1	14.07	14.93	15.27
2	14.20	14.81	15.12
3	14.32	15.85	16.09
4	24.79	15.89	15.49
5	20.71	15.38	16.23
6	16.12	15.25	15.82

Table 72 The amount of Organic C. asiatica ethanol extract crude presence in PBS at time point from 1 mg nanoparticles

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	60.15	55.32	57.74
1	70.35	74.65	76.36
2	71.00	74.06	75.61
3	71.58	79.27	80.47
4	123.95	79.43	77.45
5	103.55	76.88	81.17
6	80.60	76.27	79.10

Table 73 Stability of Organic C. asiatica ethanol extract loaded BSA nanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	60	57	53
1	59	54	48
2	59	54	49
3	59	53	47
4	52	53	48
5	55	53	47
6	58	53	48

Table 74 Result as absorbance value of Organic C. asiatica ethanol extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.102	0.112	0.109	0.107	0.101	0.098	0.098	0.115	0.107	0.159	0.177	0.161
2	0.117	0.112	0.099	0.097	0.092	0.111	0.114	0.106	0.096	0.162	0.169	0.148
3	0.107	0.103	0.114	0.098	0.112	0.092	0.116	0.081	0.123	0.102	0.112	0.109

Table 75 Solubility of Organic C. asiatica ethanol extract loaded BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	204.41	190.28	195.37	305.36
2	209.62	205.25	208.52	295.52
3	208.42	193.73	198.25	296.07

Table 76 Summary of solubility in Mean±SD

Sample	Solubility (µg/ml)
1:2	207.48 ± 2.73^{EF}
1:3	196.42 ± 7.84^{CD}
1:4	200.71 ± 6.92^{CDE}
crude	298.98 ± 5.53^{B}

2.2 Chloroform Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

$y = 0.0061x - 0.0179$ for replication 1

$y = 0.0062x - 0.0218$ for replication 2

$y = 0.0062x - 0.0200$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

.Result

Table 77 The amount of Organic chloroform extract crude presence in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	16.73	18.83	15.48
1	23.23	22.95	23.65
2	22.36	23.14	21.90
3	27.63	19.46	20.20
4	20.67	21.16	21.16
5	21.79	22.99	22.74
6	29.39	32.53	32.38

Table 78 The amount of Organic chloroform extract crude presence in PBS at time point from 1 mg nanoparticles

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	84	94	77
1	116	115	118
2	112	116	109
3	138	97	101
4	103	106	106
5	109	115	114
6	147	163	162

Table 79 Stability of Organic chloroform extract loaded BSA nanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	57	50	48
1	53	46	38
2	54	46	40
3	51	50	42
4	55	48	41
5	54	46	39
6	50	38	27

Table 80 Result as absorbance value of Organic chloroform extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.115	0.122	0.113	0.107	0.113	0.111	0.101	0.098	0.103	0.127	0.121	0.122
2	0.121	0.112	0.12	0.099	0.112	0.111	0.101	0.101	0.096	0.125	0.116	0.124
3	0.121	0.103	0.121	0.102	0.122	0.099	0.096	0.081	0.103	0.132	0.109	0.127

Table 81 Solubility of Organic chloroform extract loaded BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	225.03	214.64	198.80	235.96
2	226.67	209.73	196.61	233.22
3	222.30	210.27	186.78	234.86

Table 82 Summary of solubility in Mean±SD

Sample	Solubility (µg/ml)
1:2	224.66 ± 2.21^{CD}
1:3	211.55 ± 2.70^{CDE}
1:4	194.06 ± 6.40^{EFG}
crude	234.68 ± 1.38^{C}

2.3 Hexane Extraction

Standard curve

Using standard curve from previous experiment

From standard curve;

$y = 0.0062x - 0.0188$ for replication 1

$y = 0.0063x - 0.019$ for replication 2

$y = 0.0063x - 0.0206$ for replication 3

Where, y is the absorbance and x is represent the amount of crude in sample.

Result

Table 83 The amount of Organic Hexane extract crude presence in PBS at time point

Time	Concentration of crude inPBS		
	1:2	1:3	1:4
0	15.35	15.15	17.28
1	20.77	19.57	20.24
2	20.00	18.90	17.65
3	18.49	19.92	20.38
4	20.04	19.29	20.37
5	22.42	21.38	22.86
6	25.55	24.70	24.35

Table 84 The amount of Organic Hexane extract crude presence in PBS at time point from 1 mg nanoparticles

Time	Concentration of *C. asiatica* (µg) in PBS from 1mg nps		
	1:2	1:3	1:4
0	77	76	86
1	104	98	101
2	100	94	88
3	92	100	102
4	100	96	102
5	112	107	114
6	128	123	122

Table 85 Stability of Organic Hexane extract loaded BSA nanoparticles over period of time

Time	%Reduction		
	1:2	1:3	1:4
0	58	54	46
1	55	49	42
2	55	50	45
3	56	49	42
4	55	50	42
5	54	48	39
6	52	45	37

Table 86 Result as absorbance value of Organic Hexane extract loaded BSA nanoparticles at λmax

Replication	$OD_{\lambda max}$											
	1:2			1:3			1:4			Crude		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.098	0.081	0.098	0.084	0.083	0.084	0.096	0.077	0.087	0.149	0.108	0.123
2	0.098	0.101	0.082	0.107	0.041	0.120	0.095	0.067	0.126	0.115	0.155	0.106
3	0.112	0.085	0.107	0.074	0.077	0.093	0.077	0.095	0.081	0.145	0.112	0.132

Table 87 Solubility of Organic Hexane extract loaded BSA nanoparticles

Replication	microgram of crude extract in solution 1 ml			
	1:2	1:3	1:4	Crude
1	179.247	165.269	170.108	234.624
2	178.836	171.9577	182.5397	229.1005
3	193.545	161.799	166.561	238.519

Table 88 Summary of solubility in Mean±SD

Sample	Solubility (µg/ml)
1:2	183.88 ± 8.38^{GH}
1:3	166.34 ± 5.16^{FGH}
1:4	173.07 ± 8.39^{GH}
crude	234.08 ± 4.73^{C}

3. Antioxidant activity by Ferric reducing antioxidant potential assay (FRAP)

- **Procedure**

The modified ferric reducing antioxidant potential assay (Benzie and Strain, 1999) was used to determine FRAP value of *C. asiatica* crude extract and *C. asiatica* loaded - BSA nanopartices. The FRAP reagent was prepared using 300 mmol sodium acetate buffer at pH 3.6, 20 mmol iron chloride and 10 mmol 2,4,6-tripyridyl-s-triazine dissolved in 40 mmol hydrochloric acid at a ratio of 10:1:1 (v:v:v).The reagent was incubated at 37°C for 10 minutes before use. The 20µl of 1 mg/ml the extract and *C. asiatica* loaded - BSA nanopartices was added, followed by adding 1000µl of FRAP reagent vigorously and kept in the dark for 30 minutes. The optical density (OD) of this mixture was measured at 593 nm. FRAP values were expressed as mmol Fe^{2+}/mg of sample.

All measurements were done in triplicate and three replications independently.

Standard curve

Using 1 mM of Ferrous sulphate and diluted to make a standard curve

Table 89 Standard curve of Ferrous sulphate at OD593

Concentration (mM)	Absolute amount(mM)	Absorbance		Average
0.2	0.004	0.086	0.102	0.094
0.4	0.008	0.099	0.089	0.094
0.6	0.012	0.103	0.142	0.123
0.8	0.016	0.147	0.135	0.141
1	0.020	0.193	0.186	0.190

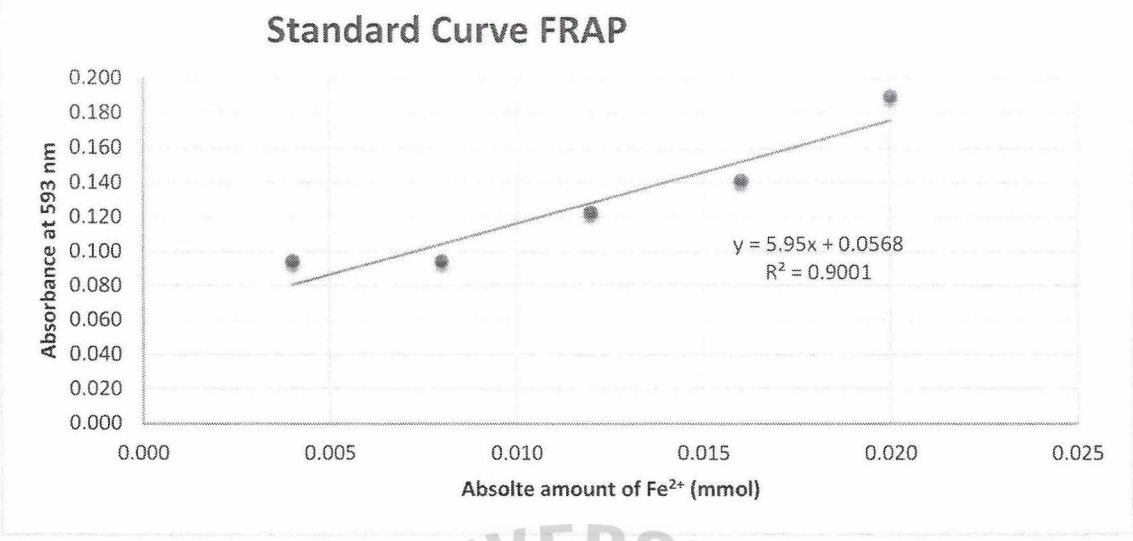

Figure 30 Standard curve plot between absorbance at 593 nm and absolute amount of Fe^{2+}

4. Antioxidant activity by DPPH radical scavenging activity

- **Procedure**

The modified DPPH radical scavenging activity (Brand-Williams et al., 1995) was used for percentage DPPH radical scavenging determination. The 100 µl of 1 mg/ml *C. asiatica* crude extract and *C. asiatica* loaded - BSA nanopartices were mixed with 3.9 ml DPPH reagent (50 µM). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 minutes. The optical density (OD) was measured at 517 nm. The results were expressed as percentage reduction of DPPH (Molyneux, 2004).

$$\text{Percentage reduction of DPPH} = 100\left(\frac{\square_0 - \square_{\square}}{\square_0}\right)$$

Where A_0 is the initial absorbance and A_c is the value for added sample concentration c. All measurements were done in triplicate and three replications independently.

Release kinetic *in Vitro*

Procedure

Release kinetic methodology was modified (Xie, et al., 2011). The release of *C. asiatica* crude extract and *C. asiatica* loaded - BSA nanopartices were done by dissolving 20 mg of *C. asiatica* loaded - BSA nanopartices in 15 ml artificial gastric juice (0.01 M PBS pH 2.0) and intestinal juice without enzymes (0.01 M PBS pH 7.4). The mixture is incubated at 37 °C at 200 rpm. At designated time points (0, 1, 2, 3, 4, 5, and 6 hours), mixture is sampled and centrifuged at 3000 rpm for 10 min. The pellet is resuspended in 100 µL of methanol to determine the amount of *C. asiatica* crude extract released by measuring optical density (OD) by UV-vis spectrophotometer

Calculation

1. Artificial gastric juice (0.01 M PBS pH 2.0)

1.1 Conventional

Table 90 Concentration of crude extract in phosphate buffer solution at pH2.0

Time	Concentration of crude in PBS (µg)								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	40.33	37.98	33.93	27.26	29.90	28.67	34.12	32.63	33.71
1	57.33	47.54	38.19	33.52	40.98	38.63	41.99	42.69	39.57
2	59.54	63.91	52.90	39.73	48.97	47.33	42.37	43.86	41.75
3	68.24	68.36	54.06	42.51	43.91	44.51	42.60	44.14	48.11
4	68.98	71.64	69.34	46.34	47.22	47.08	44.04	47.35	48.45
5	70.71	70.79	70.97	50.52	47.15	50.81	45.95	47.58	49.55
6	72.96	79.51	72.20	54.10	50.76	61.45	46.97	48.30	49.65

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Table 91 %release of C. asiatica BSA nanopartices in PBS solution at pH2.0

Time	% Release								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	9.65	9.35	7.98	4.78	8.08	10.62	9.22	8.82	12.48
1	13.72	11.71	8.99	5.88	11.08	14.31	11.35	11.54	14.66
2	14.24	15.74	12.45	6.97	13.23	17.53	11.45	11.85	15.46
3	16.33	16.84	12.72	7.46	11.87	16.48	11.51	11.93	17.82
4	16.50	17.65	16.31	8.13	12.76	17.44	11.90	12.80	17.95
5	16.92	17.44	16.70	8.86	12.74	18.82	12.42	12.86	18.35
6	17.46	19.58	16.99	9.49	13.72	22.76	12.69	13.05	18.39

Table 92 release of C. asiatica BSA nanopartices for zero order at pH2.0

Time	Zero order								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	10.20	*5.74	2.55	3.76	6.65	5.97	*4.72	6.04	3.52
2	11.53	15.56	11.38	7.48	11.44	11.20	4.95	6.74	4.82
3	16.74	18.23	12.08	9.15	8.40	9.50	5.09	6.91	8.64
4	17.19	20.20	21.25	11.45	10.40	11.05	5.95	8.84	8.85
5	18.22	19.69	22.22	13.95	10.35	13.28	7.10	8.97	9.50
6	19.58	24.92	22.96	16.10	12.52	19.67	7.71	9.40	9.56

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Table 93 release of C. asiatica BSA nanopartices for first order as log at pH2.0

Time	First order								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.35	0.22	0.12	0.21	0.32	0.30	0.21	0.27	0.16
2	0.39	0.52	0.44	0.38	0.49	0.50	0.22	0.30	0.21
3	0.53	0.59	0.47	0.44	0.38	0.44	0.22	0.30	0.36
4	0.54	0.63	0.71	0.53	0.46	0.50	0.26	0.37	0.36
5	0.56	0.62	0.74	0.62	0.46	0.57	0.30	0.38	0.39
6	0.59	0.74	0.76	0.69	0.53	0.76	0.32	0.39	0.39

Table 94 release of C. asiatica BSA nanopartices for Higuchi model at pH2.0

Time	Higuchi model								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	34.40	28.53	22.91	16.36	17.94	17.20	20.47	19.58	20.23
1	25.26	27.12	22.44	14.22	17.39	16.39	17.82	18.11	16.79
2	23.64	23.68	18.73	13.76	16.96	16.40	14.68	15.19	14.46
3	20.69	21.49	20.80	12.75	13.17	13.35	12.78	13.24	14.43
4	18.97	18.99	19.04	12.43	12.67	12.63	11.82	12.71	13.00
5	17.87	19.47	17.68	12.37	11.55	12.45	11.26	11.65	12.14
6	34.40	28.53	22.91	16.36	17.94	17.20	20.47	19.58	20.23

1.2 Orgaic farming

Table 95 Concentration of crude extract in phosphate buffer solution at pH2.0

Time	Concentration of crude in PBS (µg)								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	42.84	39.97	44.88	36.41	35.17	38.98	32.73	32.13	32.84
1	46.92	45.37	45.33	38.11	41.10	39.33	41.03	41.95	39.62
2	47.47	47.86	47.88	42.84	43.16	41.96	43.52	43.73	42.00
3	57.32	47.97	50.36	46.72	50.87	46.95	48.82	50.45	45.96
4	58.11	53.55	54.82	50.92	51.24	50.88	52.78	51.78	49.26
5	60.02	54.07	55.07	51.73	52.76	51.14	53.88	52.36	50.61
6	64.84	62.94	60.11	55.62	53.46	58.71	54.54	54.13	52.98

Table 96 %release of C. asiatica BSA nanopartices in PBS solution at pH2.0

Time	% Release								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	9.11	10.80	16.62	7.07	9.51	14.44	8.84	8.68	12.16
1	9.98	12.26	16.79	6.58	11.11	14.57	11.09	11.34	14.68
2	10.10	12.94	17.73	7.52	11.67	15.54	11.76	11.82	15.56
3	12.20	12.97	18.65	7.91	13.75	17.39	13.19	13.63	17.02
4	12.36	14.47	20.30	9.01	13.85	18.84	14.26	13.99	18.24
5	12.77	14.61	20.40	8.39	14.26	18.94	14.56	14.15	18.75
6	13.80	17.01	22.26	9.76	14.45	21.74	14.74	14.63	19.62

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Table 97 release of C. asiatica BSA nanopartices for zero order at pH2.0

Time	Zero order								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	2.45	5.40	0.45	1.02	3.56	0.21	4.98	5.90	4.07
2	2.78	7.90	3.01	3.86	4.80	1.79	6.48	6.96	5.50
3	8.69	2.60	5.04	6.19	9.42	4.78	9.66	10.99	7.87
4	9.16	13.58	9.94	8.71	9.64	7.14	12.03	11.79	9.85
5	10.31	14.11	10.20	9.19	10.55	7.29	12.69	12.14	10.67
6	22.01	22.97	15.24	11.53	10.97	11.83	13.09	13.20	12.09

Table 98 release of C. asiatica BSA nanopartices for first order as log at pH2.0

Time	First order								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.09	0.13	0.01	0.05	0.16	0.01	0.23	0.27	0.19
2	0.10	0.18	0.06	0.16	0.20	0.07	0.29	0.31	0.25
3	0.29	0.18	0.12	0.25	0.37	0.19	0.40	0.45	0.34
4	0.31	0.29	0.20	0.34	0.38	0.27	0.48	0.48	0.41
5	0.34	0.30	0.20	0.35	0.41	0.27	0.50	0.49	0.43
6	0.41	0.45	0.29	0.42	0.42	0.41	0.51	0.52	0.48

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Table 99 release of C. asiatica BSA nanopartices for Higuchi model at pH2.0

Time	Higuchi model								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	25.70	23.98	26.93	21.84	21.10	23.39	24.62	25.17	23.77
1	20.14	20.31	20.31	18.17	18.31	17.80	18.46	18.55	17.82
2	19.86	16.62	17.45	16.18	17.62	16.26	16.91	17.48	15.92
3	17.43	16.06	16.45	15.28	15.37	15.26	15.83	15.53	14.78
4	16.11	14.51	14.78	13.88	14.16	13.72	14.46	14.05	13.58
5	15.88	15.42	14.72	13.62	13.10	14.38	13.36	13.26	12.98
6	25.70	23.98	26.93	21.84	21.10	23.39	24.62	25.17	23.77

2. Intestinal juice without enzymes (0.01 M PBS pH 7.4)

2.1 Conventional

Table 100 Concentration of crude extract in phosphate buffer solution at pH7.4

Time	Concentration of crude in PBS (µg)								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	20.76	19.31	19.83	20.39	18.47	19.06	24.25	20.18	18.98
1	22.45	18.83	19.39	21.43	19.64	19.31	25.30	18.09	19.31
2	21.92	18.73	16.99	23.59	20.61	20.40	23.77	22.84	23.84
3	39.38	31.42	21.47	22.42	20.11	20.92	21.99	23.62	21.83
4	20.98	19.94	20.35	21.43	20.88	20.81	24.50	20.88	20.81
5	20.10	19.70	18.42	20.73	21.99	20.55	22.61	22.30	22.37
6	19.99	34.10	18.75	21.52	20.08	19.05	21.96	22.36	23.98

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Table 101 %release of C. asiatica BSA nanopartices in PBS solution at pH 7.4

Time	% Release								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	3.64	5.22	7.34	4.34	4.99	5.96	5.16	5.45	5.93
1	3.94	5.09	7.18	4.56	5.31	6.04	5.38	4.89	6.04
2	3.84	5.06	6.29	5.02	5.57	6.38	5.06	6.17	7.45
3	6.91	8.49	7.95	4.77	5.43	6.54	4.68	6.38	6.82
4	3.68	5.39	7.54	4.56	5.64	6.50	5.21	5.64	6.50
5	3.53	5.32	6.82	4.41	5.94	6.42	4.81	6.03	6.99
6	3.51	9.22	6.94	4.58	5.43	5.95	4.67	6.04	7.49

2.2 Orgaic

Table 102 Concentration of crude extract in phosphate buffer solution at pH 7.4

Time	Concentration of crude in PBS (µg)								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	22.80	23.77	20.26	22.89	21.90	20.96	31.25	18.29	19.55
1	22.62	19.38	19.40	21.43	19.64	19.31	22.34	16.80	19.31
2	23.19	21.77	19.91	25.03	20.61	24.24	25.64	22.45	23.84
3	27.58	24.37	22.29	22.42	22.96	20.92	20.56	20.94	21.83
4	20.98	19.94	19.59	23.87	20.88	20.81	26.12	24.46	20.81
5	21.12	20.11	22.12	20.73	21.99	22.86	22.88	25.59	24.60
6	19.99	20.32	20.08	22.56	22.36	22.22	25.96	27.29	25.88

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Table 103 %release of C. asiatica BSA nanopartices in PBS solution at pH 7.4

Time	% Release								
	Ethanol			Chloroform			Hexane		
	1:2	1:3	1:4	1:2	1:3	1:4	1:2	1:3	1:4
0	4.00	6.42	7.50	4.87	5.92	6.55	6.65	4.94	6.11
1	3.97	5.24	7.19	4.56	5.31	6.04	4.75	4.54	6.04
2	4.07	5.88	7.37	5.33	5.57	7.57	5.46	6.07	7.45
3	4.84	6.59	8.26	4.77	6.20	6.54	4.37	5.66	6.82
4	3.68	5.39	7.26	5.08	5.64	6.50	5.56	6.61	6.50
5	3.71	5.43	8.19	4.41	5.94	7.14	4.87	6.92	7.69
6	3.51	5.49	7.44	4.80	6.04	6.94	5.52	7.38	8.09

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APPENDIX B
SAS out put

1. Entrapment Efficiency

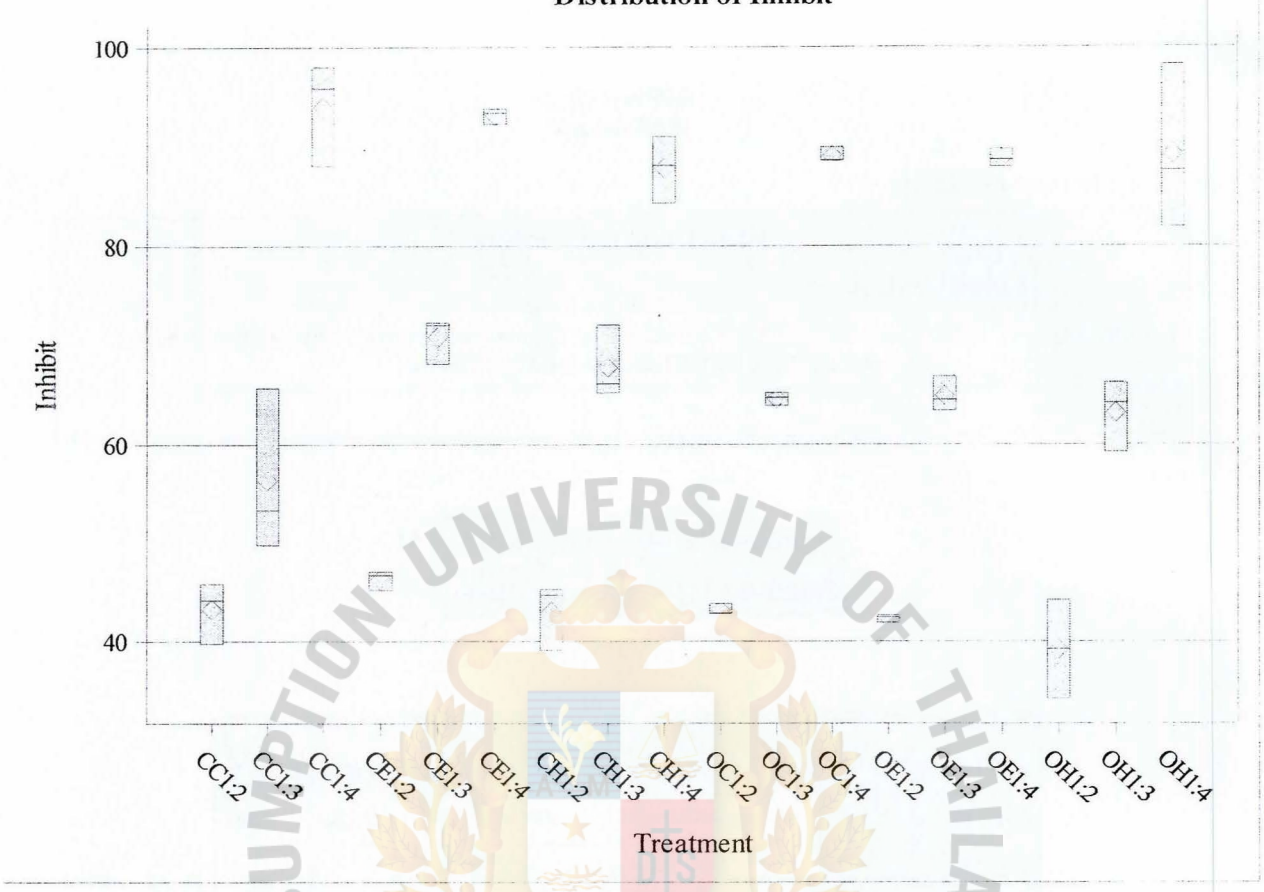
Class Level Information		
Class	**Levels**	**Values**
Treatment	18	CC1:2 CC1:3 CC1:4 CE1:2 CE1:3 CE1:4 CH1:2 CH1:3 CH1:4 OC1:2 OC1:3 OC1:4 OE1:2 OE1:3 OE1:4 OH1:2 OH1:3 OH1:4
rep	3	1 2 3

Number of Observations Read	54
Number of Observations Used	54

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	19	20896.43983	1099.81262	72.66	<.0001
Error	34	514.65630	15.13695		
Corrected Total	53	21411.09613			

Source	**DF**	**Anova SS**	**Mean Square**	**F Value**	**Pr > F**
Treatment	17	20884.15900	1228.47994	81.16	<.0001
rep	2	12.28083	6.14042	0.41	0.6697

R-Square	**Coeff Var**	**Root MSE**	**Inhibit Mean**
0.975963	5.899552	3.890623	65.94778



Level of Treatment	N	Inhibit	
		Mean	Std Dev
CC1:2	3	43.2200000	3.08991909
CC1:3	3	56.2066667	8.47758417
CC1:4	3	94.0000000	5.19935573
CE1:2	3	46.2566667	0.96572943
CE1:3	3	70.9133333	2.30989899
CE1:4	3	93.1266667	0.77938010
CH1:2	3	42.9933333	3.36607090
CH1:3	3	67.8633333	3.71685799
CH1:4	3	87.8166667	3.35529929
OC1:2	3	43.1466667	0.57448528
OC1:3	3	64.6866667	0.64127477
OC1:4	3	89.0566667	0.77371399
OE1:2	3	42.2966667	0.39247081
OE1:3	3	64.9900000	1.78123553

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Level of Treatment	N	Inhibit	
		Mean	Std Dev
OE1:4	3	88.7933333	0.89539563
OH1:2	3	39.1966667	4.96001344
OH1:3	3	63.2566667	3.65274326
OH1:4	3	89.2400000	8.26597242

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Distribution of Inhibit

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Alpha	0.05
Error Degrees of Freedom	34
Error Mean Square	15.13695

Number of Means	2	3	4	5	6	7	8	9	10	11	12	13
Critical Range	6.456	6.786	7.001	7.155	7.272	7.365	7.439	7.500	7.552	7.595	7.632	7.663

Number of Means	14	15	16	17	18
Critical Range	7.691	7.714	7.735	7.753	7.768

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	Treatment
	A	94.000	3	CC1:4
	A			
	A	93.127	3	CE1:4
	A			
	A	89.240	3	OH1:4
	A			
	A	89.057	3	OC1:4
	A			
	A	88.793	3	OE1:4
	A			
	A	87.817	3	CH1:4
	B	70.913	3	CE1:3
	B			
C	B	67.863	3	CH1:3
C	B			
C	B	64.990	3	OE1:3
C	B			
C	B	64.687	3	OC1:3
C				
C		63.257	3	OH1:3

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Means with the same letter are not significantly different.				
Duncan Grouping		Mean	N	Treatment
	D	56.207	3	CC1:3
	E	46.257	3	CE1:2
	E			
	E	43.220	3	CC1:2
	E			
	E	43.147	3	OC1:2
	E			
	E	42.993	3	CH1:2
	E			
	E	42.297	3	OE1:2
	E			
	E	39.197	3	OH1:2

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2. Loading Efficiency

Class Level Information		
Class	**Levels**	**Values**
Treatment	18	CC1:2 CC1:3 CC1:4 CE1:2 CE1:3 CE1:4 CH1:2 CH1:3 CH1:4 OC1:2 OC1:3 OC1:4 OE1:2 OE1:3 OE1:4 OH1:2 OH1:3 OH1:4
rep	3	1 2 3

Number of Observations Read	54
Number of Observations Used	54

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	19	179.9430852	9.4706887	1.86	0.0555
Error	34	172.8100185	5.0826476		
Corrected Total	53	352.7531037			

R-Square	**Coeff Var**	**Root MSE**	**Inhibit Mean**
0.510111	6.273914	2.254473	35.93407

Source	**DF**	**Anova SS**	**Mean Square**	**F Value**	**Pr > F**
Treatment	17	176.3252370	10.3720728	2.04	0.0376
rep	2	3.6178481	1.8089241	0.36	0.7031

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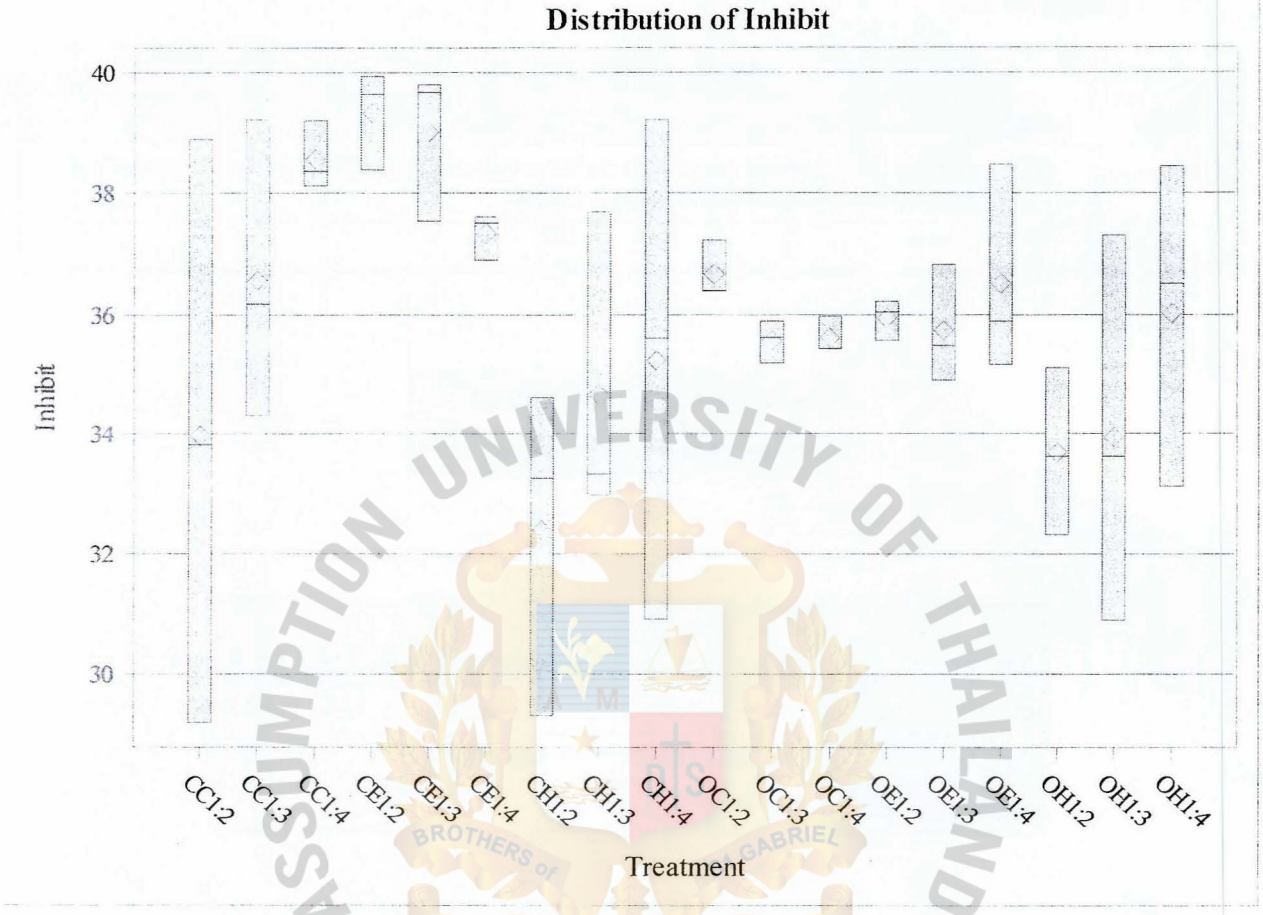

Level of Treatment	N	Inhibit Mean	Inhibit Std Dev
CC1:2	3	33.9733333	4.84182128
CC1:3	3	36.5666667	2.47277846
CC1:4	3	38.5600000	0.56709788
CE1:2	3	39.3200000	0.82776808
CE1:3	3	39.0033333	1.26894970
CE1:4	3	37.3400000	0.38431758
CH1:2	3	32.3966667	2.74864209
CH1:3	3	34.6666667	2.62412525
CH1:4	3	35.2466667	4.16257532
OC1:2	3	36.6733333	0.48211340
OC1:3	3	35.5766667	0.35303446

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Level of Treatment	N	Inhibit	
		Mean	Std Dev
OC1:4	3	35.6233333	0.30892286
OE1:2	3	35.9500000	0.33151169
OE1:3	3	35.7466667	0.98348022
OE1:4	3	36.5166667	1.72951824
OH1:2	3	33.6866667	1.40118997
OH1:3	3	33.9400000	3.22194041
OH1:4	3	36.0266667	2.69409230

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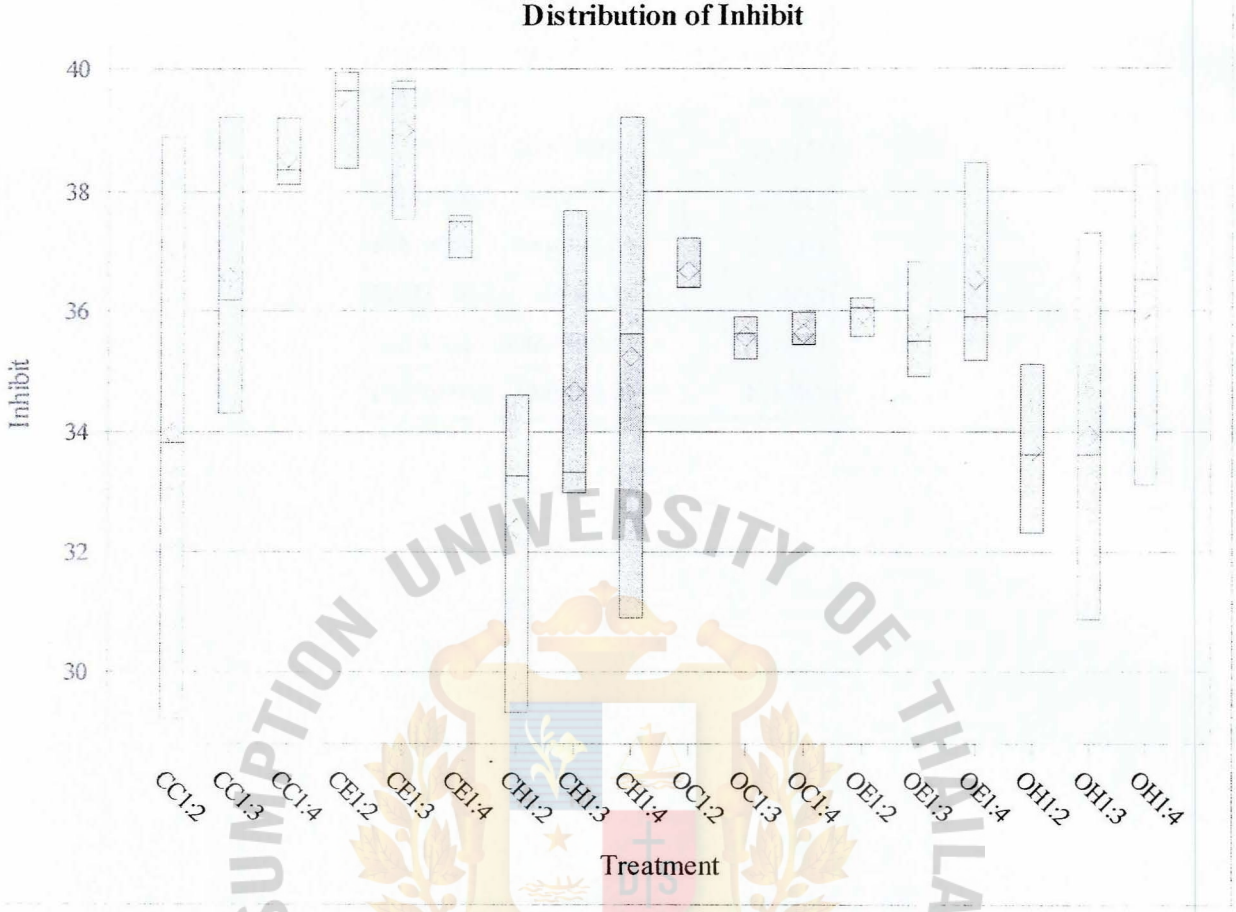

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Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	34
Error Mean Square	5.082648

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Critical Range	3.741	3.932	4.057	4.146	4.214	4.267	4.311	4.346	4.376	4.401	4.422	4.441

Number of Means	**14**	**15**	**16**	**17**	**18**
Critical Range	4.456	4.470	4.482	4.492	4.501

Means with the same letter are not significantly different.

Duncan Grouping				**Mean**	**N**	**Treatment**
		A		39.320	3	CE1:2
		A				
B		A		39.003	3	CE1:3
B		A				
B		A		38.560	3	CC1:4
B		A				
B		A	C	37.340	3	CE1:4
B		A	C			
B	D	A	C	36.673	3	OC1:2
B	D	A	C			
B	D	A	C	36.567	3	CC1:3
B	D	A	C			
B	D	A	C	36.517	3	OE1:4
B	D	A	C			
B	D	A	C	36.027	3	OH1:4
B	D	A	C			
B	D	A	C	35.950	3	OE1:2
B	D	A	C			
B	D	A	C	35.747	3	OE1:3
B	D	A	C			

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Means with the same letter are not significantly different.						
Duncan Grouping				Mean	N	Treatment
B	D	A	C	35.623	3	OC1:4
B	D	A	C			
B	D	A	C	35.577	3	OC1:3
B	D	A	C			
B	D	A	C	35.247	3	CH1:4
B	D		C			
B	D		C	34.667	3	CH1:3
	D		C			
	D		C	33.973	3	CC1:2
	D		C			
	D		C	33.940	3	OH1:3
	D		C			
	D		C	33.687	3	OH1:2
	D					
	D			32.397	3	CH1:2

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3. ANTIOXIDANT (FRAP)

Class Level Information		
Class	**Levels**	**Values**
Treatment	24	CC1:2 CC1:3 CC1:4 CE1:2 CE1:3 CE1:4 CH1:2 CH1:3 CH1:4 OC1:2 OC1:3 OC1:4 OE1:2 OE1:3 OE1:4 OH1:2 OH1:3 OH1:4 crudeCC crudeCE crudeCH crudeOC crudeOE crudeOH
rep	3	1 2 3

Number of Observations Read	72
Number of Observations Used	72

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	25	15.19492917	0.60779717	97.53	<.0001
Error	46	0.28665833	0.00623170		
Corrected Total	71	15.48158750			

R-Square	Coeff Var	Root MSE	Inhibit Mean
0.981484	10.09370	0.078941	0.782083

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	23	15.16512083	0.65935308	105.81	<.0001
rep	2	0.02980833	0.01490417	2.39	0.1028

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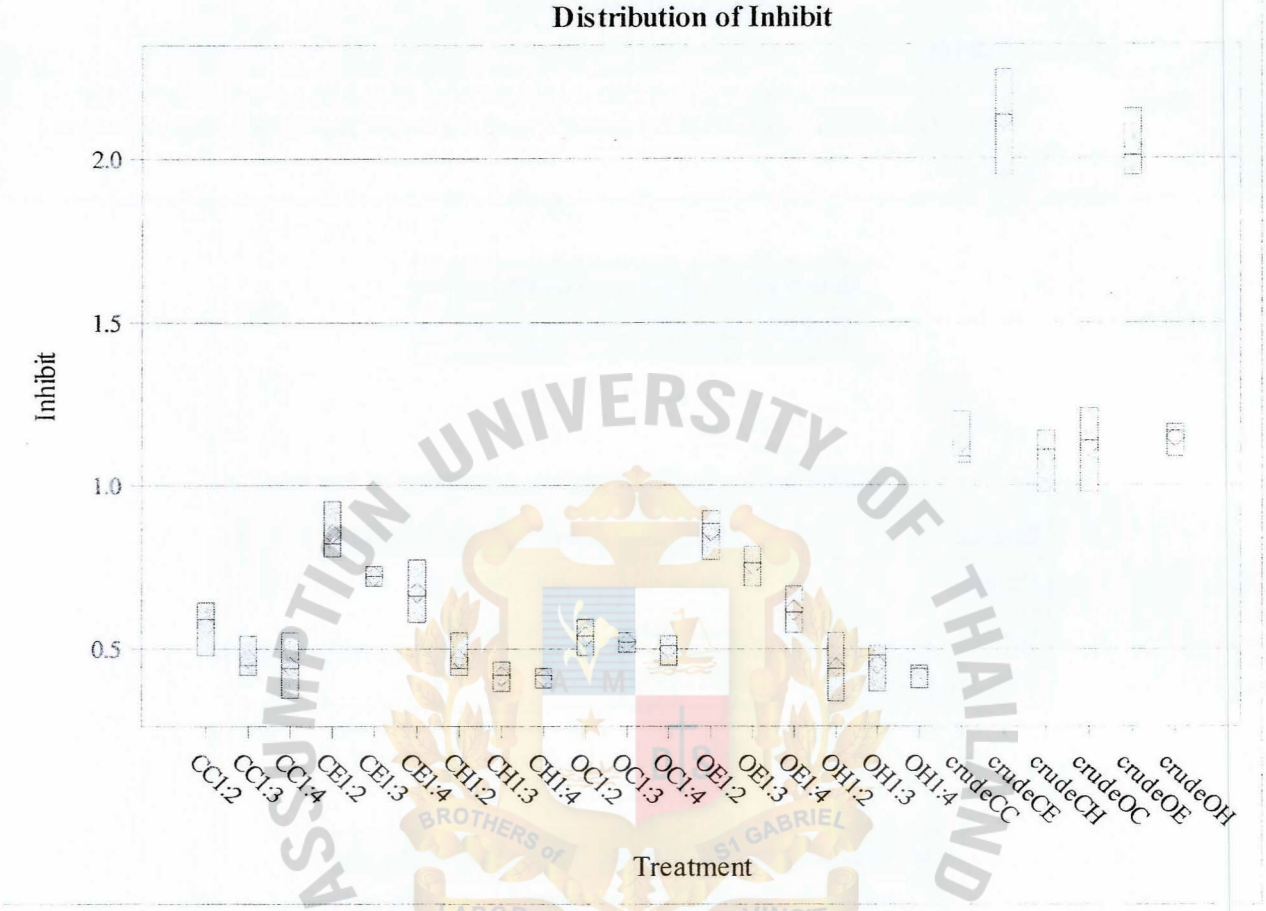

Level of Treatment	N	Inhibit	
		Mean	Std Dev
CC1:2	3	0.57000000	0.08185353
CC1:3	3	0.47000000	0.06244998
CC1:4	3	0.44666667	0.10016653
CE1:2	3	0.85000000	0.08888194
CE1:3	3	0.72000000	0.03000000
CE1:4	3	0.67000000	0.09539392
CH1:2	3	0.47000000	0.07000000
CH1:3	3	0.41666667	0.04509250
CH1:4	3	0.41333333	0.03055050
OC1:2	3	0.53666667	0.05507571
OC1:3	3	0.52000000	0.03000000

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Level of Treatment	N	Inhibit	
		Mean	Std Dev
OC1:4	3	0.50000000	0.04582576
OE1:2	3	0.85666667	0.07767453
OE1:3	3	0.75333333	0.06027714
OE1:4	3	0.61666667	0.07023769
OH1:2	3	0.44333333	0.10503968
OH1:3	3	0.45333333	0.07371115
OH1:4	3	0.42333333	0.03785939
crudeCC	3	1.13000000	0.08717798
crudeCE	3	2.11666667	0.16041613
crudeCH	3	1.08666667	0.09712535
crudeOC	3	1.12000000	0.13114877
crudeOE	3	2.03666667	0.10263203
crudeOH	3	1.15000000	0.05291503

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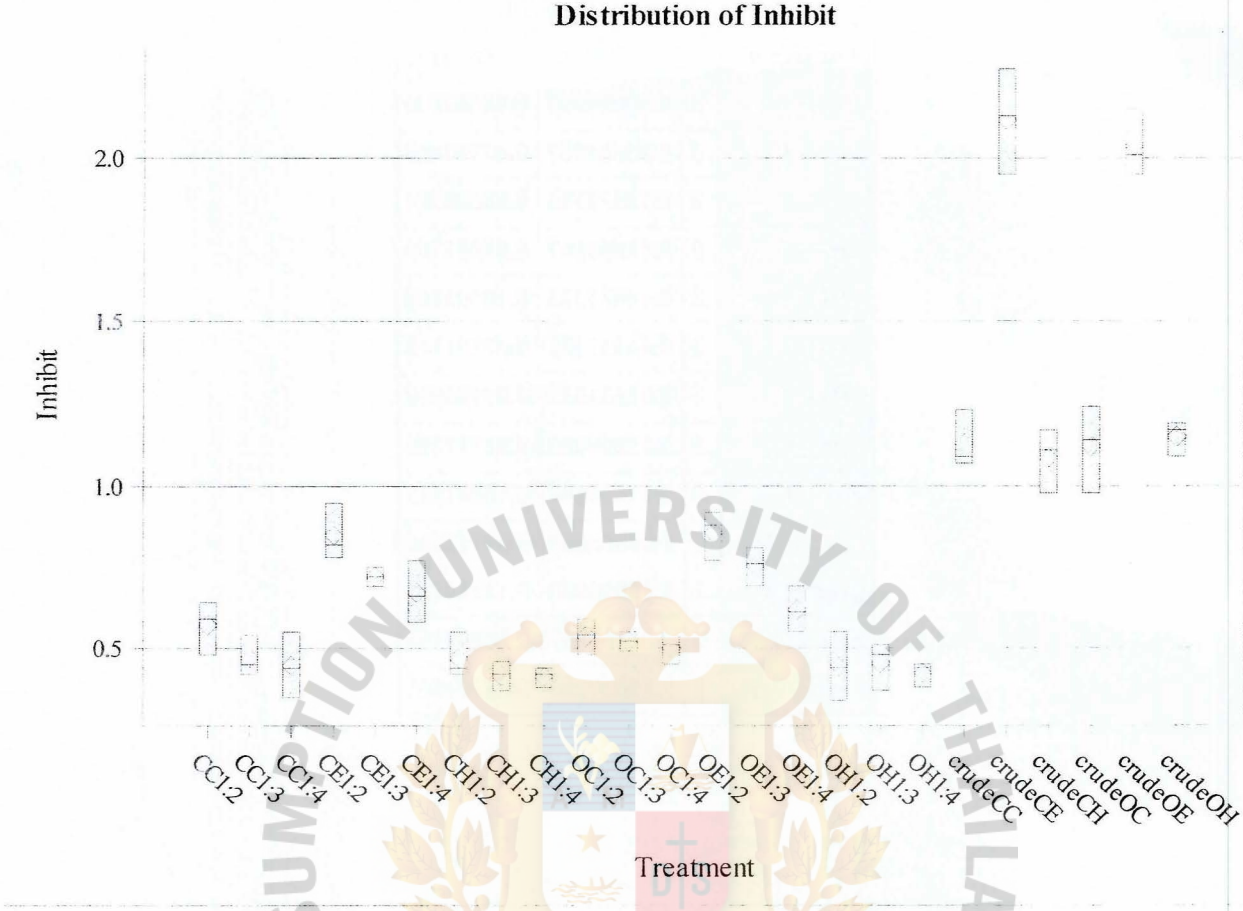

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Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	0.006232

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Critical Range	.1297	.1364	.1408	.1440	.1465	.1484	.1500	.1513	.1524	.1534	.1542	.1549	.1556

Number of Means	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Critical Range	.1561	.1566	.1570	.1574	.1578	.1581	.1583	.1586	.1588	.1590

Means with the same letter are not significantly different.

Duncan Grouping				**Mean**	**N**	**Treatment**
		A		2.11667	3	crudeCE
		A				
		A		2.03667	3	crudeOE
		B		1.15000	3	crudeOH
		B				
		B		1.13000	3	crudeCC
		B				
		B		1.12000	3	crudeOC
		B				
		B		1.08667	3	crudeCH
		C		0.85667	3	OE1:2
		C				
		C		0.85000	3	CE1:2
		C				
D		C		0.75333	3	OE1:3
D		C				
D		C		0.72000	3	CE1:3
D						

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Means with the same letter are not significantly different.						
Duncan Grouping				Mean	N	Treatment
D		E		0.67000	3	CE1:4
D		E				
D		E	F	0.61667	3	OE1:4
		E	F			
G		E	F	0.57000	3	CC1:2
G		E	F			
G	H	E	F	0.53667	3	OC1:2
G	H		F			
G	H		F	0.52000	3	OC1:3
G	H		F			
G	H		F	0.50000	3	OC1:4
G	H		F			
G	H		F	0.47000	3	CH1:2
G	H		F			
G	H		F	0.47000	3	CC1:3
G	H					
G	H			0.45333	3	OH1:3
G	H					
G	H			0.44667	3	CC1:4
G	H					
G	H			0.44333	3	OH1:2
G	H					
G	H			0.42333	3	OH1:4
G	H					
G	H			0.41667	3	CH1:3
	H					
	H			0.41333	3	CH1:4

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4. ANTIOXIDANT (DPPH)

Class Level Information		
Class	**Levels**	**Values**
Treatment	24	CC1:2 CC1:3 CC1:4 CE1:2 CE1:3 CE1:4 CH1:2 CH1:3 CH1:4 OC1:2 OC1:3 OC1:4 OE1:2 OE1:3 OE1:4 OH1:2 OH1:3 OH1:4 crudeCC crudeCE crudeCH crudeOC crudeOE crudeOH
rep	3	1 2 3

Number of Observations Read	72
Number of Observations Used	72

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	25	4458.047122	178.321885	18.27	<.0001
Error	46	449.093389	9.762900		
Corrected Total	71	4907.140511			

R-Square	**Coeff Var**	**Root MSE**	**Inhibit Mean**
0.908482	20.60529	3.124564	15.16389

Source	**DF**	**Anova SS**	**Mean Square**	**F Value**	**Pr > F**
Treatment	23	4457.495578	193.804156	19.85	<.0001
rep	2	0.551544	0.275772	0.03	0.9722

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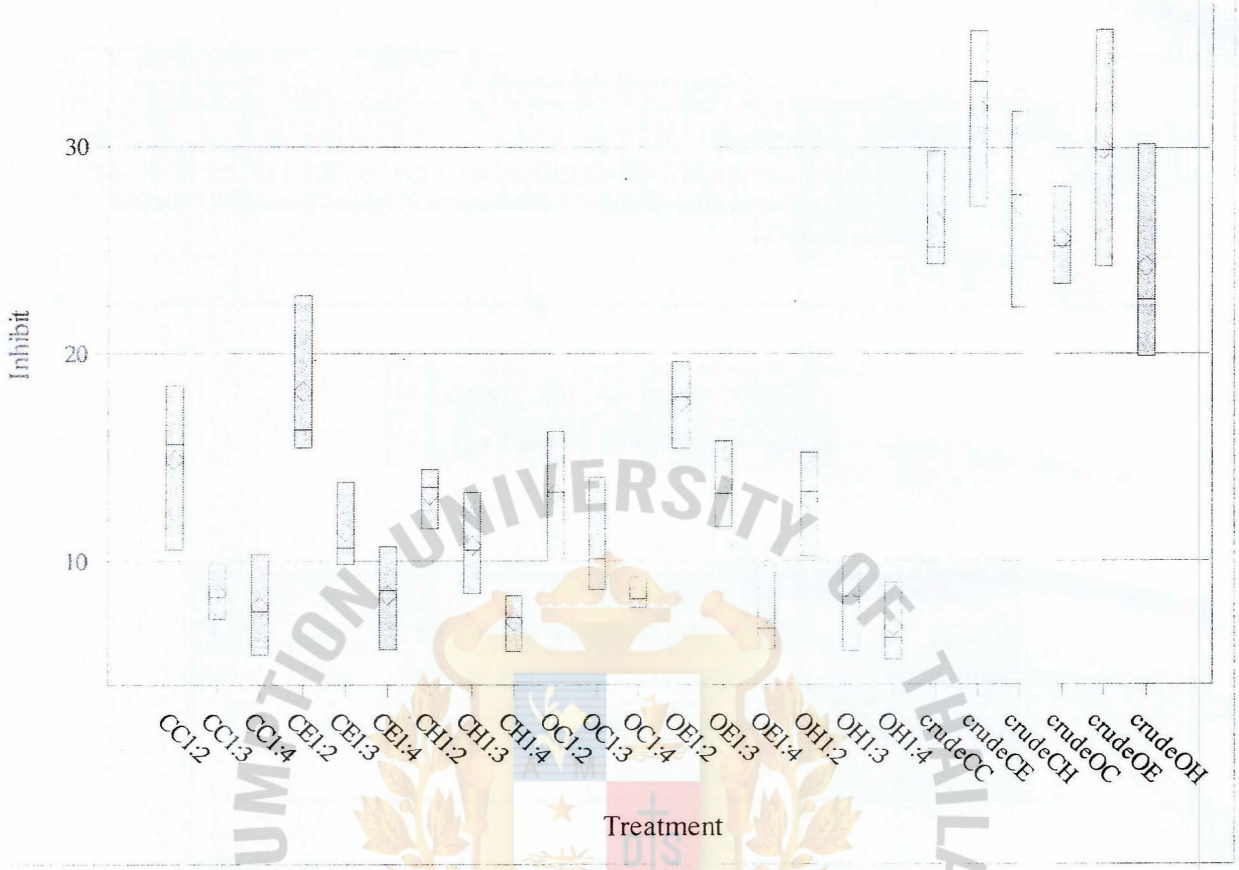
Distribution of Inhibit



Level of Treatment	N	Inhibit	
		Mean	Std Dev
CC1:2	3	14.8733333	3.99625241
CC1:3	3	8.4266667	1.37558472
CC1:4	3	7.7533333	2.48254574
CE1:2	3	18.1833333	4.00452661
CE1:3	3	11.4266667	2.08639242
CE1:4	3	8.3133333	2.54051832
CH1:2	3	13.1766667	1.46609459
CH1:3	3	10.7600000	2.45739700
CH1:4	3	7.0333333	1.38550833
OC1:2	3	13.2300000	3.04599737
OC1:3	3	10.9066667	2.79432162
OC1:4	3	8.3466667	0.77526340
OE1:2	3	17.6333333	2.09681505
OE1:3	3	13.5533333	2.07779531

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Level of Treatment	N	Inhibit	
		Mean	Std Dev
OE1:4	3	7.3600000	2.14252188
OH1:2	3	12.9233333	2.50863177
OH1:3	3	8.0033333	2.33401657
OH1:4	3	6.7566667	1.96779911
crudeCC	3	26.4233333	2.98650855
crudeCE	3	31.9366667	4.33915122
crudeCH	3	27.2200000	4.77238934
crudeOC	3	25.5533333	2.39575319
crudeOE	3	29.9133333	5.70500073
crudeOH	3	24.2266667	5.33209465

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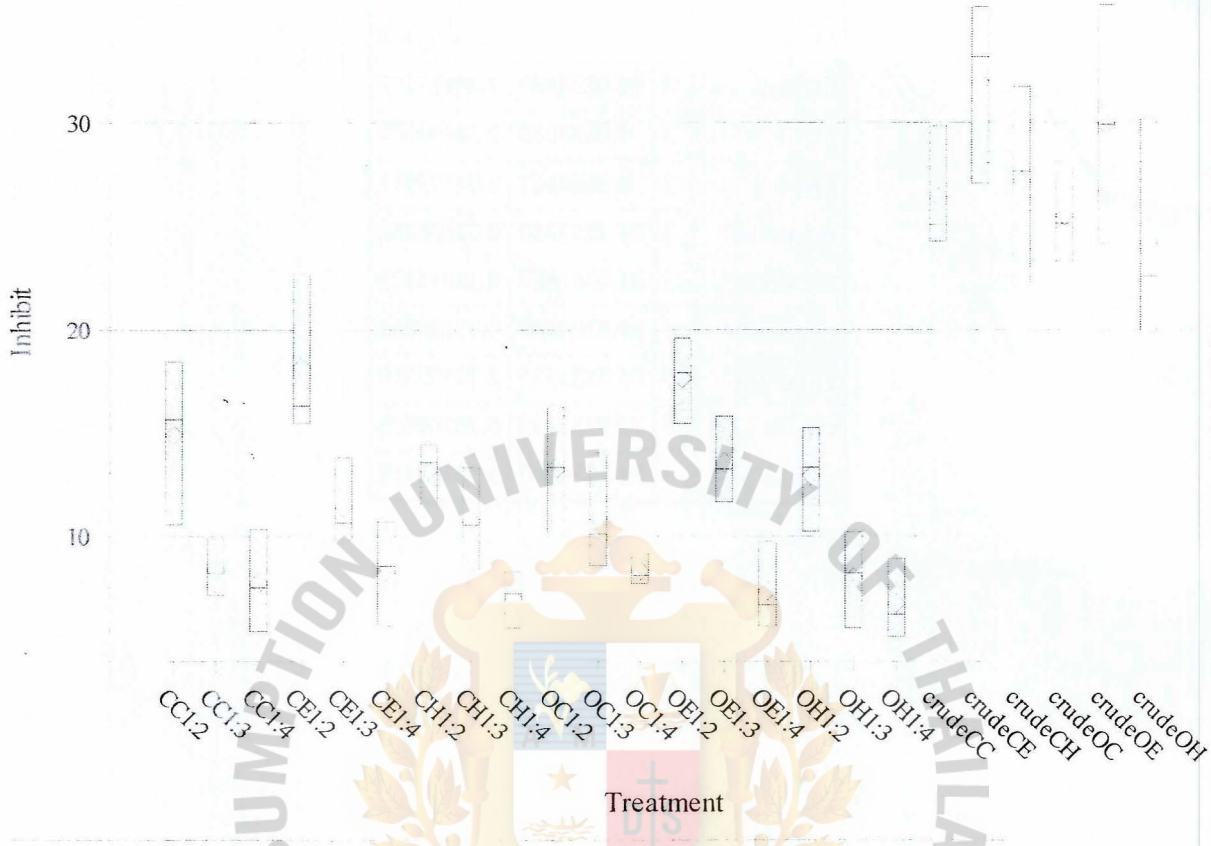
Distribution of Inhibit



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Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	46
Error Mean Square	9.7629

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Critical Range	5.135	5.401	5.575	5.701	5.797	5.874	5.937	5.989	6.033	6.071	6.104	6.132	6.157

Number of Means	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Critical Range	6.179	6.198	6.215	6.231	6.244	6.257	6.267	6.277	6.286	6.294

Means with the same letter are not significantly different.

Duncan Grouping					**Mean**	**N**	**Treatment**
			A		31.937	3	crudeCE
			A				
	B		A		29.913	3	crudeOE
	B		A				
	B		A		27.220	3	crudeCH
	B		A				
	B		A		26.423	3	crudeCC
	B						
	B				25.553	3	crudeOC
	B						
	B				24.227	3	crudeOH
			C		18.183	3	CE1:2
			C				
			C		17.633	3	OE1:2
			C				
	D		C		14.873	3	CC1:2
	D		C				
	D		C	E	13.553	3	OE1:3
	D		C	E			

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Means with the same letter are not significantly different.							
Duncan Grouping					Mean	N	Treatment
	D	F	C	E	13.230	3	OC1:2
	D	F	C	E			
	D	F	C	E	13.177	3	CH1:2
	D	F	C	E			
G	D	F	C	E	12.923	3	OH1:2
G	D	F		E			
G	D	F	H	E	11.427	3	CE1:3
G	D	F	H	E			
G	D	F	H	E	10.907	3	OC1:3
G	D	F	H	E			
G	D	F	H	E	10.760	3	CH1:3
G		F	H	E			
G		F	H	E	8.427	3	CC1:3
G		F	H	E			
G		F	H	E	8.347	3	OC1:4
G		F	H	E			
G		F	H	E	8.313	3	CE1:4
G		F	H	E			
G		F	H	E	8.003	3	OH1:3
G		F	H	E			
G		F	H	E	7.753	3	CC1:4
G		F	H				
G		F	H		7.360	3	OE1:4
G			H				
G			H		7.033	3	CH1:4
			H				
			H		6.757	3	OH1:4

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