การเตรียมบีดไคโตซานบรรจุและควบคุมการปลดปล่อยซาโปนิน
เพื่อต้านหอยเชอรี่ *Pomacea canaliculata*

นางสาวเพชรดา คุ้มทรัพย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PREPARATION OF CHITOSAN BEADS CONTAINING AND CONTROL RELEASING OF SAPONIN AGAINST GOLDEN APPLE SNAIL *Pomacea canaliculata*

Miss Phetrada Khumsup

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	PREPARATION OF CHITOSAN BEADS CONTAINING AND CONTROL RELEASING OF SAPONIN AGAINST GOLDEN APPLE SNAIL *Pomacea canaliculata*
By	Miss Phetrada Khumsup
Field of Study	Petrochemistry and Polymer Science
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เพชรดา คุ้มทรัพย์ : การเตรียมบีดไคโตซานบรรจุและควบคุมการปลดปล่อยซาโปนิน เพื่อต้านหอยเชอรี่ *Pomacea canaliculata* (PREPARATION OF CHITOSAN BEADS CONTAINING AND CONTROL RELEASING OF SAPONIN AGAINST GOLDEN APPLE SNAIL *Pomacea canaliculata*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.นาตยา งามโรจนวณิชย์, 102 หน้า.

งานวิจัยนี้ทำการเตรียมบีดไคโตซานบรรจุและควบคุมการปลดปล่อยซาโปนิน (*Camellia oleifera abel*) เพื่อต้านหอยเชอรี่ *Pomacea canaliculata* การพิสูจน์เอกลักษณ์เชิงสัณฐานวิทยาของบีดไคโตซานที่มียาซาโปนินบรรจุอยู่ ทำการพิสูจน์เอกลักษณ์ด้วยเทคนิค FTIR และ SEMจากการศึกษาพบว่าการขึ้นรูปบีด สารละลายไคโตซานที่มีความเข้มข้น 2% w/v ในสารละลายผสมโซเดียมไฮดรอกไซด์ 10% w/v, โซเดียมไตรฟอสเฟต 1% w/v, และเอธิลแอลกอฮอล์ 50% v/v บีดมีลักษณะเป็นทรงกลม ขนาดสม่ำเสมอ การบรรจุซาโปนินที่ 10% โดยน้ำหนักของซาโปนินต่อไคโตซาน ซาโปนินสามารถปลดปล่อยได้ปริมาณมากและยาวนานขึ้น มากกว่าการกักเก็บที่ 1% มากไปกว่านั้นการผสมกากชาในอัตราส่วน 1:3 โดยน้ำหนักในเมทริกซ์ไคโตซาน ส่งผลต่อสมบัติต่างๆของบีด เช่น บีดมีขนาดใหญ่ขึ้น 2 เท่า ลักษณะผิวขรุขระ การบวมน้ำมากขึ้น ทำให้ซาโปนินถูกปลดปล่อยออกมาได้ปริมาณมากขึ้นและสามารถเพิ่มระยะเวลาการปลดปล่อยมากขึ้นเมื่อเทียบกับบีดไคโตซานบริสุทธิ์บรรจุซาโปนินและซาโปนินบริสุทธิ์ และเมื่อทำการปลดปล่อยซาโปนินในสภาวะจำลอง 2 สภาวะ คือในน้ำกลั่นที่ไม่มีประจุและสารละลายแคลเซียมคลอไรด์ พบว่าในน้ำกลั่นที่ไม่มีประจุซาโปนินถูกปลดปล่อยได้เร็วกว่าในสายละลายแคลเซียมคลอไรด์ จากงานวิจัยนี้สูตรผสมที่เตรียมได้นับว่าเป็นทางเลือกใหม่สำหรับการนำไปต้านหอยเชอรี่ซึ่งเป็นศัตรูพืชในนาข้าว นอกจากนี้ได้ทำการทดสอบความเป็นพิษของโซโปนินต่อสิ่งมีชีวิตในนาข้าว โดยใช้ปลานิลและหอยเชอรี่เป็นตัวอย่าง พบว่าความเป็นพิษซาโปนินต่อปลานิลมี ค่า LC_{50} เท่ากับ 2.13 พีพีเอ็ม ที่ 72 ชั่วโมง และของหอยเชอรี่ มีค่า LC_{50} เท่ากับ 7.45 พีพีเอ็ม ที่ 96 ชั่วโมง อีกทั้งยังพบว่าต้นข้าวที่ได้รับซาโปนินที่ความเข้มข้น 10 ถึง 50 พีพีเอ็ม ต้นข้าวมีการเจริญเติมโตและให้ผลผลิตสูงเมื่อเทียบกับข้าวที่ไม่ได้รับซาโปนิน ซึ่งแสดงให้เห็นว่าซาโปนินมีฤทธิ์ในการต้านหอยเชอรี่และยังมีความเป็นมิตรต่อสิ่งแวดล้อม

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5572249723 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: CAMELLIA OLEIFERA SAPONIN / GOLDEN APPLE SNAIL / POMACEA CANALICULATA SNAIL / SAPONIN / CHITOSAN / TEA SEED MEAL

PHETRADA KHUMSUP: PREPARATION OF CHITOSAN BEADS CONTAINING AND CONTROL RELEASING OF SAPONIN AGAINST GOLDEN APPLE SNAIL *Pomacea canaliculata*. ADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., 102 pp.

Chitosan beads have been prepared to contain and control the release of saponin which used to against golden apple snails. The morphology of chitosan beads were characterized by FTIR and SEM. The formation of chitosan beads have a regular spherical shape with regular size prepared by using 2% w/v of chitosan solution with mixed solution of 10% w/v sodium hydroxide, 1% w/v of sodium tripolyphosphated and 50% v/v of ethyl alcohol. Ten percentage of saponin (w/w) was loaded into chitosan matrix found that higher amount of saponin was released when compared with loading of 1% saponin (w/w). Furthermore, mixed tea seed meal in chitosan matrix with ratio of 1:3 w/w affected beads properties such as bigger bead sizes, rough surface and more water swelling that enhanced the amount of saponin release when compared with natural chitosan beads. Release behavior of saponin were studied in deionize water and calcium chloride solution, it was found that more release of saponin in deionized water. Moreover, toxic testing of saponin with other living animals in agriculture such as *Nile Tilapia* fish and *Pomacea Canaliculata* snails showed LC_{50} values of saponin to *Nile Tilapia* fish and *P. Canaliculata* snails were 2.13 ppm in 72 hours and 7.45 ppm in 96 hours. In addition, rice that treated saponin with concenetration of 10-50 ppm had been increase growing and high productivity. Summary it can be conclude that the mixture of chitosan and tea seed meal beads containing saponin have a potential to develop as molluscicide against golden apple snails with friendly environment.

Field of Study: Petrochemistry and Polymer Science Student's Signature .....
Advisor's Signature .....

Academic Year: 2016

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LIST OF ABBREVIATIONS

CS	Chitosan
TSM	Tea seed meal
TPP	Tripolyphosphate
NaOH	Sodium hydroxide
EtOH	Ethanol
$CaCl_2$	Calcium chloride
CH_3COOH	Glacial acetic acid
min	Minute
cm	Centimeter
mm	Millimeter
h	Hour
Conc	Concentration
FTIR	Fourier Transform Infrared Spectrophotometer
SEM	Sacanning Electron Microscope
UV/Vis	Ultraviolet-visible Spectroscopy
LC_0	0% lethality concentration
LC_{50}	50% lethality concentration
LC_{100}	100% lethality concentration
mL	Milliliter
EE	Entrapment efficiency
g	Gram
kV	Kilovolt
mg	Milligram
nm	Nanometer
ppm	Part Per Million
%	Percentage
rpm	Round Per Minute
S.D.	Standard Deviation

cm^{-1}	Wavenumber (unit)
v/v	Volume by Volume
w/w	Weight by Weight
w/v	Weight by Volume
pH	Power of hydrogen ion or the negative logarithm (base ten)

CHAPTER I
INTRODUCTION

1.1 Introduction

Rice is a major agricultural exported product of Thailand. One of the major problems in rice production is golden apple snail which damages young rice seedlings, causing in low yield rice production and also economic losses [1]. There are several methods that be used to control the snails in agricultural applications such as chemical, biological, mechanical and manual methods. Among the chemical pesticides, synthetic pesticides are widely for snail controlling but they are expensive, harmful to humans, highly toxic to the environment and to non-target organisms [2]. Therefore, natural pesticide was attracted consideration. Saponins are natural glycosides of triterpene or steroid which contain both a sugar moiety (water-soluble) and sapogenin (fat-soluble) subunits, found in various plants and some animal kingdoms. Saponins are used as pesticides because of their advantages such as high efficiency, biodegradability, biocompatibility, eco-friendly, low cost and especially harmless to humans. However, saponins are instability and rapid degradation in water lead to minimize its efficiency, and increase production costs [3]. Then, saponins were designed as encapsulated drug by using biodegradable polymers to improve stability, increase the potential application and protect the drug. Chitosan (CS) which is a natural linear polysaccharide, used for drug delivery applications due to its low toxicity, good biodegradability, and good biocompatibility [4]. However, the drawbacks of chitosan for drug delivery system are poor solubility in neutral and alkaline solution, high crystallinity that resist drug transfer and low surface area [5-7], so tea seed meal (TSM) which widely uses as feed filler was selected to overcome these drawbacks. In this research, we focused on bead preparation from chitosan for encapsulation of *Camellia oleifera* saponin in order to maximize its efficiency and control releasing against *Pomacea. canaliculata* snail.

1.2 Objectives of this research

- Evaluation activity of *C. oleifera* saponin

- To prepare the optimal chitosan beads for encapsulation of *C. oleifera* saponin

- To study the *in vitro* swelling and controlled-release behaviors of saponin-containing chitosan beads in deionized water and calcium chloride solution

1.3 The scope of research

- Literature review

- Part I: Evaluation activity of *C. oleifera* saponin (Figure 1.1)

    1. Molluscicidal test on *P. canaliculata* snail
    2. Toxicity test on Tilapia fish
    3. Phytotoxic test on rice (*Oryza sativa* L.) cultivar RD41

- Part II: Preparation of chitosan and the mixture of chitosan and tea seed meal beads with the optimal size and shape for encapsulating saponin using the combination of the *ionotropic* gelation and neutralization methods (Figure 1.2). The parameters are investigated as following:

    1. Effect of chitosan concentration
    2. Effect of sodium tripolyphosphate (TPP) concentration
    3. Effect of sodium hydroxide (NaOH) concentration
    4. Effect of ethanol concentration
    5. Effect of the amount of tea seed meal
    6. Effect of saponin content

- Part III: Evaluation of chitosan and the mixture of chitosan and tea seed meal beads

    1. Characterization of the obtained chitosan and the mixture of chitosan and tea seed meal beads to study size and morphology by vernier caliper and scanning electron microscope.
    2. Determination of the saponin encapsulation efficiency (%EE).

3. Study the *in vitro* swelling behavior of the beads in deionized water and calcium chloride solution for 28 days.

4. Study the *in vitro* release behavior of the beads in deionized water and calcium chloride solution for 28 days.

- Report, Discussion and writing up thesis.

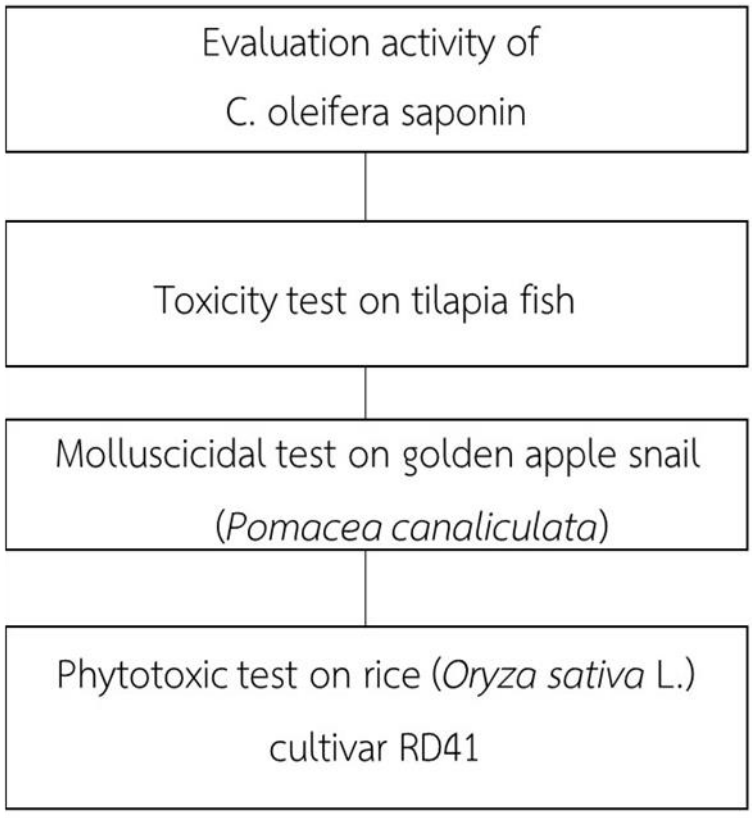


Figure 1.1 Evaluation activity of C. oleifera saponin

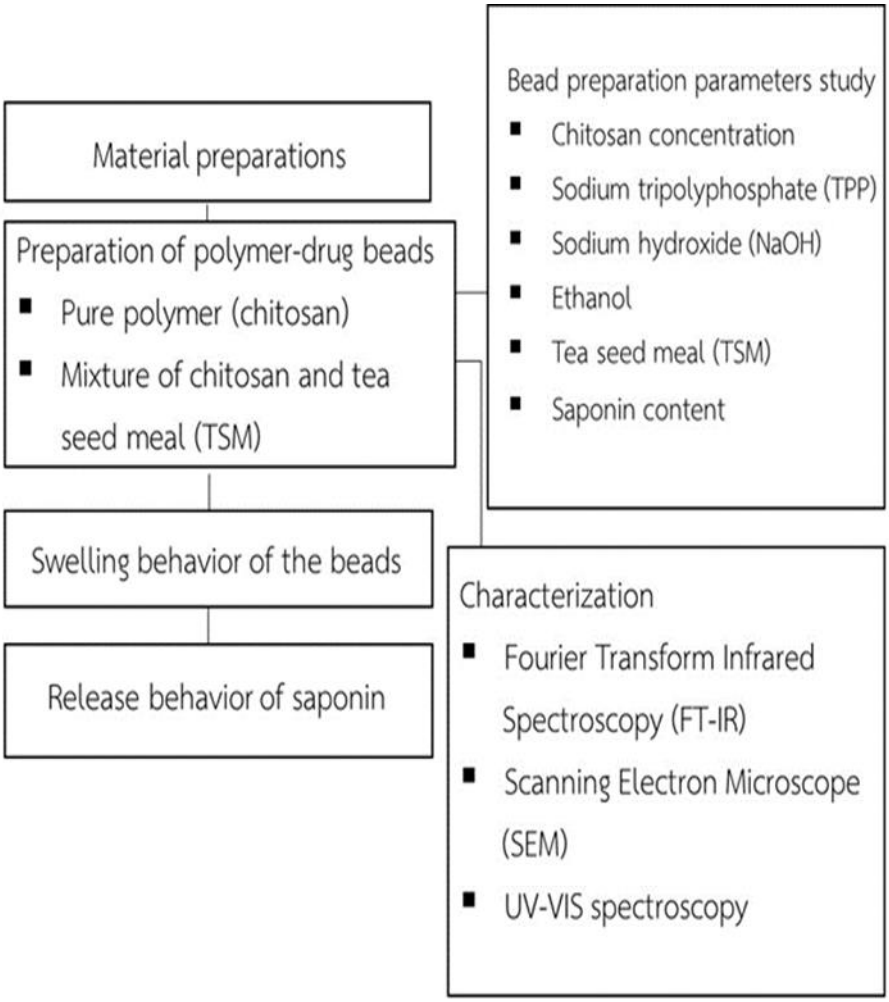


Figure 1.2 Method for preparation of chitosan beads and the mixture of chitosan and tea seed meal (TSM)

CHAPTER II
THEORY AND LITERATURE REVIEWS

2.1 Golden apple snail (GAS)

Golden apple snails (*Pomacea canaliculata*) are freshwater snails from the family Ampullaridae which have round shape and large size like an apple. Their body color are yellow, green, or dark brown with and without spiral bands. The first observation of *P. canaliculata* snails is presented by their bright pink or orange egg masses. Their habitat eat a wide range of succulent leafy plants such as rice seedlings, ponds, swamps, rivers and also well adapted to live on dry land [8].

Figure 2.1 (a) golden apple snail *P. canaliculata* and (b) golden apple snail eggs

2.1.1 Golden apple snail's life cycle

The life cycle of golden apple snails consists of three stages which are eggs, juvenile and adult stage, respectively.

Eggs stage is the first stage that adult females lay 25-500 eggs in bright pink masses over firm substrata to protrude from the water. Hatching is the next stage and takes one to two weeks. The second stage is juvenile stage which newly hatched snails look like a miniature version of an adult snail. They fall into the water and feed on soft aquatic plants. Juveniles that grown to shell height about 15 millimeters beginning to consume plant material. The final stage is adult stage. The adult apple snail has a

tight brown shell and separates sexes of male and female by their operculum curve. Adult females can reproduce once a week or throughout the year after hatching 2-3 months. They are active when water is available and hibernate deep into the soil during dry season [9].

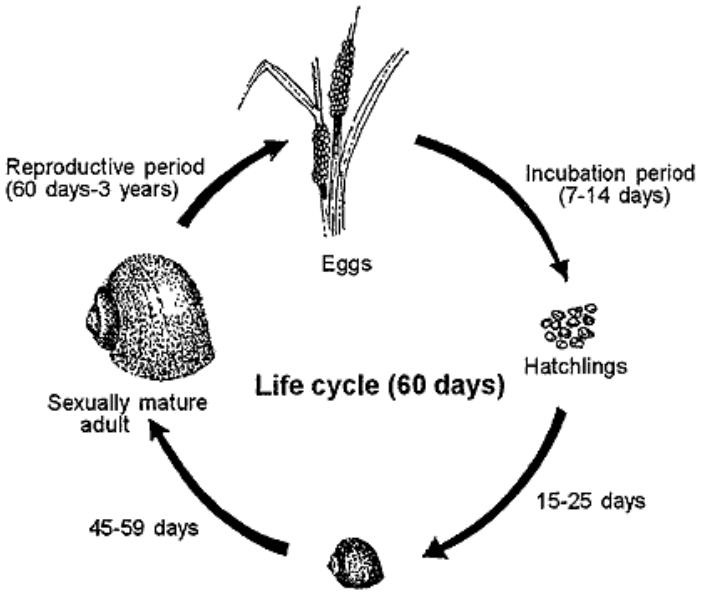


Figure 2.2 Golden apple snail's life cycle [9]

2.1.2 Impacts of golden apple snails

Golden apple snails are a major serious pest of rice because of fast growth, high reproduction and the ability to aestivate underground more than 6 months. They damage young rice plants cause to rice crop production as follows [1, 10]:

- Yield decrease of rice production
- Cost of using chemical control.
- Effect of synthetic molluscicides such as harmful to humans and non-target species, moreover, contaminate on the product and environment.

2.1.3 Golden apple snail control methods

There are several methods for controlling the golden apple snails including chemical, biological, cultural, mechanical and manual methods. Chemical method is general method for farmers. The synthetic molluscicides such as niclosamide and metaldehyde are usually used due to high control and protection efficiency in short time against the snails. However, these synthetic molluscicides are expensive, long degradation period, harmful and high toxic to human, the environment and non-target organisms [11, 12]. The synthetic chemical molluscicides problems lead to the development of natural molluscicides which can reduce these disadvantages of synthetic chemical molluscicides. Moreover, natural molluscicides can reduce the production cost.

2.2 Natural molluscicides

Natural molluscicides are carbon-based compounds and derived from natural sources such as plants which used to control slugs and snails. Nowadays, they are widely used because they are inexpensive and friendly to environment than the synthetic molluscicides [13, 14].

2.2.1 Saponins

Saponins are natural glycosides of triterpene, steroids or steroid alkaloid which contain both a sugar moiety (water-soluble) and sapogenin (fat-soluble) subunits, saponins are found in various plants and some animal kingdom. They are excellent natural non-ionic surfactant, and they can be used in widespread applications, such as cholesterol-lowering activity in humans, anti-cancers, surfactants, pesticides and molluscicides. In 2000, Molgaard reported that an aqueous extracted saponin of *P. dodecandra* was completely degraded within 10 days in aquatic environments under aerobic conditions [15].

San Martin et al. reported that quinoa husks were treated with basic solution in order to convert biodesmosidic saponins to more effective monodesmosides form. It killed 100% golden apple snails at 24 hours. It was suggested that more the

hydrophobic compounds, that higher affinity with the cholesterol in golden apple snail gills [16].

Joshi et al. found that saponin shows lower golden apple snail mortality than niclosamide which is synthetic chemical molluscicide at 24 hours. However, niclosamide completely exhibited rice seedling development but the plants treated with saponin solutions showed normal development [17].

Huang et al. reported that extracts of soapnut showed molluscicidal effects against *P. canaliculata* snail with LC_{50} values of 85, 22, and 17 ppm after treating 24, 48, and 72 h, respectively [18].

Adewumi et al. tested molluscicidal activities against the eggs, juvenile and adult snails with the *sasanqua* saponin extracted from *Camellia oleosa*. The mortality rate of juvenile and adult snails was 93% and 96% and no hatching of eggs at saponin 10 ppm for 48 hours [19].

González-Cruz et al. reported that three plants with a high content of saponins, which are *Camellia oleifera*, *Gleditsia amorphoides* and *Quillaja saponaria*, showed toxicity against the grey field slug [20].

Francis et al. found that dietary from *quillaja* saponin has ability to change the sex-ratio in *Nile tilapia* fish [21].

Saha et al. reported that the saponins derived from *S. mukorossi* and *D. butyracea* had effect in maize than in rice. The active compounds from *D. butyracea* could be used as ecofriendly plant growth regulators [22].

Park et al. indicated that saponin extracted from the starfish has toxicity and potential as a repellent against insects [23].

2.2.2 Camellia oleifera saponin

Camellia oleifera Abel. (Figure 2.3-2.4) is commonly knew as tea seed oil and extracted from tea seed which is native plant from China. It is widely used to control golden apple snails because of its high efficiency, biodegradability, and biocompatibility. *Camellia saponin* was reported that can control the golden apple snail and no rice plant damage was detected [20, 24]. Moreover, It is able to eliminate unwanted fish in prawn ponds [25]. However, Sun (2009) has reported that saponin is

unstable and degradation in water, lead to decrease the efficiency [3]. Table 2.1 shows physical and chemical properties of *C. oleifera* saponin.

Minsalan et al. presented that tea seed powder from camellia seeds at 15 ppm was sufficient to kill fish within 6 hours [26].

Andresen reported that physiological effects depend on dose of tea seed powder on plants [27].

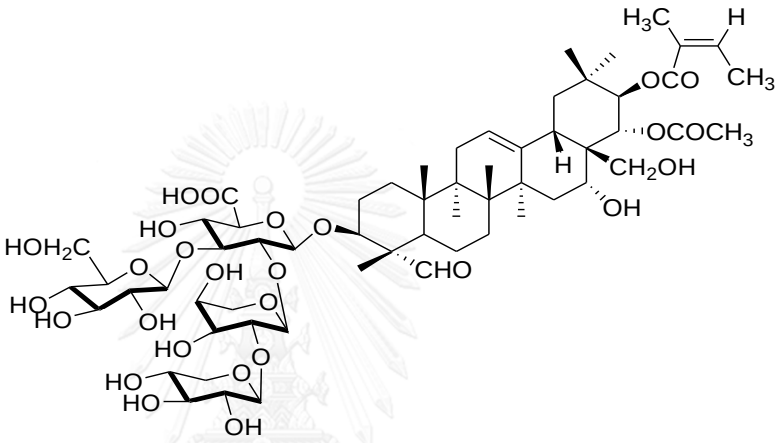


Figure 2.3 Chemical structure of *C. oleifera* saponin

(a) (b)

Figure 2.4 (a) *C. oleifera* Abel. characterization and (b) seed of *Camellia* was used in the experiment [28]

Table 2.1 Physical and chemical properties of *C. oleifera* saponin [29]

Parameter	Value
Physical state	Amorphous powder
Color	Light yellow solid
Odor	Characteristic odor
Melting point	224 °C
Solubility	Easily soluble in water and alcohol
Flammability	Non-flammable
Explosive properties	Non-explosive

2.2.3 Tea seed meal

Tea seed meal is a residue of camellia seeds from tea seed oil production, containing more granular and powder size < 5 mm. It is widely used in agriculture and aquaculture area such as a feed filler, natural organic fertilizer promote plant growth and pesticide to kill golden apple snail without harm to the plants. Moreover, it is a porous material which used as a moisture-absorbing device [30].

Figure 2.5 Tea seed meal from camellia seeds

2.3 Controlled release system

Controlled release system is used to maintain concentrations of active ingredients such as pesticides and molluscicides which usually encapsulated by polymers. The advantages of controlled release molluscicides are decreased application costs due to less frequent applications require, save to the user, prolong effective amount of molluscicides and reduce toxicity to non-target organisms for the same period with non-controlled release molluscicides. Molluscicides are easily decomposed by environment factors including microbes, light and moisture. Controlled release system in agriculture applications is used for controlling various pests such as insects, mites and golden apple snails [31]. Controlled devices are varied in size of particles from micrometers to centimeters depend on the objectives and properties. Biodegradable polymers such as chitosan, starch and alginate are widely used for controlled release due to their properties such as degrade and leave no harmful residues to the environment. Preparation methods are effects on release profile. Neutralization is a reaction between an acid and a base, which consisted of covering continuous phase, core material and coating material phase. The *ionotropic* gelation method is non-toxic and based on the ionic interactions between positively charged and negatively charged groups. The reasons for using controlled release system are decreased application [32].

2.4 Biodegradable polymer

Biodegradable polymers are synthesized or formed in natural environment that break down and decompose into nontoxic smaller substances such as carbon dioxide, water, inorganic compounds biomass. Degradable polymers can be used in various applications consist of medicine, packaging and agriculture. In agricultural application, they are used as the drug delivery devices to control drug release in effective amount for minimizing the side effect. Biodegradable polymers should be nontoxic, maintained effective properties and capable to controlled degradation rates. Degradation rate of biodegradable polymers are affected by various parameter for material chemistry,

molecular weight, hydrophobicity, surface charge, percent crystallinity and water adsorption [33].

2.4.1 Chitosan

Chitosan is a natural cationic polysaccharide which derived from crustacean shell such as shrimps and crabs and obtained by partial alkaline N-deacetylation of chitin. The repeating unit of chitosan is glucosamine. Chitosan is soluble in dilute acid solutions below pH 6 and insoluble in water and alkaline media. At low pH, the primary amino groups of chitosan get protonated that makes chitosan can soluble in water. The solubility of chitosan depends on pH and the degree of deacetylation (DD). It is used in many field such as agriculture, medicine, cosmetics and textile industries. Chitosan has attracted attention as a device for drug delivery in agriculture applications because it is a natural polymer, low toxicity, biodegradability, and good biocompatibility [4]. However, several drawbacks of chitosan for using as a drug device are poor solubility in neutral and alkaline solution, high crystallinity that resist drug transfer [5-7]

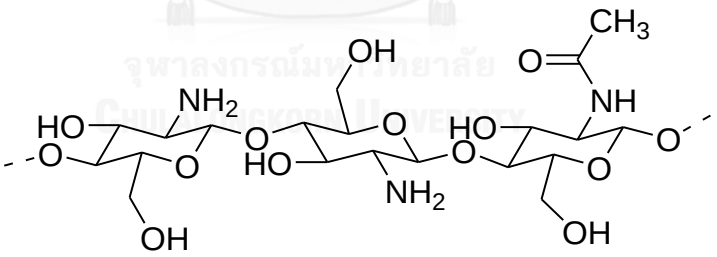


Figure 2.6 Chemical structure of chitosan

In 1997, Fernandez-Hervas et al. prepared the alginate-chitosan beads containing diclofenac hydroxyethypyrrolidine in order to achieve an enteric formulation. All formulations have high encapsulation efficiency. Alginate-chitosan beads showed dense homogeneous internal structure due to interpolymeric complex [34].

In 2008, Lathuder et al. showed that chitosan is the best gel to coat monolith for immobilization of PGA because it is stable and suitable with monolith channels. The reaction rate of the PGA beads is 50% lower than free enzyme [35].

In 2008, Elzatahry et al. reported that alginate-chitosan beads containing theophylline prepared by the *ionotropic* hydrogelation method achieved to control release of theophylline over 24 hours. The release rate depended on the component polymers, bead composition and its interactions [36].

In 2012, Nnamonu et al. exhibited that reinforced alginate improved matrix strength and prevented leaking of imazaquin [37].

In 2013, Chen et al. found that suberoyl chloride crosslinking agent succeeded to decrease the permeability of various plant nutrients from chitosan beads. This is significant potential for agricultural application [38].

CHAPTER III

EXPERIMENTAL

3.1 Materials

3.1.1 Model drug against golden apple snail

The tea saponin powder of *Camellia oleifera* was obtained from Changsha Huakang Biotechnology Development Co., Ltd. (China).

3.1.2 Polymer

Chitosan with molecular weight of 500 kDa and a degree of deacetylation (DD) of 85% (Seafresh Chitosan (Lab) Company Limited, Thailand).

3.1.3 Plant material

Rice (*Oryza sativa L.*) cultivar RD41 seeds were obtained from Phitsanulok Rice Research Center, Thailand.

Tea seed meal (TSM) was obtained from local suppliers.

3.1.4 Chemicals

1. Sodium hydroxide, commercial grade (NaOH) (Merck, Germany)

2. Sodiumtripolyphosphate, commercial grade ($Na_5P_3O_{10}$) (Union chemical 1986, Thailand)

3. Glacial acetic acid 100%, AR grade (CH_3COOH) (Merck, Germany)

4. Calcium chloride, AR grade ($CaCl_2$) (Unilab, Australia)

5. Ethanol was commercial grade purchased from local suppliers and used without purification.

3.2 Instruments

1. UV/Vis Spectrophotometer (HP 8435)
2. Fourier Transformed Infrared Spectrophotometer (FT-IR, Nicolet 6700, Thermo Scientific)
3. Scanning Electron Microscope (SEM, XL30CP, Philips)
4. Analytical balance (AL204, Mettler Toledo)
5. Micropipette (50-1,000 µL, Volumate, Mettler Toledo)
6. pH indicator trips (Merck, Germany)
7. Incubator shaker (KS 4000 is control, IKA)
8. Hotplate stirrer and magnetic bar (SMH5-6, Wisd Laboratory Instruments)
9. Oven (UFE 500, Memmert)
10. Vernier caliper
11. Stainless steel ruler

3.3 Evaluation activity of *Camellia oleifera* saponin

3.3.1 Molluscicidal test

To find the optimal dosage and time of *C. oleifera* saponin against the golden apple snails in order to less dose of saponin used for saving cost and environment. The LC_{50} value of *C. oleifera* saponin against the golden apple snails (GAS) on mortality of *P. canaliculata* snails was investigated as a function the exposure time from 24 to 96 hours [24].

3.3.1.1 Experimental golden apple snails

The golden apple snails or *P. canaliculata* were collected from Amphoe Srithep, Phetchabun Province, selected size in the range of shell length around 2.5-4.0 centimeters, as shown in Figure 3.1. Before testing, the tested snails were acclimatized to laboratory conditions by using dechlorinated tap water and left at room temperature at least 24 h before used and fed them with the leaves of morning glory.

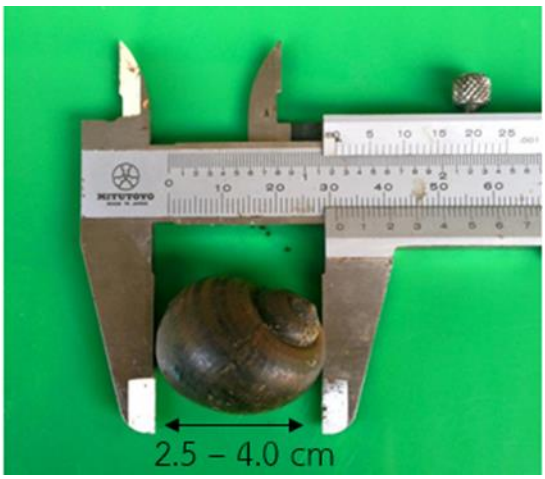


Figure 3.1 The golden apple snails P. canaliculata in the range of shell length about 2.5-4.0 centimeters were used for molluscicidal test

3.3.1.2 Molluscicidal test

In this study, acute toxicity activity and determined effect of median lethal concentration (LC_{50}) of *C. oleifera* saponin against golden apple snail (*P. canaliculata*) were studied, it was measured in terms of the highest concentration that killed none of fish (LC_0), the median lethal concentration which causes fish death of 50% (LC_{50}) and the lowest concentration that killed all fish (LC_{100}), as outlined by USEPA (2005). In the concentration range-finding test was guided to find the appropriate concentration which consisted of five different concentrations of *C. Oleifera* saponin solutions were 5, 10, 15, 20 and 30 ppm. Deionized water without any chemicals addition was used as an untreated control (0 ppm).

The snails were kept and submerged in 1.5 L of covered-net plastic bottle containing 300 mL of each saponin different concentration solutions with triplicate experiment (5 snails for each). Solution medium and dead snails were renewed and removed daily to maintain initial concentration in a semi-static system and water quality during experimental period 96 h (Figure 3.2). The number of dead snails was recorded after 24, 48, 72 and 96 hours, respectively.

Figure 3.2 The snails were kept and submerged in a covered-net 1.5 L plastic bottle for 96 hours

3.3.2 Toxicity test

This preliminary study was determined the minimum lethal concentration of *C. oleifera* saponin as a molluscicide on mortality of Nile tilapia fish as a function of time (24, 48 and 72 hours) to select a safe concentration to be harmless on tilapia fish [39, 40].

3.3.2.1 Experimental fish

Disease-free healthy Nile tilapia fingerlings with an average total weight and length of 1.80 ± 0.51 g and 4.6 ± 0.6 cm, respectively. They were purchased from a commercial hatchery at Chatuchak market, Bangkok, Thailand. The tested fish were acclimatized in laboratory conditions by using dechlorinated tap water and leaving at room temperature at least 24 hours before used and were fed with commercial pelleted feed twice a day. Furthermore, during experimental period (72 hours), they were not fed in order to maintain water quality and initial concentration.

Figure 3.3 The fingerlings of Nile tilapia *Oreochromis niloticus*

3.3.2.2 Toxicity test on fish

In this study, acute toxicity activity and determined effect of median lethal concentration (LC_{50}) of *C. oleifera* saponin against Nile tilapia fish *O. niloticus* were studied. It was measured in terms of the highest concentration that killed none of fish (LC_0), the median lethal concentration which causes fish death of 50% (LC_{50}) and the lowest concentration that killed all fish (LC_{100}) [41]. The concentration range-finding test was guided to find the LC_{50} value which consisted of four different concentrations of *C. Oleifera* saponin solutions were 1, 3, 5 and 10 ppm. Deionized water without any chemicals addition was used as an untreated control (0 ppm).

The tilapia fingerlings were kept and submerged in 10 cm wide x 10 cm long x 9 cm deep covered-net plastic box containing 300 mL of each saponin various concentration solutions with triplicate experiment (10 fishes for each). Solution medium and dead tilapia fishes were renewed and removed daily to maintain initial concentration in a semi-static system and water quality during experimental period 72 hours (Table 3.1). The number of dead fishes was recorded after 24, 48, and 72 hours, respectively.

Table 3.1 The toxicity tests consisted of four various concentrations of *C.oleifera* saponin (10 fishes for each). The fingerlings of Nile tilapia *O. niloticus* were kept in the test solutions

Tank	Concentration (ppm)				
	Control	1	3	5	10
A					
B					
C					

3.3.3 Phytotoxic test

Besides the effects of saponin on the fish and snails were studied, we also studied effect on *O. sativa* L. rice growth between rice untreated (DI water) and treated with saponin solutions, as shown in Figure 3.4.

3.3.3.1 Rice seeds sample

Rice (*Oryza sativa* L.) cultivar RD41 seeds were obtained from Phitsanulok Rice Research Center, Thailand. The seeds were soaked in water and wrapped with moist cloth until germination.

3.3.3.2 Preparation of tested plant material

Rice seeds were soaked in water for 48 h and wrapped with moist cloth until germination was observed. The germinated seeds were cultured in a nursery seedling tray containing wet clay, then 3 week-old seedlings were transplanted into 10 cm wide x 10 cm long x 9 cm deep square plastic pot and then 40 day-old rice plants were transplanted into bigger plastic pot and maintained until they had produced grains [42].

3.3.3.3 Effect of saponin on the growth of rice plants

The effect of *C. oleifera* saponin on the growth of *O. sativa* L. cultivar was studied. The germinated seeds were grown in a nursery seedling tray containing wet clay with triplicate replication (6 seeds per replicate). Plants of each treatment were treated for 30 days with 20 mL of four different saponin concentrations (0, 10, 30 and 50 ppm) using DI water as an untreated control (0 ppm). The growth was recorded by measuring shoot height (in cm) of rice plants with a ruler.

Figure 3.4 (a) O. sativa L rice seeds and (b) The germinated rice seeds after soaking in water and wrapped with moist cloth

3.4 Preparation of chitosan beads containing saponin

3.4.1 Preparation of chitosan beads containing saponin

Figure 3.5 shows the preparation procedure of drug-containing polymer beads which prepared by the combination of the *ionotropic* gelation and neutralization method crosslink with triphosphate (TPP). Two grams of chitosan (CS) were dissolved in 1% glacial acetic acid (100 mL) to prepare 2% chitosan and then stirred by using three-blade stirrer at 500 rpm until chitosan completely soluble. The clear yellow chitosan solution was mixed with saponin. The mixture was stirred by magnetic stirrer for 30 min at room temperature. The chitosan beads with various concentrations of saponin loading of 0.5, 1, 5, 10 and 20% (w/w) were formed by dropping with syringe above mixture solution of 1% (w/v) sodium tripolyphosphate (TPP), 10% (w/v) sodium hydroxide (NaOH), 50% (v/v) ethanol (EtOH) and then stirred slowly at 100 rpm for 15

min to complete crosslinking. After that, the chitosan beads were washed several times with deionized water until the rinsed water was neutral. Finally, the beads were dried in the oven at 60ºC until reaching a constant weight and stored in a desiccator.

3.4.2 Preparation of the mixture of chitosan and tea seed meal (TSM) beads containing saponin

This process was prepared as same as saponin-containing chitosan (3.4.1) but added chitosan and tea seed meal (TSM) in ratio of 1:3 after drug was completely mixed with the chitosan solution.

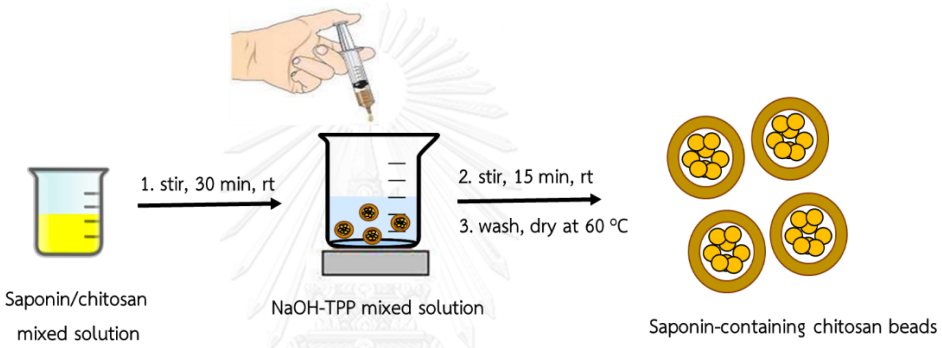


Figure 3.5 The preparation procedure of chitosan beads containing saponin

3.5 Characterization of drug-containing polymer beads

3.5.1 Fourier Transformed Infrared Spectrophotometer (FT-IR)

To determine the present of functional groups in the bead samples. The FTIR spectra of all conditions were measured by ATR method on a Perkin Elmer spectrometer from 400 to 4,000 cm^{-1}.

3.5.2 Scanning Electron Microscope (SEM)

To investigate the morphology of the bead samples which prepared by using double carbon tap to stick on the stub, coated by gold and then operated voltage at 15 kV to observe the surface.

3.5.3 UV/Vis Spectrophotometer

A HP-8435 UV visible spectrophotometer was used for studying of saponin encapsulation efficiency and *in vitro* drug release.

3.5.4 Bead size measurement

The size of wet and dried beads of chitosan and the mixture of chitosan/tea seed powder was determined by a Vernier caliper and SemAFore 5.21 software with three-dimensional position of beads for the average sizes (in mm). Each measurement was repeated three times.

3.6 Drug encapsulation efficiency

3.6.1 Calibration curve of *Camellia oleifera* saponin

3.6.1.1 Standard Solution

The standard saponin solution of 500 ppm was prepared by dissolving 25 mg of saponin in 50 mL of Deionized water.

3.6.1.2 Determination of maximum wavelength (λ_{max})

The standard solution was scanned by UV/Vis spectrophotometer between 200 to 800 nm. Saponin showed maximum absorption at 265 nm.

3.6.1.3 Calibration curve of saponin

Five hundred ppm of saponin standard solution was diluted to the concentration of 100, 200, 300 and 400 ppm with Deionized water in volumetric flask. by using UV/Vis spectrophotometer data, the maximum absorption at 265 nm gave 5 points calibration graph.

3.6.2 Determination of drug encapsulation efficiency

An accurate weight of saponin-containing chitosan and chitosan/tea seed meal beads were ground and maintained in DI water at room temperature for 24 h under stirring at 140 rpm to determine the amount of saponin in polymer matrix. The solution

of each formulation was measured by UV/Vis spectrophotometer at 265 nm. The percentage of encapsulation efficiency (EE) was calculated according to equation (1) below [43]:

$$EE\ (\%) = \frac{\text{Actual amount of saponin loaded in the beads}}{\text{Theory amount of saponin loaded in the beads}} \times 100 \qquad (1)$$

3.7 *In vitro* swelling studies on beads of the mixture chitosan/tea seed meal

Twenty milligrams of dried hydrogel beads of each condition crosslinked by 1, 3 and 5% w/v TPP) in duplicate measurements were immersed in vial containing 10 mL of deionized water and calcium chloride solution. The wet hydrogel beads were weighted after using tissue paper wipe of the liquid at 1, 3, 5, 7, 14 and 28 days, respectively. The percentage of swelling ratio was calculated by using the equation (2) below [44]:

$$\text{Swelling ratio}\ (\%) = \frac{\text{Wt-Wd}}{\text{Wd}} \times 100 \qquad (2)$$

W_t is weight of the swollen beads at time interval (t)

W_d is weight of dry beads at the beginning of time (t_0)

3.8 *In vitro* saponin release profile

Drug release study used DI water and calcium chloride solution as dissolution medium. Beads (200 mg) of each formulation were immersed in 20 mL dissolution medium at room temperature. At time intervals, 5 mL of sample solution was withdrawn with replacement of fresh medium (5 mL) and then analyzed the amount of saponin by UV/Vis spectrophotometer at 265 nm in triplicate. The amount of saponin release was calculated from a calibration curves. The actual amount of saponin release without degradation at time (A_t) was calculate by the equation (3) [45]. The percentage of cumulative saponin release was calculated by using the equation (4) below:

$$A_t = D_t + C_0(1\text{-}e^{-k2t}) \quad (3)$$

At is measured amount of saponin at time (t)

C_0 is the initial amount of saponin prior to degradation

K_2 is the degradation rate constant

$$\text{Cumulative release } (\%) = \frac{\text{Actual amount saponin release}}{\text{Theoretical amount saponin release}} \times 100 \quad (4)$$

3.9 Statistical analysis

3.9.1 Mortality of experimental fish and snails

Percentage of mortality was the ratio number of deaths during a specific period of time per total population during the period. An equation of mortality was used as equation (5) below [10]:

$$\% \text{ Mortality} = \frac{\text{number of deaths}}{\text{number of total population}} \times 100 \quad (5)$$

The number of deaths is the number of dead animals in chemical test solutions during specific period.

The number of total population is the total number of exposed animals chemical test solutions during specific period.

The mortality data after 24 h were calculated by One-way ANOVA using Graphpad Prism software to determine the LC_{50} value.

3.9.2 Measurement of rice growth

In this study rice growth promotion was defined as an increase of rice height with increasing of time [46, 47]. After planting for estimating the plant growth

promotion, the heights of rice plants were measured by stainless steel ruler from the base to the tip of rice plants at different intervals.

3.9.3 Statistical analysis

Statistical analysis was calculated by one-way ANOVA using Microsoft excel (Microsoft Corporation) with $p < 0.05$ considered to indicate statistical significance.

CHAPTER IV
RESULTS AND DISCUSSION

This work focused on the preparation of chitosan beads for containing and studying release behaviors of *Camellia oleifera* saponin from chitosan beads in order to increase the molluscicide activity and stability of saponin. The morphology and size of the prepared beads were determined by SEM, Vernier caliper and SemAfore 5.21 software program. Using FTIR to confirm that saponin was encapsulated in chitosan beads. UV/Vis spectrophotometer was used to determine the amount of saponin encapsulated in chitosan beads and *in vitro* saponin release behavior. In this work, the effect of water hardness was studied by using calcium chloride solution ($CaCl_2$). The saponin release in water and $CaCl_2$ solution for 28 days were investigated. Moreover, the biological effect of saponins on *Oryza sariva* L rice growth and mortality of *Tilapia* fish and *Pomacea canaliculata* snails had been studied.

4.1 Biological activity tests of *C. oleifera* saponin

4.1.1 *In vitro* molluscicidal activity test of saponin on mortality of golden apple snail

The effect of saponin concentration on the molluscicidal activity was first preliminary testing. Various concentrations of saponin solution from 0 to 30 ppm were used for molluscicidal activity test against 5 of *P. canaliculata* snail with the size of shell length about 2.5-4.0 cm. The dead snails were observed as a function of the exposure time from 24 to 96 h and used for mortality calculation of *C. oleifera* saponin. The results of molluscicidal activity test with LC_{50} show in Figure 1A (appendix A) and Table 4.1-4.2.

Concentrations of 0, 5, 10, 15, 20 and 30 ppm showed mortality percentage of 0.00, 0.00, 33.33, 40.00, 66.67 and 73.33 at 48 h, respectively, as shown in Table 4.1. The effect of saponin concentrations on snail mortality showed that there was no

mortality (0%) on snails after testing in 5 ppm saponin solution and control during experimental period from 24 to 96 h. It exhibited that 5 ppm saponin was the concentration that killed none of snails (LC_0). The concentration of 30 ppm saponin showed the highest mortality. Causing of high snail mortality in higher saponin concentration, it is related to increase in the hydrophobic interactions between triterpenoid part of saponin and membrane lipid of snail gills, caused an increase in the permeability of cell membranes [16, 24, 48, 49].

Furthermore, the LC_{50} values are calculated by a one-way ANOVA using Graphpad Prism 5 software. The LC_{50} value on mortality of *P. canaliculata* snails was investigated as a function of the exposure time from 24 to 96 h. The results showed that LC_{50} values of saponin against the snails were decreased with increasing the exposure time, as shown in Table 4.2. The exposure time at 96 h showed the highest mollusccicidal activity with lowest LC_{50} value of 7.45 ppm. It might be due to prior intoxication during proceeding hours, which enhanced in subsequent hours increase in time [50]. An exposure time at 72 h had lower molluscicidal activity with 9.94 ppm than those of exposure time at 24 and 48 h with the LC_{50} value of >30 and 15.02 ppm, respectively. The highest of the LC_{50} value was observed at 24 h. It might be due to the snails closed their operculum instantly when they were immersed in saponin solutions for protection and reducing penetration into the body from the toxicant [17]. Abnormal behavior responses of the snails were observed when they were immersed in saponin concentrations such as 10, 15, 20 and 30 ppm which are greater than 5 ppm. The snails became inactive by closing operculum and dragging the body inside the shell in experimental period then eventually died. The dead snails had opened operculum, rigid body and unpleasant smell, shown in Figure 1A

The experiment results showed dose and time dependent effects on mortality of golden apple snail. Increasing concentrations of saponin solutions and exposure times increased snail mortality. The minimum of LC_{50} value is 7.45 ppm for the exposure time of 96 h, suggesting that this exposure time is optimal condition against the golden apple snails due to less dose of saponin used for saving cost and environment.

Table 4.1 Molluscicidal activity test of C. saponin against *P. canaliculata* snails during 96 hours

Concentration (ppm)	Total snails	24h		48h		72h		96h	
		Dead snails ± S.D.	%Mortality ± S.D.	Dead snails ± S.D.	%Mortality ± S.D.	Dead snails ± S.D.	%Mortality ± S.D.	Dead snails ± S.D.	%Mortality ± S.D.
0	5	0	0	0	0	0	0	0	0
5	5	0	0	0	0	0	0	0	0
10	5	0	0	2 ± 0.6	33.33 ± 66.7	3 ± 0.6	55.33 ± 0.0	5 ± 0.0	100.00 ± 0.0
15	5	0	0	2 ± 0.0	40.00 ± 0.0	5 ± 0.0	100.00 ± 0.0	5 ± 0.0	100.00 ± 0.0
20	5	0	0	3 ± 0. 6	66.67 ± 6.7	5 ± 0. 0	100.00 ± 0.0	5 ± 0.0	100.00 ± 0.0
30	5	0	0	4 ± 0. 6	73.33 ± 6.7	5 ± 0. 0	100.00 ± 0.0	5 ± 0.0	100.00 ± 0.0

Data are shown as the mean ± SD and derived from triplicate replications (5 snails for each)

Table 4.2 Lethal concentration (LC_{50}) value of *C. Oleifera* saponin against snails

Time (h)	The LC_{50} value (ppm)
24	>30
48	15.02 ± 0.58
72	9.94 ± 0.10
96	7.45 ± 0.24

4.1.2 Preliminary *in vitro* toxic activity test of saponin on mortality of fish

The effect of saponin concentration on the toxic activity was first preliminary testing. Various concentrations of saponin solutions from 0 to 10 ppm were used for toxic activity test against 10 of Nile tilapia fish *O. niloticus* with the size of body length about 4.6 ± 0.6 cm and approximate weight of 1.80 ± 0.51 g. The dead tilapia fishes were observed as a function of the exposure time from 24 to 72 h and used to determinate the minimum lethal concentration of *C. oleifera* saponin as shown in Figure 2A (Appendix A) and Table 4.3-4.4.

Concentrations of 0, 1, 3, 5 and 10 ppm showed mortality percentage of 0.00, 0.00, 36.67 56.67 and 100.00 at 24 h, respectively, as shown in Table 4.4. The effect of saponin concentrations on tilapia fish mortality showed that there was no mortality (0%) on tilapia fish after testing in 1 ppm saponin solution and control during experimental period from 24 to 72 h. It exhibited that 1 ppm saponin was the concentration that killed none of fish (LC_0) and and concentration of 10 ppm was the concentration that killed all fish (LC_{100}). High fish mortality was observed when they were exposed into higher saponin concentration solutions because increasing in the hydrophobic interactions between triterpenoid component of saponin and their grill membranes which caused swelling of the respiratory epithelium and increased the permeability of erythrocyte, haemoglobin and O_2 uptake volume levels [49, 51-55].

Furthermore, the LC_{50} values are calculated by a one-way ANOVA using Graphpad Prism 5 software. The LC_{50} value on mortality of tilapia fish was investigated

as a function of the exposure time from 24 to 72 h. Table 4.3 shows the LC_{50} values were 3.10, 2.72 and 2.13 ppm for 24, 48 and 72 h. exposure time, respectively. The results showed that LC_{50} values of saponin against tilapia fish were decreased with increasing the exposure time. It might be due to prior intoxication during proceeding hours, which enhanced in subsequent hours increase in time [50].

In addition, the behavior responses of the Nile tilapia fish were studied. There was significantly observed when they were exposed into high saponin concentration. After 30 mins the highest concentration of 10 ppm, fish had begun abnormal behaviors such as swimming quickly, jerky and uncoordinated movements, trying to jump out the container and swimming upside down. Sometimes, they floated near the surface of the water, twitched, sinked to the bottom, laid and gasped on the bottom of the container and then eventually died. In addition, all fish died after 24 h.

Besides, saponins have effected on arthropods such as the insects, crabs, shrimps and scorpion, by interacting with steroid in their intestine which was reduced absorption [56].

The experiment results showed dose and time dependent effects on mortality of tilapia fish. Increasing concentrations of saponin solutions and exposure times increased fish mortality. Therefore, to select a safe concentration to be harmless on tilapia fish, we suggested that using in minimum dose of saponin less than 2.13 ppm at 72 h.

Table 4.3 Lethal concentration (LC_{50}) value of C. oleifera saponin against Tilapia fish

Time (h)	The LC_{50} value (ppm)
24	3.10 ± 0.49
48	2.72 ± 0.11
72	2.13 ± 0.13

Table 4.4 Toxicity activity test of *C. oleifera* saponin on *Tilapia* fish in 72 hours

Concentration (ppm)	Total fish	24h		48h		72h	
		Dead fish ± S.D.	%Mortality ± S.D.	Dead fish ± S.D.	%Mortality ± S.D.	Dead fish ± S.D.	%Mortality ± S.D.
0	10	0	0	0	0.00 ± 0.00	0	0
1	10	0	0	0	0.00 ± 0.00	0	0
3	10	4 ± 1.5	36.67 ± 15.3	6 ± 0.6	66.67 ± 5.8	9 ± 0.0	90.00 ± 0.0
5	10	6 ± 1.5	56.67 ± 15.3	9 ± 0.6	93.33 ± 5.8	10 ± 0.0	100.00 ± 0.0
10	10	10 ± 0.0	100.00 ± 0.0	10 ± 0.0	100.00 ± 0.0	10 ± 0.0	100.00 ± 0.0

Data are shown as the mean ± SD and derived from triplicate replications (10 fish for each)

4.1.3 Effect of saponin on the growth of rice plants

Besides the effects of saponin on the fish and snails were studied. The effect on *O. sativa* L. rice growth between rice untreated (deionized water) and treated with saponin solutions was studied. Rice growth was recorded by measuring shoot height in centimeter. Furthermore, the effect of various saponin concentrations of 0, 10, 30 and 50 ppm on rice was determined. The results of saponin effect on rice are shown in Figure 4.1-4.5 and Table 4.5.

Figure 4.1 and 4.2 show rice growth and development. After 30 days, plants treated with all concentrations grew up gradually with increase in height and size of tillers and leaves. Increasing plant heights were observed significantly when they were treated with saponin concentration of 10 ppm. From 30 to 150 days the plants had been growing up increasingly, this period they had long, large green leaves and straight strong tillers. Moreover, after more than 90 days, all treatments were found in during booting stage which leaf sheaths had been swelling and extending (Figure 4.3). At 120 days, the tips of rice panicles which emerged from the stem rice and continued grow, were found in all treatments and then harvested after 150 days, as shown in Figure 4.4. The average heights of 5-month old rice plants treated with saponin concentration of 0, 10, 30 and 50 ppm were recorded at 136.5, 148.0, 145.5 and 141.5 cm, respectively, are shown in Table 4.5.

The experiment results showed there were no significant differences ($p>0.05$) in height when rice plants treated with various concentrations of saponin. Higher concentration of saponin showed no effect on rice growth during the growth period of 5 months. The growth of rice plants treated with saponin solutions was similar to the control (untreated saponin). These results showed that saponin had no effect on rice plants.

Figure 4.1 Effect of saponin on rice plant growth after 30, 60, 90, 120 and 150 days. Plants were treated with various saponin solutions of 10, 30 and 50 ppm and compared to untreated control

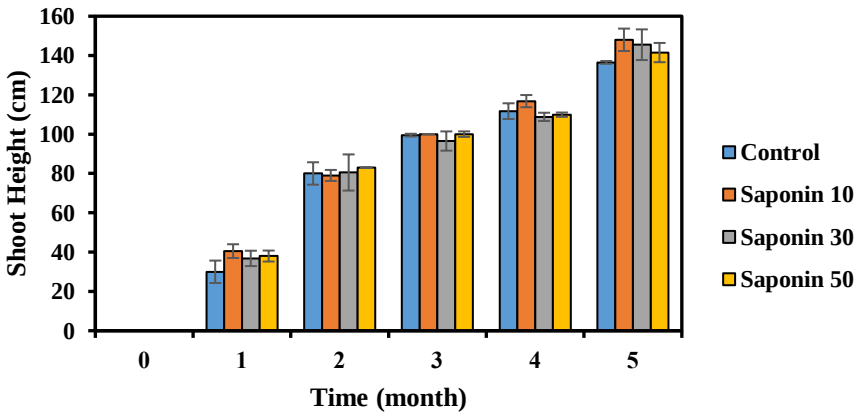


Figure 4.2 Shoot height of young O. sativa L.rice after treating with different saponin solutions compare with the control treatments after 150 days.

Table 4.5 Mean (±SD) of plant heights (in cm) of rice after treating with *C. oleifer* saponin solutions

Concentration (ppm)	No. of seedlings	Shoot height (cm)				
		30 days	60 days	90 days	120 days	150 days
0	6	30.0±5.7	88.8±2.1	104.1±4.0	113.9±3.2	136.5±0.7
10	6	40.5±3.5	90.0±1.3	105.6±2.5	119.2±2.5	148.0±5.7
30	6	36.8±3.9	85.4±1.8	103.0±1.8	110.3±3.0	145.5±7.9
50	6	38.0±2.8	84.1±3.3	102.0±0.4	111.9±1.6	141.5±4.9

Data are shown as the mean ± SD and derived from triplicate replications (6 seeds for each)

Figure 4.3 Rice plants during booting state after 90 days.

Figure 4.4 Rice panicle after 130 days.

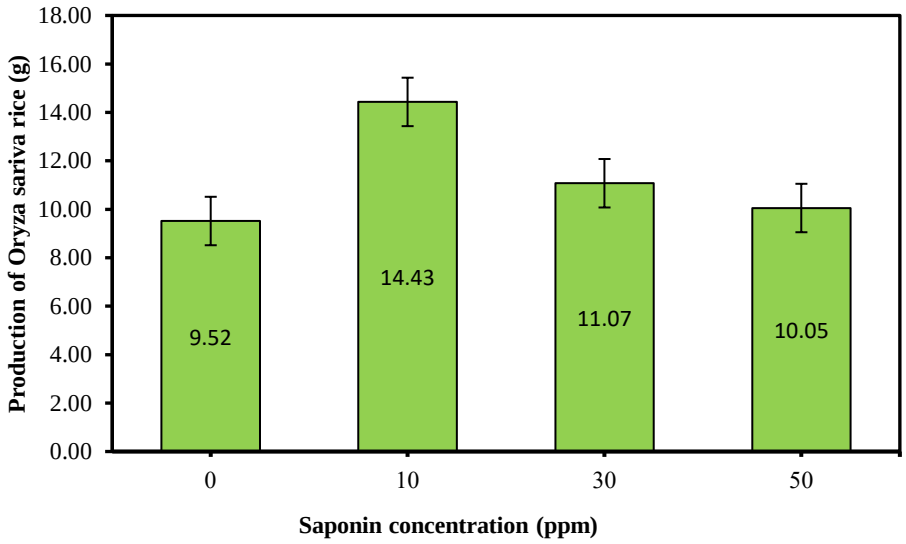


Figure 4.5 Production of O. sativa L. rice after treating with various saponin solutions compare with the control treatments, after 150 days

4.2 Preliminary preparation and formation studies of chitosan beads

In preliminary study, to evaluate the effects of various parameters of bead preparation on the formation and size of chitosan beads. The beads were prepared by the combination of the *ionotropic* gelation and neutralization methods.

Experiments conducted on optimized value of that specific parameter. The optimized value was used for further studies.

1. Effect of chitosan as a marix polymer
2. Effect of sodium tripolyphosphate (TPP) as a crosslinking agent
3. Effect of sodium hydroxide (NaOH) as a neutralizer
4. Effect of ethanol (EtOH) as a mixed solvent
5. Effect of mass ratios of chitosan and tea seed meal (TSM) as a filler
6. Effect of saponin content as a model drug against golden apple snail

The results showed that all parameters affected the formation of the beads as shown in Figure 4.6-4.10 and Table 4.6-4.7. The preparation beads of all formulations were left 15 minutes in crosslinking step to prevent the drug loss to the surrounding medium.

4.2.1 Effect of chitosan concentration

This research focused on the bead preparation of biodegradable polymer for containing and controlled releasing of *C. oleifera* saponin against *P. canaliculata* snails. The beads were prepared by the combination of the *ionotropic* gelation and neutralization methods. The shape and formation chitosan beads of three different chitosan concentrations 1, 2 and 3% (w/v) in 1% (v/v) acetic acid were studied, as shown in Figure 4.6A-C. The experiment showed the concentrations of 1% and 3% chitosan solution were not able to form bead. The chitosan solution of 2% w/v was the optimal concentration because it formed durable and perfect spherical beads with average size of 4.9±0.1 mm. The successful spherical beads were affected by the intermolecular hydrogen bonding. The hydrogen bonding increases with increasing the concentration of chitosan caused strong intermolecular crosslinking of polymer leading

to the strong bead formation [57-59]. The gel droplets at low concentration (1%) could not form in bead, it broke when dropped onto the disperse phase. It might be decreasing in viscosity of chitosan solution cause of weak intermolecular crosslinking which increased the solubility, the external solution can penetrate easily through the bead and then the bead rupture [60, 61]. In addition, chitosan concentration of 3% was not able to drop in spherical droplets. The gel had various sizes and long line due to the extent of entanglement which resisted the mobility when the chitosan concentration was increased [62-64]. The results exhibited that the shape of beads depended on the viscosity of chitosan solution. Thus, the best chitosan concentration was selected as 2% (w/v).

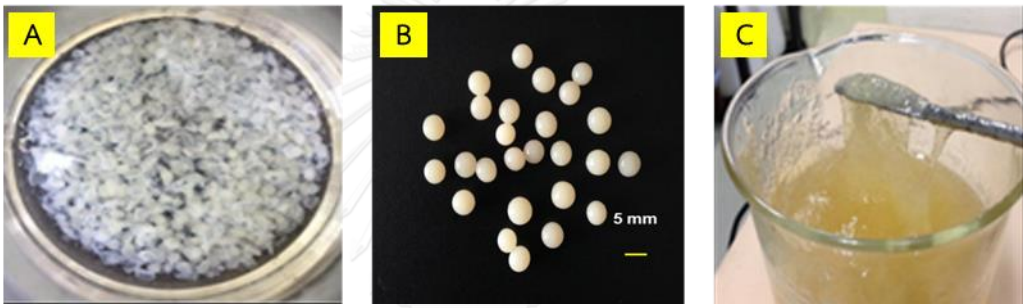


Figure 4.6 Effect of chitosan concentration at (A) 1, (B) 2 and (C) 3% (w/v) on bead formation for controlled release saponin

4.2.2 Effect of sodium tripolyphosphate crosslinking agent

Sodium tripolyphosphate (TPP) is a non-toxic polyanion which can form a gel bead by ionic interaction between positively charged amino groups of chitosan and negatively charged phosphoric ions of TPP, forming either intermoleculaor or intramolecular bonds [65]. Moreover, TPP can increase the encapsulation efficiency and prolong the release of saponin [66, 67]. The effects of TPP concentration at 1, 3 and 5% (w/v) on bead formation were studied (Figure 4.8A-C). Increasing in TPP concentration from 1 to 5% (w/v), the gel droplets rapidly formed dense and spherical structures due to increase of crosslinking density between TPP and chitosan led to more rigid network structure. However, no significant difference in bead shape and size of all formulations, as shown in Figure 4.8. The average sizes of the chitosan beads

crosslinked with 1, 3 and 5% TPP were 4.3±0.2, 4.2±0.1 and 3.9±0.1 mm, respectively. In addition, a change of the physical states of the chitosan to form beads was indicated by the appearance of the gel droplets changed from clear droplets to opalescent droplets [61]. The results showed that the formation of droplets depended on TPP concentration. Thus, the best TPP concentration was selected as 1% (w/v) because chitosan beads had well-form spherical shape similar to higher concentration of 3 and 5% and reduced usage of TPP content.

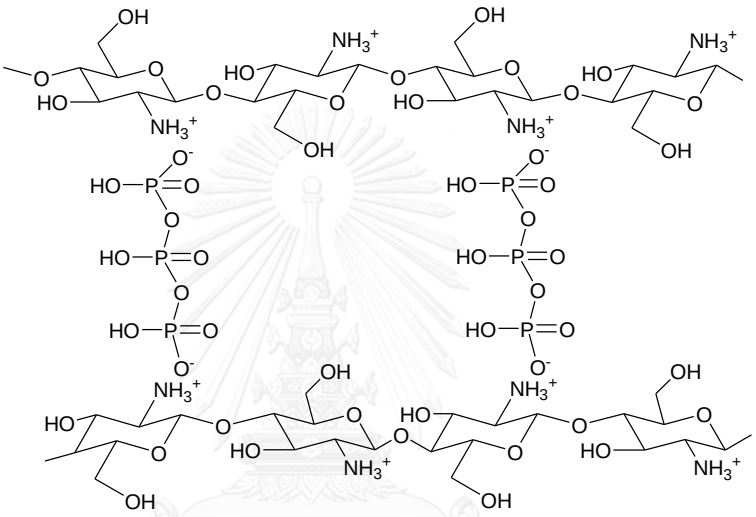


Figure 4.7 Chitosan crosslinked in NaOH-TPP mixed solution

Figure 4.8 Effect of TPP concentration (A) 1, (B) 3 and (C) 5% (w/v) on bead formation for controlled release saponin

4.2.3 Effect of sodium hydroxide

The preparation of chitosan beads by the ionic gelation in TPP solution gave soft and weak beads, the beads could not stay in strong and perfectly spherical shape after washing step. Sodium hydroxide (NaOH) is a neutralization agent which deprotonate the NH_3^+ groups of chitosan to the NH_2. The Effect of NaOH concentration from 5, 10 and 15% (w/v) on bead formation was studied, as shown in Figure 4.9A-C. The results showed that after washing process, chitosan beads of all formulations were more rigid, compact and less deformable because chitosan was aggregated by deprotonation of the OH groups in alkaline solution [68-70]. The optimum concentration was 10% NaOH, the beads from this formulation had well uniform and spherical shape than other formulations (5 and 15%).The beads of 5% had oval and spherical shape, and 15% had water drop shape and short tails. The average sizes of the chitosan beads in 5, 10 and 15% NaOH were 4.9±0.2, 4.93±0.1 and 7.74±0.6 mm, respectively. The results showed that the formation of droplets depended on NaOH concentration. Thus, the best NaOH concentration was selected as 10% (w/v).

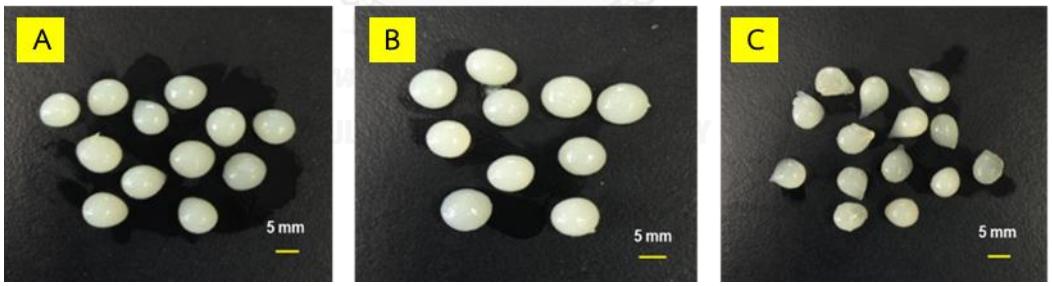


Figure 4.9 Effect of sodium hydroxide concentration (A) 5, (B) 10 and (C) 15% (w/v) on bead formation for controlled release saponin

4.2.4 Effect of ethanol

For bead preparation process, the surface tension of disperse solution is an important physical property. Ethanol is a low surface tension substance (0.022 N/m) and very popular short-chain alcohol solvent on surface adsorption. Mixed solvents for using in chitosan bead preparation were prepared in terms of volume ratios (v/v). Figure 4.10A-D shows different percentage compositions of ethanol in water of 0, 30, 50 and 70% (v/v), respectively, were studied for the formation of beads. When chitosan droplets were dropped onto disperse solution without ethanol (0%), they floated on solution surface causing the droplets were not able to form in strong and spherical beads. The spherical beads floated near solution surface and stuck to another when they were dropped in 30% ethanol. In mixed solvent of 50%, the beads randomly floated and formed in strong and perfectly spherical. It can be suggested that the average interaction between solvent molecules was reduced with increasing in ethanol content [71]. The oval beads sank rapidly and overlap each other in 70% ethanol. Increasing concentration of ethanol from 30-70% (v/v) cause of the gel droplets penetrated into disperse solution easily leading to the spherical beads formed. It might be due to the high surface tension of water, the molecules of water bind together through hydrogen bonds cause water has a high surface tension and difficult to separate. The results exhibited that the shape of beads count on the compositions of ethanol and water. Thus, the best percentage compositions of ethanol in water was selected as 50% (v/v).

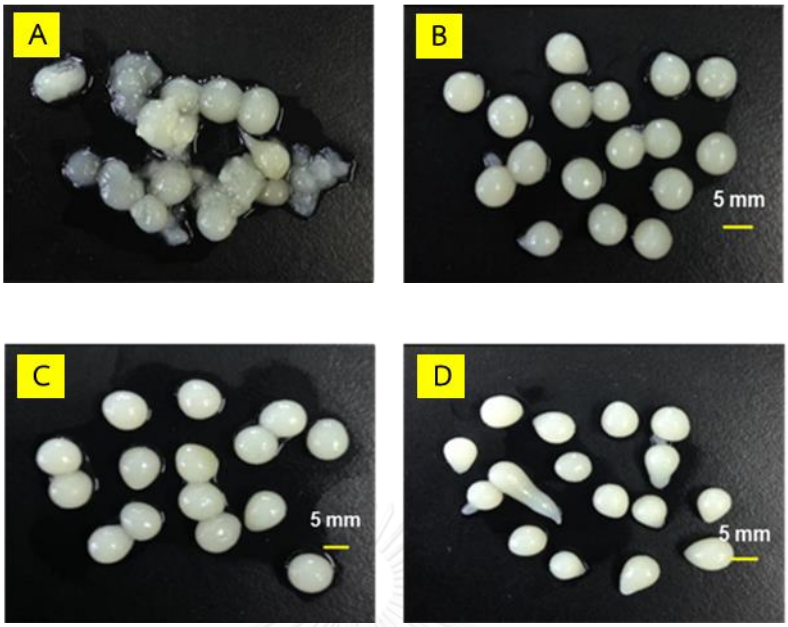


Figure 4.10 Effect of ethanol concentrations (A) 0, (B) 30, (C) 50 and (D) 70% (v/v) on bead formation for controlled release saponin

4.2.5 Effect of tea seed meal content

Agnihotri has reported that large beads of the gellan increased amount of drug in the beads lead to increase the release rate [72]. In this study, tea seed meal was mixed into the polymer solution as a filler in order to maximize size of chitosan beads for more sustained and prolonged release of saponin. The effect of tea seed meal content on bead size and formation was studied. Table 4.6 shows four mass ratios of chitosan/tea seed meal which were mixed at 1:1, 1:2, 1:3 and 1:4 (CS/TSM1-CS/TSM4), respectively. Tea seed meal at the ratio of 1:1, 1:2 and 1:3 (CS/TSM1- CS/TSM3) was homogenously distributed within the chitosan solution. An obvious increase in bead size was observed when increased tea seed meal content. Low tea seed meal content at the ratio of 1:1 (CS/TSM1) and 1:2 (CS/TSM2) showed that the dry beads had smaller sizes than 1:3 (CS/TSM3) and 1:4 (CS/TSM4). Dry beads of CS/TSM3 had more spherical shape and uniform size than other formulations. The highest tea seed meal content of CS/TSM4 indicated an excess value. The chitosan beads broke easily and had various sizes due to the heterogeneous dispersion of tea seed meal within the solution and

excessive masses led to a low total surface area, making the gel elasticity and strength decreased [73, 74]. The bead size and shape depended on the tea seed meal contents. Thus, the best mass ratio of chitosan/tea seed meal was selected as 1:3 (w/w).

4.2.6 Effect of drug content

Various saponin contents loaded to chitosan solution from 10 to 70% (w/w) for studying bead shape. Table 4.7 shows that the shape of the beads was slightly broader and larger when more drug loading of 10 to 50% (w/w) was added. However, the beads were no significant difference in size after drying. In addition, the spherical beads could not be prepared at saponin loading greater than 50% (w/w) because the droplets had broad, long tails and various sizes. This could be ascribed to the dispersion and viscosity in the solution increased with increasing saponin content [75, 76]. The results showed that the shape of beads depended on loading of saponin content. The size of beads proportionally increased when the content of saponin increased. Thus, the optimal saponin content for loading in chitosan solution was from 10 to 50% (v/v).

In summary chitosan beads were prepared by the combination of the ionotropic gelation and neutralization method. From preliminary parameters such as concentration of chitosan solution, TPP concentration, NaOH concentration, ethanol concentration, tea seed meal and saponin contents were examined to obtain an optimum condition for chitosan bead preparation. The results showed that round spherical chitosan beads were obtained at mixed solutions of 2% (w/v) chitosan, 1% (w/v) TPP, 10% (w/v) NaOH, 50% (v/v) EtOH and saponin loading from 10 to 50% (v/v).

Table 4.6 Wet and dry beads of four mass ratios of chitosan/tea seed meal

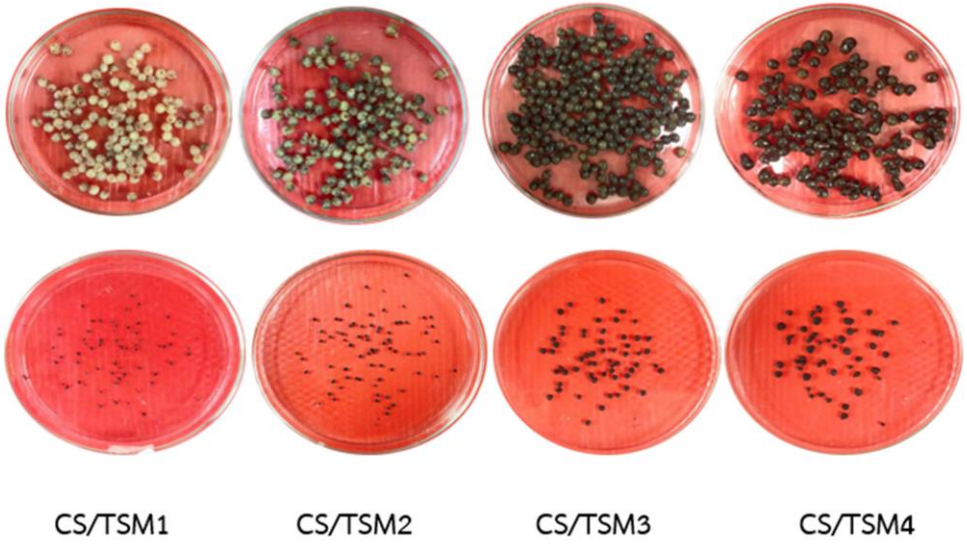
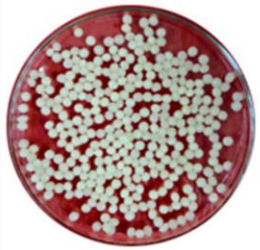
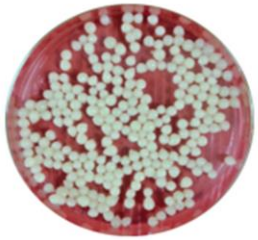
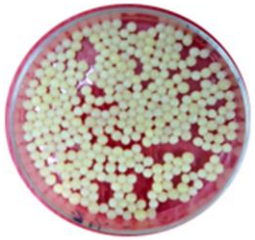
mass ratios of chitosan/tea seed meal (w/w)			
1:1	1:2	1:3	1:4
			
CS/TSM1	CS/TSM2	CS/TSM3	CS/TSM4

Table 4.7 Effect of saponin content from 10 to 70% by weight on bead shape and formation for controlled release saponin (before drying)

Saponin content (% w/w)			
10	30	50	70
			
SA10	SA30	SA50	SA70

4.3 Chitosan beads containing saponin

4.3.1 Determination of saponin encapsulation efficiency (EE)

Saponin is unstable and rapid degradation in water lead to decrease in the efficiency**.** In this study, the effect of loaded amount of saponin on encapsulation efficiency was studied. The percentages of saponin encapsulation efficiency are shown in Table 4.8.

The amount of saponin encapsulated in chitosan beads was determined by UV spectrophotometry at 265 nm in deionized water. The standard curve with the equation of the linear regression (y=0.0015x+0.0267, where y and x are the absorbance and saponin concentration (ppm)), as shown in Table 1B and Figure 1B (Appendix B). Table 4.8 shows that there was no significant difference in encapsulation efficiency (EE) of 0.5-10% (w/w) saponin loading (52-64%) Loading of 1% (w/w) saponin in chitosan matrix showed the highest encapsulation efficiency that was 64%. Moreover, saponin content significantly affected to the encapsulation efficiency. The EE of 1% saponin loading into chitosan solution was six times higher than 20% saponin loading. Loading of 20% (w/w) saponin showed 9.92% percent.

It suggested that saponin loading ratio of 1% (w/w) is the optimum condition for encapsulation in chitosan solution.

Table 4.8 Encapsulation efficiency of saponin loaded into chitosan

Formulation	%Encapsulation
CS-saponin-0.5%	59.89 ± 6.36
CS-saponin-1.0%	64.28 ± 7.43
CS-saponin-5.0%	54.09 ± 3.86
CS-saponin-10.0%	52.30 ± 2.99
CS-saponin-20.0%	9.92 ± 0.57

Chitosan/TSC- saponin-10%	70.12 ± 1.23

* Data are shown as the mean ± SD and derived from three independent repeats

4.3.2 *In vitro* release studies of saponin

In previous encapsulation efficiency study showed that saponin loading ratio of 1% (w/w) is the optimum condition for encapsulation in chitosan matrix due to it has the highest encapsulation efficiency. However, Shan-Ting showed that more Rhodamine loading into polylactic acid (PLA) increased amount and release rate of the Rhodamine [77] so the *in vitro* release behavior of 10% (w/w) saponin loading in chitosan beads was studied comparison with loading of 1% saponin loading and pure saponin for 28 days at room temperature due to the highest loading of saponin and no difference in encapsulation efficiency compared with 1% saponin loading. The exponential equation of saponin calibration curve is $y = 3.4598e^{-0.137x}$ and $R^2 = 0.9882$

The saponin was completely degraded within 10 days in aquatic environments under aerobic conditions so the degradation factor is an important factor to correct release value of saponin [15].

Figure 4.11 shows the characteristic degradation behavior of *C. oleifera* saponin in deionized water for this experiment.

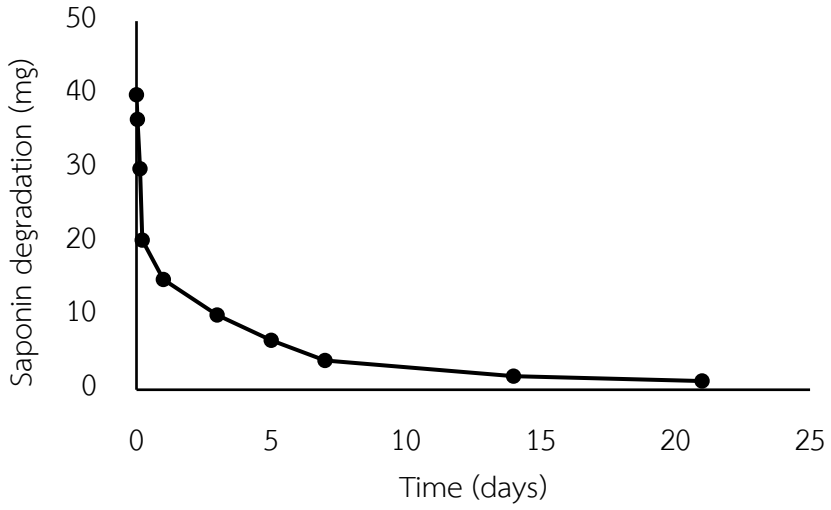


Figure 4.11 Degradation of *C. oleifera* saponin in deionized water

Figure 4.12 presents the degradation rate constant in days (k_2)(-0.137) following the first-order kinetics which could be calculated from the slope of the exponential line plot [78].

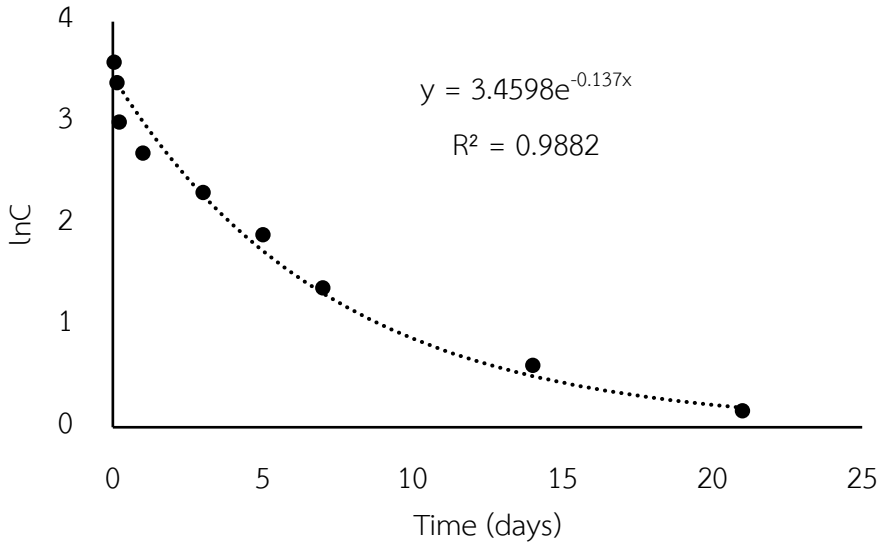


Figure 4.12 The degradation rate constant of *C. oleifera* saponin

Figures 4.13 and 4.14 show the plots of the saponin release in deionized water before and after correction by the degradation factor.

The pure saponin content completely released 99% within 1 hour and then the release rate had declined steadily due to saponin was degraded by aquatic environments. The initial burst release of chitosan beads loading 1% and 10% (w/w) saponin was lower than the pure saponin with release of 27 and 40% in an hour, then, gradually decreased and prolong the release to 28 days. An initial burst release due to dissolved saponin move with water and stored on the surface of chitosan beads. The results showed that saponin encapsulation can prolong and reduce amount of saponin release from 1 to 14 days.

Moreover, it showed that the cumulative release increased as saponin concentration increased because more saponin molecules can connect with each other which allow for quickly saponin diffusion into the release medium.

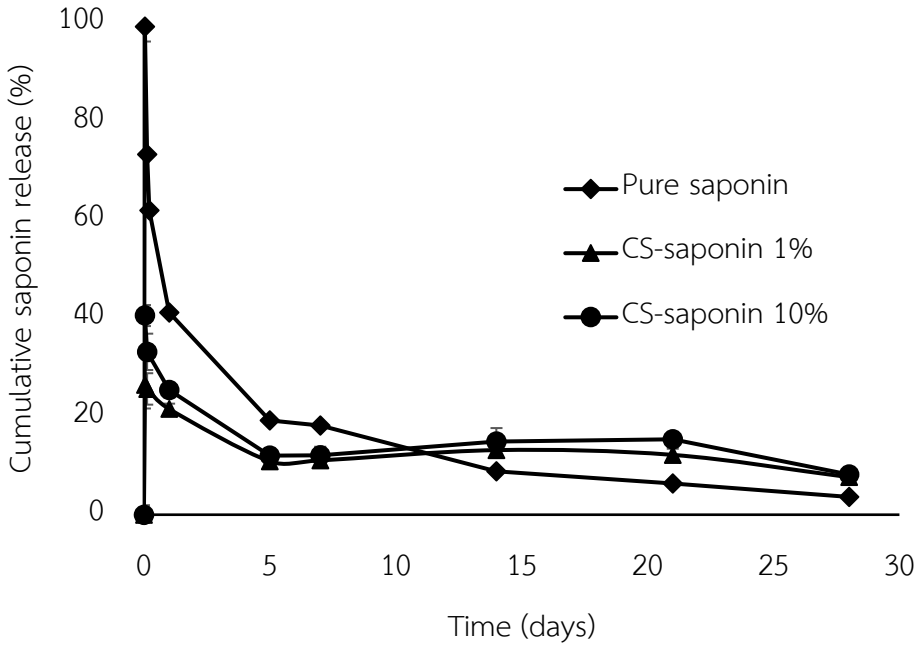


Figure 4.13 Release profiles of 1% and 10% (w/w) saponin from chitosan beads in deionized water before correcting by the degradation factor (average±S.D., n=3)

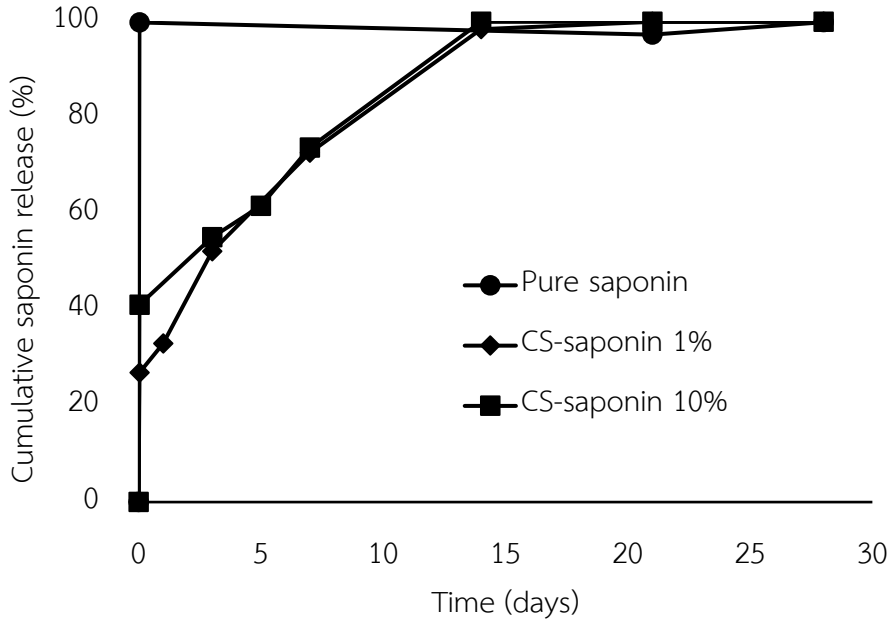


Figure 4.14 Release profiles of 1% and 10% (w/w) saponin from chitosan beads in deionized water (average ± S.D., n=3)

Thus, chitosan beads containing 10% (w/w) saponin is chosen as an optimum condition against the golden apple snails because of more amount of saponin release at long period time and promote its efficacy.

High molluscicidal activity has been observed for monodesmosidic saponins [16]. Camelliasaponin 1, Theasaponin E1 and Theasaponin E2 were molluscicidal compounds from C. *oleifera* Abel which displayed the highest molluscicidal activity with LC_{50} value of 6.52 ppm after 24 h (Figure 4.15-4.16). Both the aglycone and the sugar moiety played an important role in the biological activity of saponin. These compounds interact with membrane lipid leading to membrane destabilization [24, 79]. Decrease in saponin contents were found in aqueous solution because of the degradation of saponin via oxidation reaction. Camellia saponin is a monodesmosidic saponin which has a sugar chain linked to the C-3 position of its aglycone [80]. The degradation products of this saponin has not been investigated in detail. Camellia saponin contains both of triterpene glycoside and sugar moiety similar to quillaja saponin but they are different in number of sugar chain. Moreover, the degradation of quillaja saponin in aqueous solution was reported that is proposed in Figure 4.17. The QS saponin molecule was brokendown into quillaja acid which is the aglycone part through the hydrolysis of ester bond and the sugar moieties of glucuronic acid, xylose, fucose, apiose, phamnose, galactose, arabinose and fatty acyl units were also degraded [80-82]. It is suggested that the mechanism of camellia saponin breakdown is the linked bond between aglycone and sugar chain would be broken via ester bond hydrolysis as mentioned in the degradation of quillaja saponin and the intact aglycone was then left in the medium [83]. A hydrolysis of ester bonds that hydrolyze rapidly at room temperature, generating by-products that do not have molluscicidal activity [84].

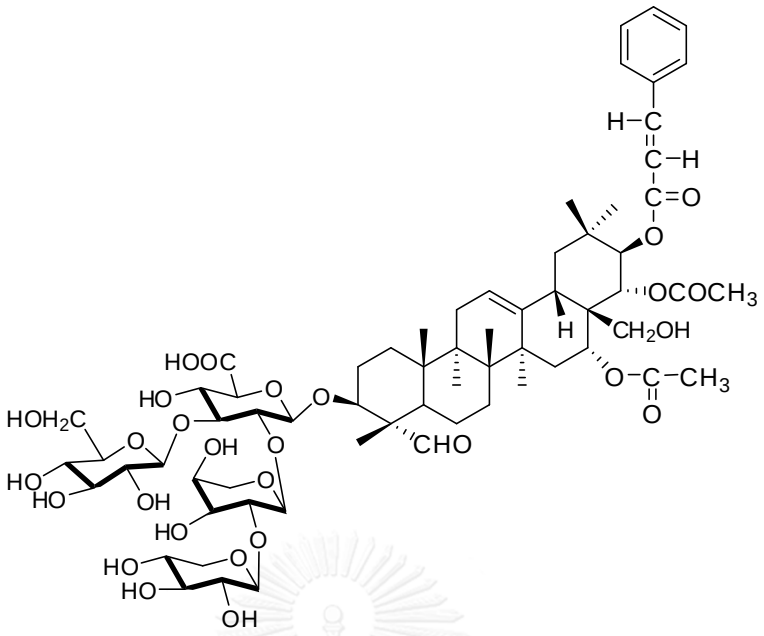
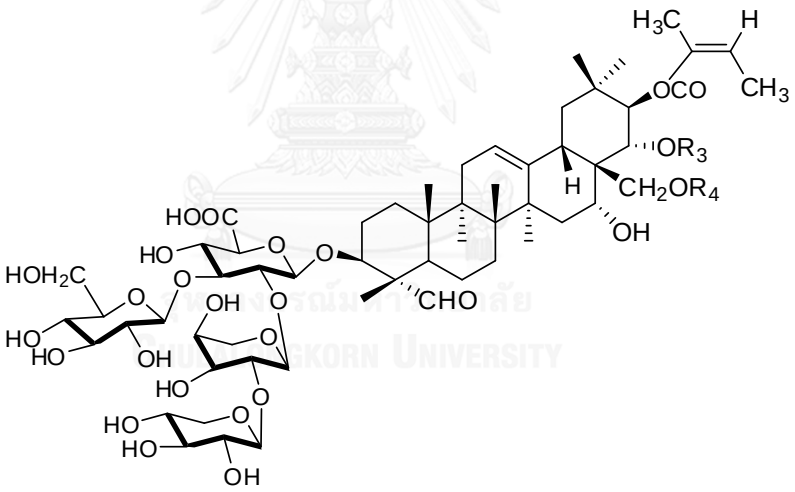


Figure 4.15 The structure of Camelliasaponin 1



	R_1	R_2	R_3	R_4	R_5
Theasaponin E1	CHO	Ang	Ac	H	H
Theasaponin E2	CHO	Ang	H	Ac	H

Figure 4.16 The structure of Theasaponin E1 and Theasaponin E2

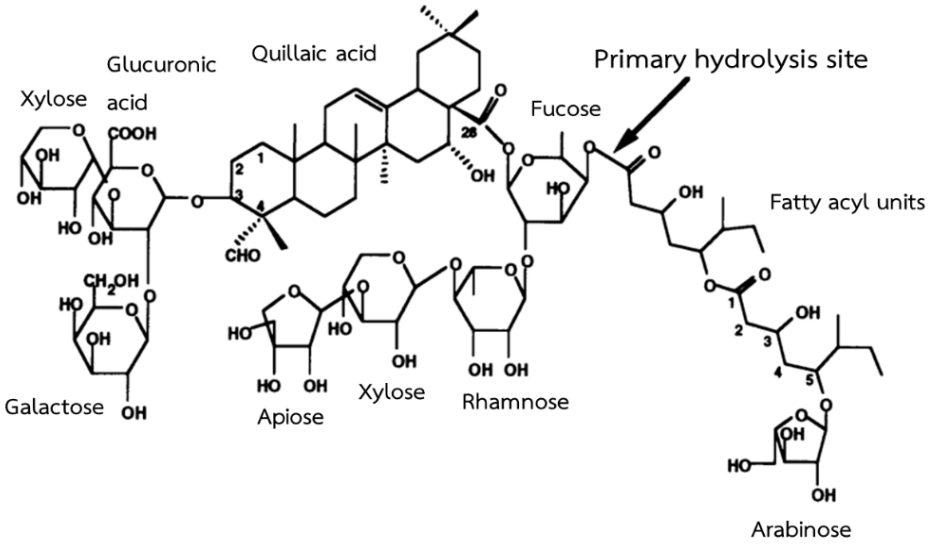


Figure 4.17 The primary degradation site of quillaja saponin in aqueouse solution

4.4 Beads of the mixture of chitosan and tea seed meal containing saponin

4.4.1 Determination of saponin encapsulation efficiency (EE)

Previous study (Chapter 4.2.5) showed that the best mass ratio of chitosan/tea seed meal 1:3 (w/w) was selected as a filler for more sustained and prolonged release of saponinn. For encapsulation studies of chitosan beads indicated that the optimum saponin content was 10% (w/w) so encapsulation efficiency of saponin with content of 10% (w/w) loaded into chitosan/teas seed meal beads was studied. It showed 70.12% encapsulation efficiency (Table 4.8).

4.4.2 *In vitro* release studies of saponin

To compare the release of saponin in deionized water as a function of time for 28 days between beads of the mixture of chitosan/tea seed meal and chitosan beads which containing 10% saponin were studied to obtain the best condition for using against golden apple snails.

The release of saponin from two formulations had the same behavior, as shown in Figure 4.18. The release rate was observed after 1 hour with 52% saponin release from beads of the mixture of chitosan and tea seed meal while the release from chitosan beads was 38%. After that the release amount of saponin was increased gradually and completely released in 14 days. However, beads of the mixture of chitosan and tea seed meal showed higher release rate and amount of saponin than chitosan beads. It might be related to more porosity and large surface area after mixing tea seed meal into chitosan lead to more amount of water diffuse into the beads and more amount of saponin was encapsulated, causing high penetration of saponin into the release medium.

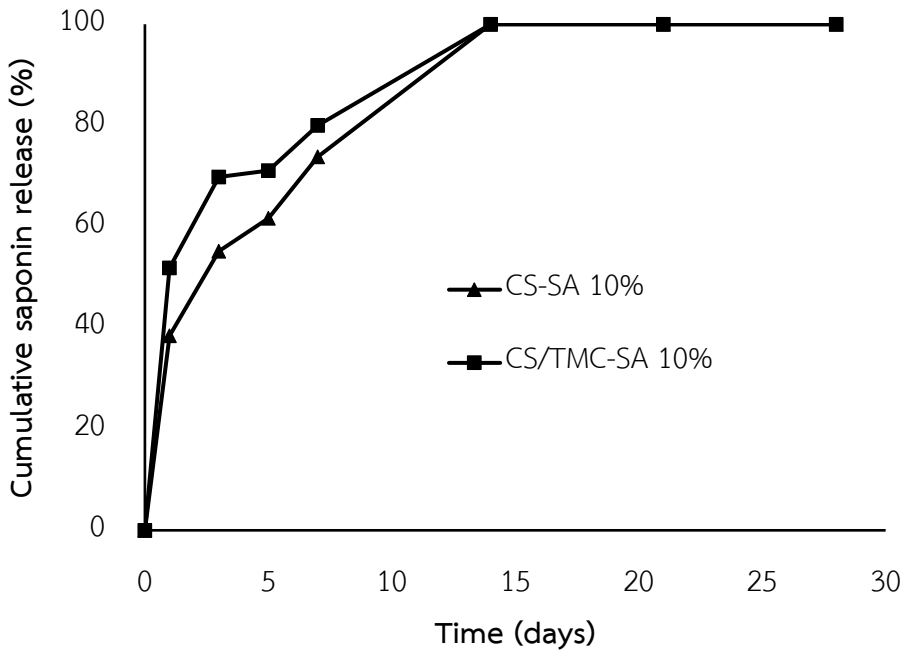


Figure 4.18 Release profiles of 10% (w/w) saponin from chitosan beads and beads of the mixture of chitosan and tea seed meal in deionized water

4.4.3 Effect of calcium chloride solution on saponin release

In this work, the effect of water hardness was studied by using calcium chloride solution ($CaCl_2$) because of ground water contains dissolved minerals especially calcium and magnesium. Different dissolution medium (deionized water and $CaCl_2$ solution) on the release rate of saponin from beads of the mixture of chitosan/tea seed meal containing 10% (w/w) saponin was investigated for 28 days (Figure 4.19). The result showed that the release rate of saponin in $CaCl_2$ solution was lower than in DI water. An initial drug release in $CaCl_2$ solution was about 30% that lower than 2 times compared with deionized water (60%). Low amount of water content in $CacCl_2$ solution decreased saponin mobility so the release rate decreased. The release amount of saponin in two conditions was increased gradually from 1 to 28 days.

4.4.4 Effect of tripolyphosphate crosslinking agent on saponin release

The release behavior of 10% (w/w) saponin loading with various concentrations of TPP crosslinking agent (1, 3 and 5% (w/v)) in DI water was studied. Figure 4.20 shows that an initial drug release of 5% TPP concentration (CS/TSM-TPP5) was about 20% that lower than 2 times compared with 1% and 3% TPP concentrations (CS/TSM-TPP1 and CS/TSM-TPP3). At 1% and 3% (w/v) TPP (CS/TSM-TPP1 and CS/TSM-TPP3) presented in an initial release about 43% and 36% saponin, respectively. There was no significant difference in release behaviors among of 1% to 5% (w/v) TPP concentrations after 1 day. The release rates gradually increased to 14 days and then sustained for 28 days. The saponin release rate decreased at the beginning with increasing crosslink density of TPP due to increase in crosslink density of the beads leading to reduce the hole free volume and mesh size that prevented drug release [85]. Thus, 1% TPP concentration (CS/TSM-TPP1) was the best condition for being the crosslinking agent against golden apple snails due to less amount of TPP used for saving cost and environment.

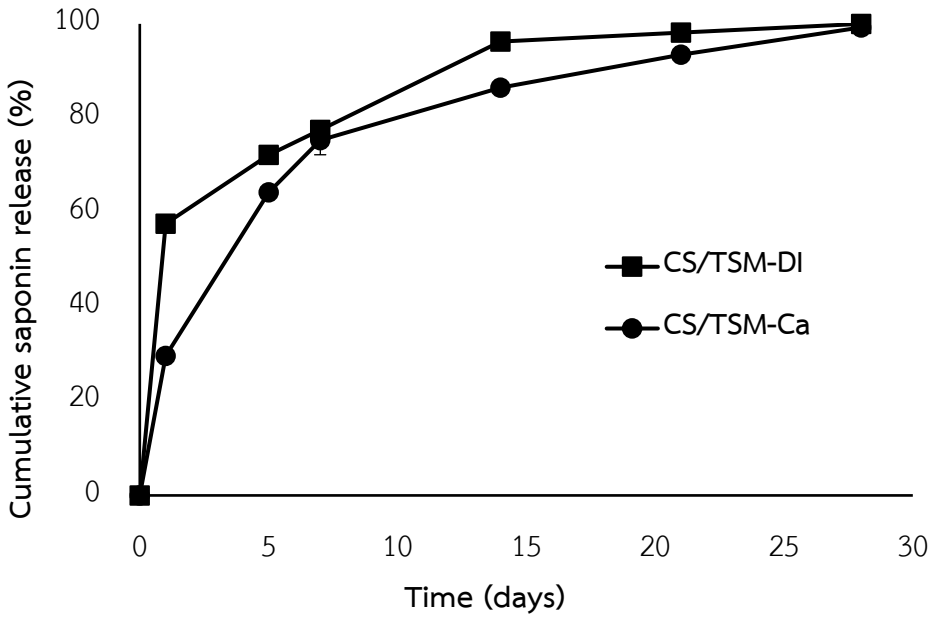


Figure 4.19 Release profiles of 10% saponin from beads of the mixture of chitosan and tea seed meal (TSM) in deionized water and $CaCl_2$ solution

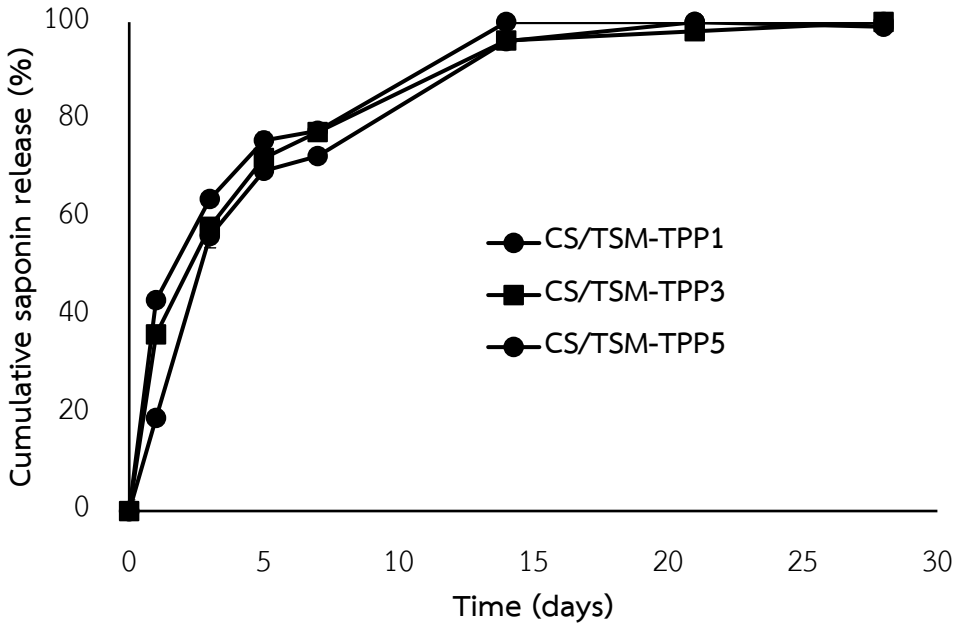


Figure 4.20 *In vitro* release of saponin from beads of the mixture of chitosan and tea seed meal (TSM) in deionized water at various TPP concentrations (1-5%)

4.4.5 Effect of tripolyphosphate crosslinking agent on swelling

The swelling behaviors of the beads of the mixture chitosan and tea seed meal containing 10% (w/w) saponin crosslinked with various TPP concentrations (1, 3 and 5% w/v) were studied by immersing them in deionized water and calcium chloride ($CaCl_2$) solution for 28 days, as shown in Figure 4.21-4.22.

Figures 4.21 and 4.22 show the swelling curves of the beads immersed in deionized water and $CaCl_2$, respectively. The swelling ratio of swollen beads was 1 and 2 times greater than dried beads in both of $CaCl_2$ solution and DI water. There was no significant difference in swelling when the beads were crosslinked with various TPP concentrations in both dissolution medium. However, those beads crosslinked with higher TPP concentration, the swelling ratio tend to lower in comparison to lower concentration. In both dissolution medium showed that the formulation of CS/TSM-TPP5 had the minimum value of the swelling ratio due to high crosslinking agent concentration increased in the gel strength, causing the solute permeability decreased through the beads [66]. The water uptake was fast and reached the maximal value within 7 h. The beads in DI water were fully swollen and the weight is slightly decreased. It might be penetration of saponin into the release medium. [86]. The swelling ratios of all formulation in $CaCl_2$ solution reached the maximal value after 7 h, gradually decreased to 3 days and after that the swelling remained constant to 28 days.

The swelling behavior of chitosan/tea seed meal beads depended on the dissolution medium. It was observed that the swelling of $CaCl_2$ solution was lower than in DI water and reached an equilibrium around 1 day. It may be ascribed to increase in ionic strength of $CaCl_2$ solution reduced the osmotic pressure that cause of solvent diffusion decreased [87].

In summary chitosan beads were prepared by the combination of the *ionotropic* gelation and neutralization method. From preliminary parameters such as concentration of chitosan solution, TPP concentration, NaOH concentration, ethanol concentration, tea seed meal, saponin contents, effect of TPP concentration on the

swelling and release behavior and dissolution medium were examined to obtain an optimum condition for bead preparation of chitosan containing saponin in order to use against the golden apple snails. The results showed that beads containing the mixture of chitosan and tea seed meal is the best condition and obtained at mixed solutions of 2% (w/v) chitosan, 1% (w/v) TPP, 10% (w/v) NaOH, 50% (v/v) EtOH, mass ratio of chitosan and tea seed meal (1:3), and 10% (w/w) saponin loading.

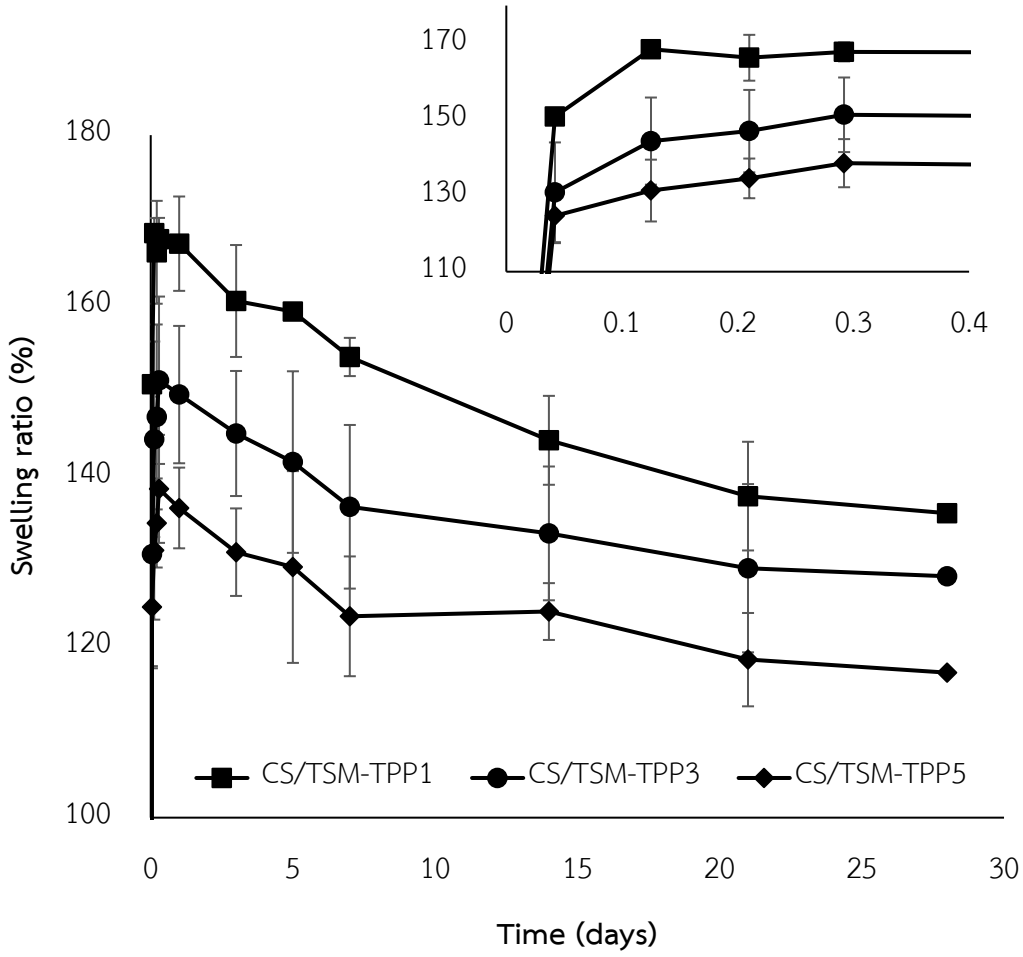


Figure 4.21 Swelling ratios of beads of the mixture of chitosan and tea seed meal crosslinked with various TPP concentration immersed in deionized water. (CS/TSM-TPP1: TPP concentration of 1% (w/v), CS/TSM-TPP3: TPP concentration of 3% (w/v), CS/TSM-TPP5: TPP concentration of 5% (w/v))

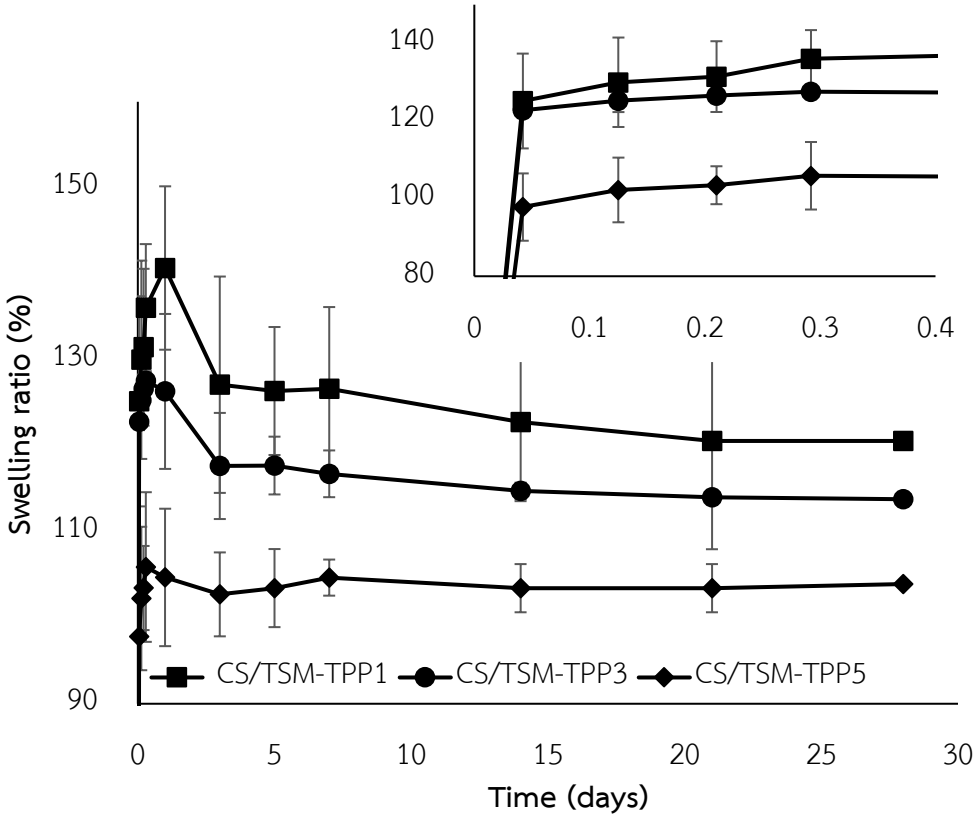


Figure 4.22 Swelling ratio of beads of the mixture of chitosan and tea seed meal (TSM) crosslinked with various TPP concentration immersed in $CaCl_2$. (CS/TSM-TPP1: TPP concentration of 1% (w/v), CS/TSM-TPP3: TPP concentration of 3% w/v, CS/TSM-TPP5: TPP concentration of 5% (w/v))

4.5 Characterizations

4.5.1. Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR spectra were recorded to confirm the saponin in chitosan and to understand the interaction of saponin and chitosan beads as shown in Figure 4.23.

In Figure 4.23a, the broad band positioned at 3320 cm^{-1} attributed to amine group of chitosan (-NH_2 stretching). The peak at 2898 cm^{-1} corresponded to alkyl groups of chitosan (-CH_2 stretching). The vibration of amide groups (C=O strectching and –NH_2 bending) were observed at 1649 and 1585 cm^{-1} respectively. The skeletal vibration of saccharides (C-O stretching) observed at 893 and 1059 cm^{-1} [88, 89]. The FTIR spectrum of saponin was shown in Figure 4.23b: the strong –OH stretching vibration and antisymmetric –CH stretching vibration of CH_2 or CH_3 groups were ascertained at 3317 and 2927 cm^{-1} respectively, evidences the saccharide units (sugar) in saponin. The benzene ring skeleton of aglycone was confirmed by the stretching vibrations of CH_3, C=C and C=O observed at 1376, 1588 and 1713 cm^{-1} respectively. The glycosidic linkage was confirmed from the C-O stretching vibration at 1036 cm^{-1} [90, 91]. The IR spectra of saponin-loaded chitosan beads with variable saponin concentration were recorded and shown in the Figure 4.23c to 4.23g. The IR spectrum of saponin loaded beads of the mixture of chitosan and tea seed meal (TSM) shows in Figure 4.23h. The presence of saponin in the chitosan beads was confirmed by the stretching vibrations of C=C (1580-1590 cm^{-1}) and and the broadening of hydroxyl group also evidences the intermolecular hydrogen bonding of chitosan and saponin [92, 93].

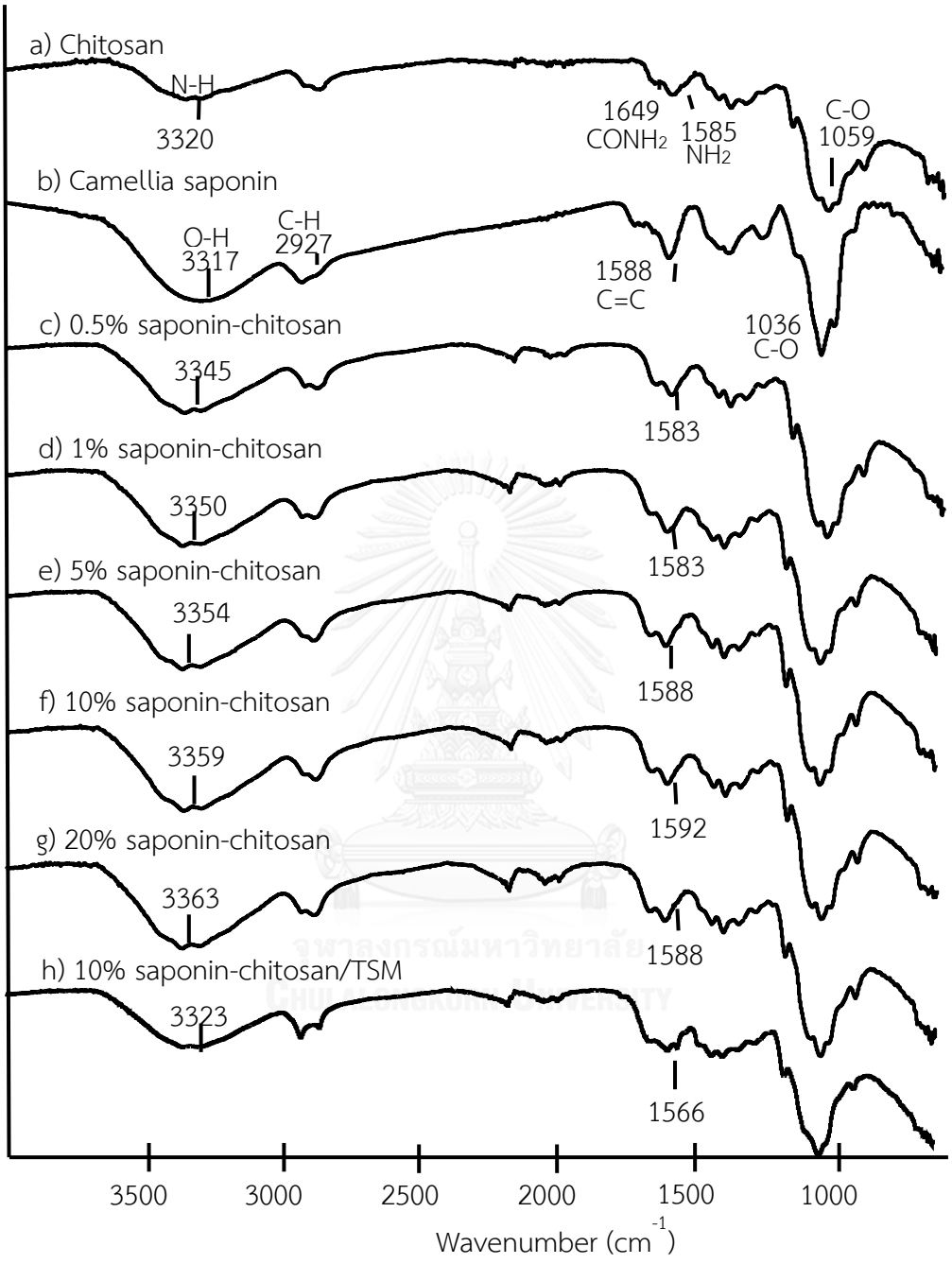


Figure 4.23 Representative FTIR spectra of saponin-containing chitosan beads with different saponin concentration (w/w), (a) pure chitosan, (b) *C. oleifera* saponin, (c) 0.5% saponin-chitosan, (d) 1% saponin-chitosan, (e) 5% saponin-chitosan, (f) 10% saponin-chitosan, (g) 20% saponin-chitosan beads and (h) 10% saponin-chitosan/tea seed meal

4.5.2. Bead size of chitosan and beads of the mixture chitosan and tea seed meal

Thirty wet and dry beads were determined with three-dimensional position of beads for the average sizes (in mm) by using a Vernier caliper for wet beads and SemAFore software for dried beads. Each measurement was repeated three times. The result showed that average sizes of the prepared wet and dried of chitosan beads were 4.2 ± 0.1 mm and 1.4 ± 0.1 mm, respectively, as shown in Figure 4.24A-A*. The average sizes of the prepared wet and dried of chitosan/tea seed meal beads were 3.9 ± 0.2 mm and 2.4 ± 0.1 mm, respectively, as shown in Figure 4.24B-B*. They suggested that the bead size was affected by tea seed meal.

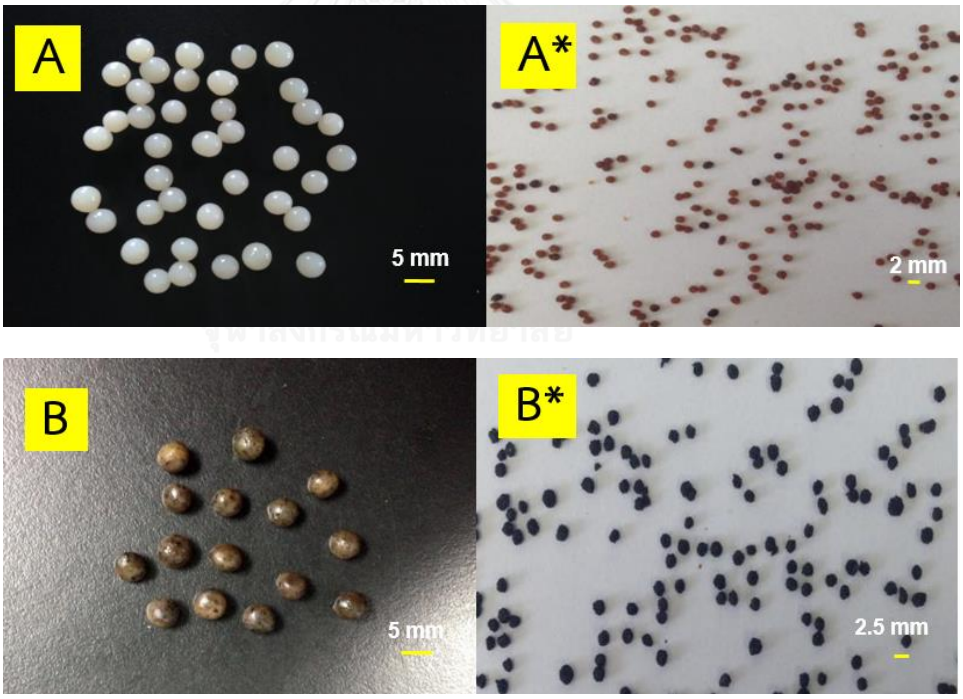


Figure 4.24 Images of saponin encapsulation in chitosan beads and beads of the mixture of chitosan and tea seed meal; (A) wet beads of CS-saponin 10% with its dried beads (A*) and (B) wet beads of CS/TSM-saponin 10% with its dried beads (B*)

4.5.3. Morphology of the beads

The spherical, and cross-sectional morphology of chitosan beads and beads of the mixture of chitosan/tea seed meal was investigated by SEM. The samples were prepared by sticking them on double carbon tap to stick on the stub. SEM operated at 15 kV to observe the surface morphology of the beads. SEM images of spherical, and a cross-sectional chitosan beads and beads of the mixture of chitosan and tea seed meal are shown in Figures 4.25, 4.26, 4.27 and 4.28, respectively.

Figures 4.25A-F, 4.26A-F and 4.26A*-F* show the spherical, cross-sectional and its magnification morphologies of chitosan beads without saponin compared with chitosan beads containing various saponin concentrations from 0.5% to 20% (w/w). The results exhibited that the beads had spherical structure and rough surface when the saponin content increased due to dissolved saponin moved to the outside with the water and stored on the bead surface. However, the internal structures were smooth and dens because of the homogenous saponin-chitosan dispersion was uniform [94]. This might be expected to the fact that saponin was successfully entrapped and embedded in a dense chitosan bead.

Figures 4.27A, 4.27A* and 4.27a show the spherical, cross-sectional and its magnification morphologies of the mixture of chitosan and tea seed meal bead containing 10% saponin. The bead had more rough, porous surface and larger than those chitosan beads without tea seed meal because of the hardness and large size of tea seed meal as the filler.

Figure 4.28 shows effects of various TPP concentrations from 1% to 5% (w/v) on the morphologies of chitosan beads (A-C) and the beads of the mixture of chitosan and tea seed meal (D-F). The beads prepared with 5% TPP concentration were spherical in shape and had a less porosity when compared with 1% and 3% due to the increasing strong crosslink of electrostatic interactions between positive charge of chitosan and negative charge of TPP leading to decrease the size of interconnecting pores [95]. The results exhibited that TPP concentration had effect on bead surface and formation.

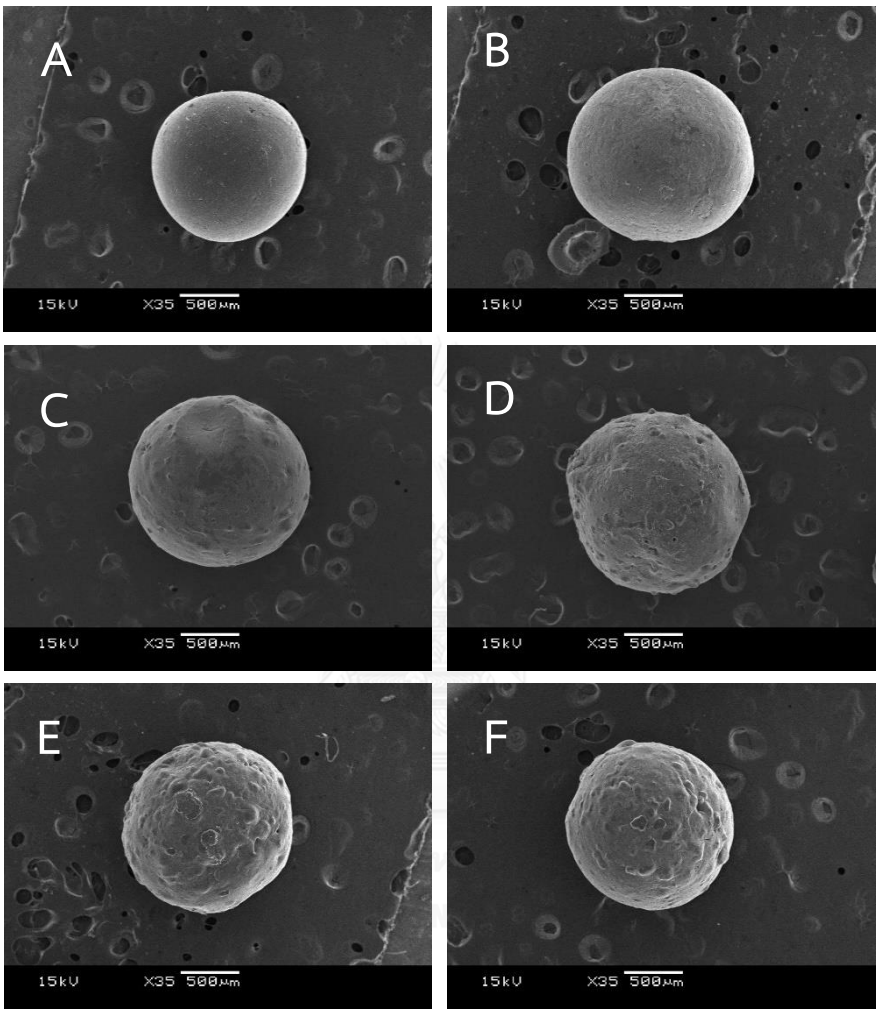


Figure 4.25 Representative SEM images of spherical beads; (A) chitosan (B) 0.5% saponin-chitosan, (C) 1.0% saponin-chitosan, (D) 5.0% saponin-chitosan, (E) 10.0% saponin-chitosan and (F) 20.0% saponin-chitosan beads

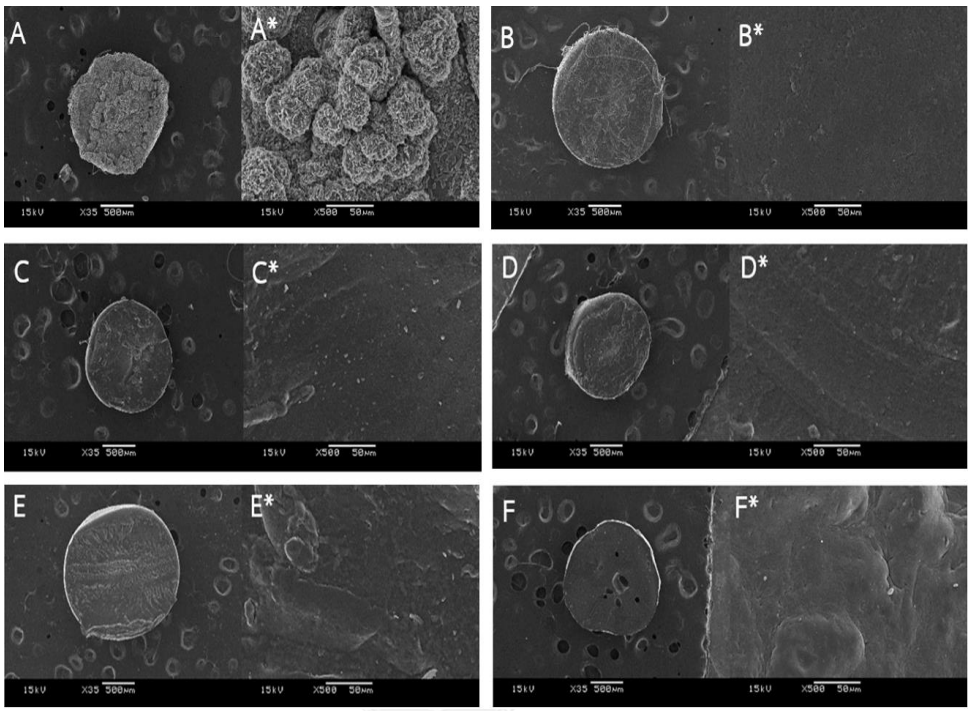


Figure 4. 26 Representative cross-sectional SEM images; (A) chitosan with its magnification (A*), (B) 0.5% saponin-chitosan with its magnification (B*), (C) 1.0% saponin-chitosan with its magnification (C*), (D) 5.0% saponin-chitosan with its magnification (D*), (E) 10.0% saponin-chitosan with its magnification (D*) and (F) 20.0% saponin-chitosan with its magnification (F*)

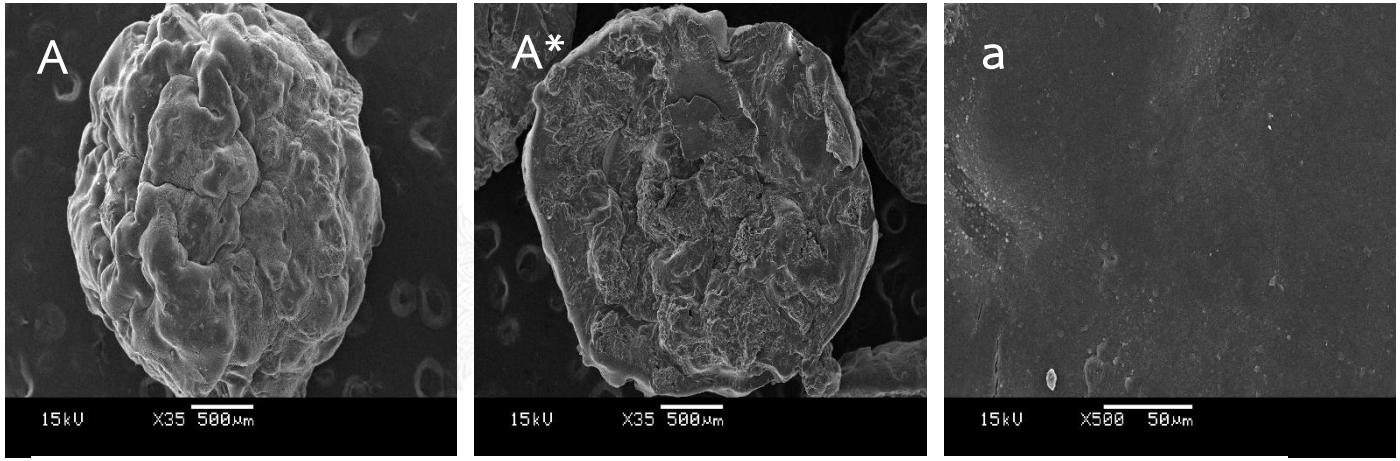


Figure 4.27 Representative spherical and cross-sectional SEM images; (A) bead of the mixture of chitosan and tea seed meal containing 10% saponin with its cross section (A*) and its magnification (a)

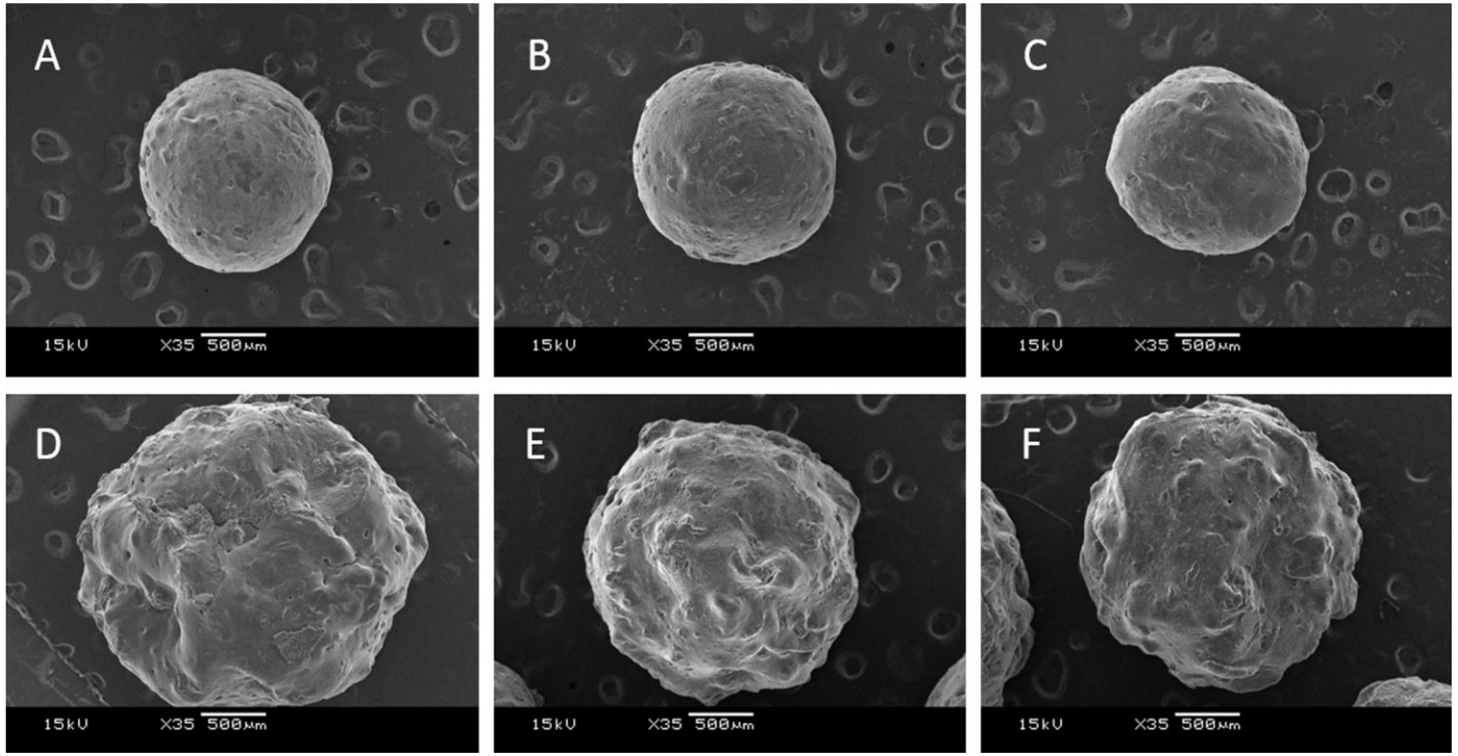


Figure 4.28 SEM micrographs. Surface morphology of chitosan and chitosan/tea seed meal beads prepared by adding different % (w/v) TPP (A) 1%TPP-chitosan; (B) 3%TPP- chitosan; and (C) 5%TPP- chitosan; (D) 1%TPP-chitosan/tea seed meal; (E) 3%TPP- chitosan/tea seed meal; and (F) 5%TPP- chitosan/tea seed meal beads.

CHAPTER V
CONCLUSION AND SUGGESTION

Conclusion

In this work, the chitosan beads were successfully prepared by the combination of the *ionotropic* gelation and neutralization methods in order to controlled release of saponin against the golden apple snail (*Pomacea canaliculata*). First, the effects of various parameters of chitosan bead preparation on the formation and size of chitosan beads were evaluated. From preliminary parameters such as the chitosan concentration solution, TPP concentration, NaOH concentration, ethanol concentration, tea seed meal and saponin contents were examined to obtain an optimum condition for chitosan bead preparation. The results showed that the optimum condition of spherical chitosan beads with regular in size and shape were obtained at mixed solutions of 2% (w/v) chitosan, 1% (w/v) TPP, 10% (w/v) NaOH and 50% (v/v) EtOH.

Camellia oleifera saponin was successfully loaded into chitosan beads by the combination of the *ionotropic* gelation and neutralization methods. One percent of saponin loaded chitosan showed the highest encapsulation efficiency around 64.28 ± 7.43 % and the saponin loading ratio of 0.5, 5, 10 and 20% saponin loaded chitosan were 59.89 ± 6.36, 54.09 ± 3.86, 52.30 ± 2.99 and 9.92 ± 0.57%, respectively. However, chitosan beads loading 10% saponin is chosen as an optimum condition because of more amount of saponin release to prolong release and promote its efficacy. The morphologies of the chitosan beads with and without saponin were investigated by SEM. Moreover, FTIR was recorded to confirm the saponin in chitosan beads

Tea seed meal (TSM) was mixed into the chitosan solution as a filler in order to size maximization of chitosan beads for more amount of saponin released, sustained and prolonged release of saponin. The results exhibited that the beads of the mixture

of chitosan and tea seed meal showed higher release rate and amount of saponin than pure chitosan beads.

The release profiles of saponin encapsulation in chitosan could prolong and sustain longer than the pure saponin. On the other hand, the effect of TPP as a crosslinking agent on the swelling and release profile were studied. There were no significant differences on release and swelling behaviors among of 1% to 5% (w/v) TPP concentrations.

Moreover, the biological effect of saponins on *O. sariva* L rice growth and mortality of *Tilapia* fish and *P. canaliculata* snails had been studied. The minimum of LC_{50} value is 7.45 ppm at 96 h, suggesting that this exposure time is optimal condition against the golden apple snails due to less dose of saponin used for cost saving and environment. A safe concentration for Tilapia fish, we suggested that using the minimum dose of saponin less than 2.13 ppm. Saponin had no effect on rice plants. It can be concluded that the mixture of chitosan and tea seed meal beads containing saponin have a potential to develop as molluscicide against golden apple snails with friendly environment.

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APPENDIX

Appendix A

Evaluation activity of *Camellia oleifera* saponin

a) b)

Figure 1A Characteristic of a) alive golden apple snail b) dead golden apple snail

Figure 2A Tilapia fish were kept in a covered-net plastic box

Table 1A The fingerlings of Nile tilapia *O. niloticus* were left after 24 h

Tank	Concentration (ppm)				
	Control	1	3	5	10
A					
B					
C					

Appendix B

Calibration curve of saponin

The concentration versus absorbance of saponin determined by UV/Vis spectrophotometer as the same conditions described in Chapter III is presented in Table 1B. The plot of calibration curve of saponin is illustrated in Figure 1B

Table 1B Absorbance of various saponin concentrations determined by UV/Vis spectrophotometry at 265 nm in deionized water

Concentration (ppm)	Absorbance at 265 nm
100	0.1450
200	0.3867
300	0.4754
400	0.6329
500	0.7859

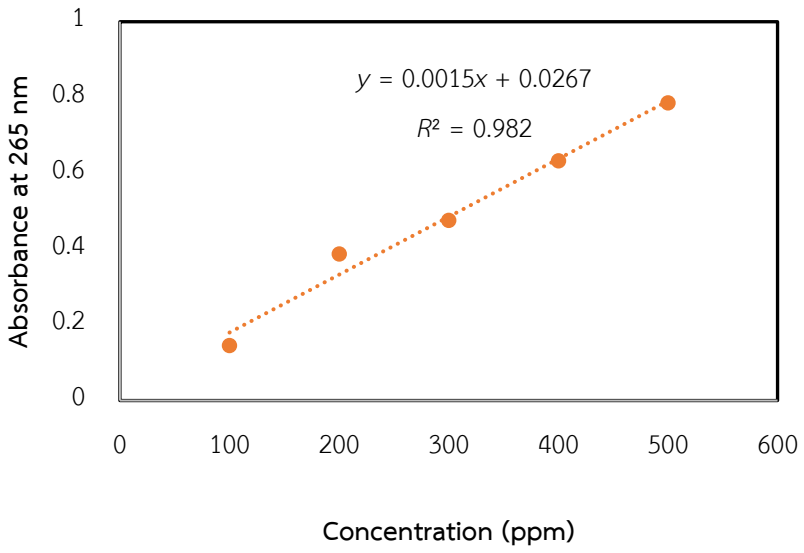

Figure 1B Standard calibration curve of *C. oleifera* saponin

Table 2B Absorbance of various saponin concentrations determined by UV/Vis spectrophotometry at 265 nm in calcium chloride solution

Concentration (ppm)	Absorbance at 265 nm
100	0.2324
200	0.3652
300	0.5172
400	0.6750
500	0.8173

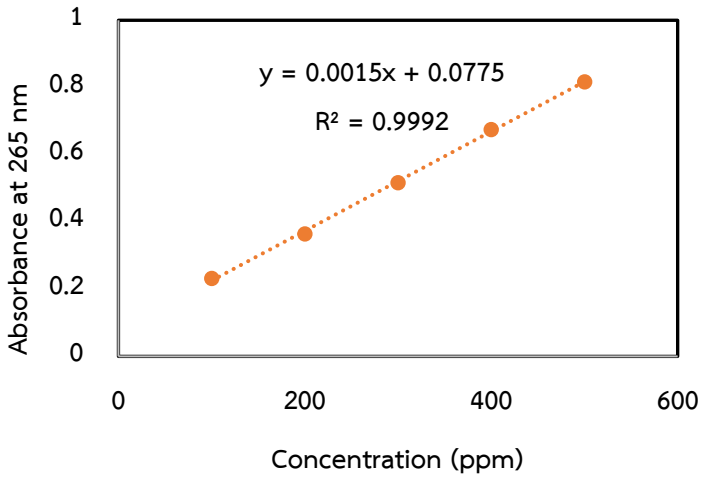

Figure 2B Standard calibration curve of *C. oleifera* saponin

Appendix C

Encapsulation and Cumulative Drug Release

Table 1C Cumulative of pure saponin in deionized water before correcting by the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
0.04	98.443	100.110	99.443	99.332	0.839
0.13	62.477	61.443	61.777	61.899	0.527
0.21	70.660	72.660	76.660	73.327	3.055
1	41.150	41.110	41.213	41.158	0.052
3	38.660	37.443	37.777	37.960	0.629
5	18.669	18.907	19.933	19.170	0.672
7	17.694	18.762	17.988	18.148	0.551
14	8.400	8.656	9.444	8.833	0.544
21	5.797	6.559	6.647	6.334	0.468
28	3.590	3.433	3.793	3.606	0.181

Table 2C Cumulative of 1% saponin chitosan beads in deionized water before correcting by the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
0.04	26.938	27.011	25.325	26.425	0.953
0.13	27.613	28.111	28.625	28.116	0.506
0.21	18.577	17.110	17.844	17.844	0.733
1	21.166	19.311	20.044	20.174	0.935
3	38.660	37.443	37.777	37.960	0.629
5	18.669	18.907	19.933	19.170	0.672
7	17.694	18.762	17.988	18.148	0.551
14	8.400	8.656	9.444	8.833	0.544
21	5.797	6.559	6.647	6.334	0.468
28	3.590	3.433	3.793	3.606	0.181

Table 3C Cumulative of 10% saponin chitosan beads in deionized water before correcting by the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	22.871	24.272	29.874	25.672	3.706
3	20.070	21.471	22.871	21.471	1.401
5	10.414	12.915	12.835	12.055	1.421
7	11.938	12.556	11.814	12.102	0.397
14	14.867	16.102	13.631	14.867	1.236
21	12.316	17.752	15.888	15.319	2.762
28	7.157	8.221	9.202	8.193	1.023

Table 4C Cumulative of pure saponin in deionized water corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
0.04	98.990	100.660	99.990	99.880	0.840
0.13	76.420	76.990	76.720	76.710	0.285
0.21	53.540	53.690	53.540	53.590	0.087
1	50.260	50.230	50.190	50.227	0.035
3	58.920	59.060	59.160	59.047	0.121
5	66.270	66.300	66.360	66.310	0.046
7	71.360	71.700	71.660	71.573	0.186
14	89.570	90.100	90.090	89.920	0.303
21	97.300	97.330	97.330	97.320	0.017
28	99.890	99.950	99.910	99.917	0.031

Table 5C Cumulative of 1% saponin in deionized water corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
0.04	27.480	27.560	25.870	26.970	0.953
0.13	20.340	18.880	19.610	19.610	0.730
0.21	30.450	30.950	31.460	30.953	0.505
1	33.970	32.110	32.850	32.977	0.936
3	52.740	50.810	53.010	52.187	1.200
5	55.040	56.350	56.310	55.900	0.745
7	73.420	73.250	71.480	72.717	1.074
14	97.700	98.540	99.120	98.453	0.714
21	106.590	106.680	106.350	106.540	0.171
28	104.230	106.730	105.490	105.483	1.250

Table 6C Cumulative of 10% saponin in deionized water corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	35.670	37.070	42.680	38.473	3.710
3	53.770	55.170	56.570	55.170	1.400
5	60.010	62.510	62.430	61.650	1.421
7	73.610	74.230	73.490	73.777	0.397
14	100.180	101.410	98.940	100.177	1.235
21	106.690	112.120	110.260	109.690	2.760
28	105.000	106.060	107.040	106.033	1.020

Table 7C Cumulative of 10% saponin loaded chitosan/tea seed meal beads in deionized water corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	51.610	51.840	52.100	51.850	0.245
3	69.450	69.690	70.390	69.843	0.488
5	70.830	71.120	71.290	71.080	0.233
7	79.720	80.410	79.930	80.020	0.354
14	112.980	113.290	113.920	113.397	0.479
21	112.040	111.680	112.540	112.087	0.432
28	107.280	108.000	107.550	107.610	0.364

Table 8C Effect of deionized water on cumulative of 10% saponin loaded chitosan/tea seed meal corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	36.12	36.02	36.14	36.10	0.06
3	57.22	58.47	58.61	58.10	0.76
5	71.37	72.48	72.77	72.21	0.74
7	77.28	77.54	77.63	77.48	0.18
14	94.53	97.06	97.12	96.24	1.48
21	98.29	98.18	98.01	98.16	0.14
28	100.81	99.93	100.61	100.45	0.46

Table 9C Effect of calcium chloride solution on cumulative of 10% saponin loaded chitosan/tea seed meal corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	28.879	29.874	29.949	29.567	0.597
3	54.266	56.522	56.569	55.786	1.316
5	63.498	64.555	64.724	64.259	0.665
7	71.769	77.059	77.116	75.315	3.071
14	85.681	86.373	87.386	86.480	0.858
21	93.949	93.085	93.310	93.448	0.449
28	99.284	98.852	99.621	99.252	0.386

Table 10C Effect of 1% TPP crosslinking concentration on cumulative of 10% saponin loaded chitosan/tea seed meal corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	45.725	42.248	41.488	43.154	2.259
3	63.406	64.076	64.012	63.831	0.370
5	75.577	75.917	75.975	75.823	0.215
7	79.659	76.707	76.850	77.739	1.665
14	99.462	100.453	100.604	100.173	0.620
21	100.935	101.001	100.867	100.934	0.067
28	99.003	98.441	99.754	99.066	0.659

Table 11C Effect of 3% TPP crosslinking concentration on cumulative of 10% saponin loaded chitosan/tea seed meal corrected with the degradation factor

Time (days)	Release	Release	Release	AVG	SD
0	0.000	0.000	0.000	0.000	0.000
1	36.121	36.023	36.144	36.096	0.064
3	57.222	58.470	58.608	58.100	0.764
5	71.374	72.479	72.771	72.208	0.737
7	77.276	77.541	77.631	77.483	0.185
14	94.535	97.064	97.115	96.238	1.475
21	98.286	98.180	98.007	98.158	0.141
28	100.808	99.933	100.612	100.451	0.459

Table 12C Effect of 5% TPP crosslinking concentration on cumulative of 10% saponin loaded chitosan/tea seed meal corrected with the degradation factor

Time (days)	Release	Release	Release	Release	AVG
0	0.000	0.000	0.000	0.000	0.000
1	27.733	27.726	27.863	27.774	0.077
3	57.920	55.151	55.529	56.200	1.501
5	72.292	67.921	68.496	69.570	2.375
7	73.640	72.616	71.463	72.573	1.089
14	96.256	96.010	96.044	96.104	0.134
21	103.376	102.779	102.963	103.040	0.306
28	101.463	100.462	99.340	100.422	1.062

Appendix D

The weight and swelling ratio of beads

Table 1D The weight of chitosan beads in deionized water

Time	Weight of beads (mg)					
	CS/TSM-TPP1		CS/TSM-TPP3		CS/TSM-TPP5	
	1	2	1	2	1	2
0 h	0.0	0.0	0.0	0.0	0.0	0.0
1 h	50.1	50.2	48.2	48.3	47.0	43.8
3 hrs	53.1	54.3	52.5	49.6	48.7	44.8
5 hrs	52.7	53.8	52.8	50.4	49.5	45.3
7 hrs	53.6	53.5	53.6	51.4	49.9	46.5
1 day	53.0	53.9	53.1	51.2	49.6	45.9
3 days	52.1	52.1	51.9	50.5	48.3	45.1
5 days	52.0	51.7	51.1	49.9	48.0	44.7
7 days	50.1	51.5	50.5	48.3	47.7	42.7
14 days	47.8	49.9	49.7	47.8	47.2	43.4
21 days	46.1	49.0	48.6	47.2	45.5	42.8
28 days	45.5	48.8	48.7	46.7	45.5	42.2

Table 2D The swelling ratio of chitosan/tea seed meal beads in deionized water

Time	Weight of beads (mg)					
	CS/TSM-TPP1		CS/TSM-TPP3		CS/TSM-TPP5	
	1	2	1	2	1	2
0 h	0.0	0.0	0.0	0.0	0.0	0.0
1 h	154.3	147.3	132.9	128.9	128.2	121.2
3 hrs	169.5	167.5	153.6	135.1	136.4	126.3
5 hrs	167.5	165.0	155.1	138.9	140.3	128.8
7 hrs	172.1	163.5	158.9	143.6	142.2	134.8
1 day	169.0	165.5	156.5	142.7	140.8	131.8
3 days	164.0	156.7	150.7	139.3	134.5	127.8
5 days	164.0	154.7	146.9	136.5	133.0	125.8
7 days	154.3	153.7	144.4	128.9	131.6	115.7
14 days	142.6	145.8	140.1	126.5	129.1	119.2
21 days	134.0	141.1	134.8	123.7	120.9	116.2
28 days	131.2	140.2	135.3	121.3	120.9	113.1

Table 3D The weight of chitosan beads in calcium chloride solution

Time	Weight of beads (mg)					
	CS/TSM-TPP1		CS/TSM-TPP3		CS/TSM-TPP5	
	1	2	1	2	1	2
0 h	0.0	0.0	0.0	0.0	0.0	0.0
1 h	47.8	45.2	43.3	45.6	41.7	43.8
3 hrs	49.4	45.6	43.8	46.1	42.3	44.8
5 hrs	49.6	46.0	44.6	45.8	42.5	45.3
7 hrs	50.2	47.3	44.4	46.4	42.5	46.5
1 day	50.9	48.5	44.2	46.1	42.8	45.9
3 days	48.4	45.4	41.2	45.7	42.3	45.1
5 days	48.7	44.8	41.6	45.3	42.0	44.7
7 days	48.0	45.6	41.8	44.7	42.2	42.7
14 days	47.5	44.5	41.5	44.2	41.6	43.4
21 days	47.0	44.1	41.6	43.8	41.7	42.8
28 days	47.5	43.6	41.6	43.7	41.8	42.2

Table 4D The swelling of chitosan/tea seed meal beads in calcium chloride solution

Time	Weight of beads (mg)					
	CS/TSM-TPP1		CS/TSM-TPP3		CS/TSM-TPP5	
	1	2	1	2	1	2
0 h	0.0	0.0	0.0	0.0	0.0	0.0
1 h	130.9	119.4	122.1	123.5	105.4	90.2
3 hrs	138.6	121.4	124.6	126.0	108.4	96.1
5 hrs	139.6	123.3	128.7	124.5	109.4	97.6
7 hrs	142.5	129.6	127.7	127.5	109.4	102.4
1 day	145.9	135.4	126.7	126.0	110.8	98.5
3 days	133.8	120.4	111.3	124.0	108.4	97.1
5 days	135.3	117.5	113.3	122.1	106.9	100.0
7 days	131.9	121.4	114.4	119.1	107.9	101.5
14 days	129.5	116.0	112.8	116.7	104.9	102.0
21 days	127.1	114.1	133.3	114.7	105.4	101.5
28 days	129.5	111.7	113.3	114.2	105.9	102.0

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