CHAPTER III

EXPERIMENTAL

Materials

Rosmarinic acid microparticles and freeze dried patch

Alginic acid sodium salt, from brown algae (Lot no. BCBG6637V, Sigma, Germany)

Calcium chloride (B/no. 1201424, Ajax Finechem, Australia, 2011)

Deionized Water

Mannitol (B/no. AF509221, Ajax Finechem, Australia, 2011)

Polysaccharide Gel Extract from Durian Fruit-Hulls (Lot no. CP372, pH2.43, 09-06-55)

Standard rosmarinic acid 96% (Lot no. BCBF9162V, Aldrich, UK)

Thunbergia laurifolia callus culture

T. laurifolia leaves

Agar

Modified basal medium w/ Gamborg vitamins (Lot no. 11M0404016E, Phytotechnoloty Laboratories, USA)

alpha-Naphthaleneacetic acid (NAA) (Lot no. 1405095, Fluka analytical, China)

6-Benzyl Aminopurine (BA) (Lot no. 11C080003, Phytotechnoloty Laboratories, USA)

Sucrose

HCL

Sodium hydroxide

Chemicals

95% Ethanol (Lot no. 2C170613, 2013)

Trisodium citrate (B/no. 0911213, Ajax Finechem, Australia, 2011)

Sodium hydroxide (B/no. F2C273, Ajax Finechem, Australia, 2011)

Dipotassium hydrogen orthophosphate (B/no. F2A246, Ajax Finechem, Australia, 2011)

Methanol (Lot no. M9QG1H, Burdick&Jackson, Korea)

Methanol (Lot no. L4AG1H Burdick&Jackson, Korea)

Glacial acetic acid (Lot no. K34739317521 VMR, International limited, England)

Equipments

Analytical balance (Model XP205, Mettler Toledo, Switzerland)

Analytical balance (Model PB3002, Mettler Toledo, Switzerland)

Analytical balance (Model A2005, Sartorius analytic, Germany)

Autoclave (Model SS320, Tomy)

Differential Scanning Calorimetry (DSC) (DSC822e, Mettler Toledo, Switzerland)

Franz diffusion cell

Freezer (Model MF-U14E, Mitsubishi, Thailand)

Freeze dryer (Model ST4B, LYOLAB, USA)

Fourier Transform Infrared Spectrometer (Model Nicolet iS10, Thermoscientific, USA)

Laminar air flow (Model HLF120, German Sciences)

Microwave oven

HPLC (Model RF -10AXL-Shimadzu, Shimadzu, Japan)

Hot air oven (Memmert, Germany)

Magnetic stirrer (Model MSH-300, Biosan., Ltd.)

Particle size analyzer (Hydro 2000MU, Malvern Mastersizer 2000, UK)

pH meter (Model FE201FG2, Mettler Toledo, Switzerland)

Rotary evaporator (Model R-200, BUCHI Rotavapor, BUCHI, Switzerland)

Scanning Electron Microscope (Model JSM-5410 LV, JEOL, Japan)

Sieve mesh No. 30

Sonicator (Model 570H, Elmasonic, Germany)

Spray dryer (Model B290 BUCHI Mini spray dryer, BUCHI, Switzerland)

X-ray powder diffractometer (Model MiniFlex II, RIGAKU, Japan)

Miscellaneous

Aluminium foil

Beaker

Cellulose acetate membrane filter (Lot no. 0610111060903263, Sartorius, Biolab Product)

Centrifuge tube (Corning, Mexico)

Cylinder

HPLC column (K58046, 4.6X25, 5C-18-AR-II, Cosmosil® C18, Japan)

Magnetic bar

Nylon membrane filter pore size 0.2 micron (Lot no. 110217002, , MS Nylon)

Nylon syringe filter pore size 0.2 micron 12 mm (B/no. 1108122B01-01, Filtrex, Singapore)

Parafilm®

Petri dish

Plant tissue culture bottle

Methods

1. Preliminary of rosmarinic acid encapsulation

1.1 HPLC analysis and method validation of rosmarinic acid

High Pressure Liquid Chromatography (HPLC) was used to analyze the rosmarinic amount for the experiment, using Cosmosil®C18 column. The condition of HPLC analysis was referred to Suwanchaikasem method [15]. The mobile phase consisted of methanol and water at the ratio 35:65 with 0.1% v/v of acetic acid. The flow rate of mobile phase was controlled to be at 1 ml/min and at 35°C, 10 µl of the filtered solution was injected into HPLC equipped with UV detector at the wavelength of 320 nm. Analytical method validation was carried out for accuracy, precision, specificity and linearity.

For the sample preparation, standard rosmarinic acid was weighed and spike into methanol in volumetric flask for the concentrations at 5, 25, 50, 100, 150, 200 µg/ml which polymer DG, AG and mannitol at the ratio of 1:1:1 were added into each

volumetric flask for the final concentrations at 20, 100, 200, 400, 600, 800 µg/ml. Calibration curves were constructed by plotting between the peak area and concentration added of standard samples and statistical analysis was performed. To establish the within-day and between-day accuracy and precision of the method, triplicate of standard rosmarinic acid solutions at three different concentrations (25, 50 and 100 µg /mL) were assayed on one day and three separate days. To determine limit of detection and limit of determination, the very small amount of rosmarinic acid was spiked in the solution as 2 µg/ml and repeated by ten times. The limit of quantification was calculated from 10 times of standard deviation of the concentration found from spiked small amount of rosmarinic acid. The limit of detection was calculated from 3 times of standard deviation of the concentration found from spiked small amount of rosmarinic acid.

1.2 Stress test of rosmarinic acid

1.2.1 Photostability

For photostability tesing, rosmarinic acid was exposed to UV light with GE black light clue F20T12/BLB emitted at 368 nm and OSRAM LUMILUX cool white L18W/840 in photostability box, Samples were collected by 1 mg and dilute to final concentration at 100 µg/ml for analyzing the remaining of rosmarinic acid amount and observed for degradation product peak at time interval of 1, 3, 6, 9, 18 hours respectively.

1.2.2 Temperature

For temperature stability tesing, rosmarinic acid was exposed to heat at 60°C in hot air oven. Samples were collected by 1 mg and dilute to final concentration at 100 µg/ml and analyzed at time interval of 1, 3, 6, 9, 18 hours, respectively.

1.2.3 Humidity

For humidity stability, rosmarinic acid was kept in desiccator with saturated sodium chloride to control humidity at 75%RH. The samples were collected by 1 mg and dilute to final concentration at 100 µg/ml and analyzed at time interval of 1, 3, 6, 9, 18 hours, respectively.

1.2.4 Oxidation

For humidity stability, rosmarinic acid was added into 1ml of 10%v/v H_2O_2 solution. The samples were collected every hours and diluted to 100 µg/ml for analysis.

For analyzing the remaining of rosmarinic acid amount, Cosmosil® C18 column was used. The mobile phase consisted of methanol and water at the ratio 35:65 with 0.1% v/v of acetic acid. The flow rate of mobile phase was controlled to be at 1 ml/min and at 35°C. 10 µl of the filtered solution was injected into HPLC and equipped with UV detector at the wavelength of 320 nm. The stability testing was investigated until rosmarinic acid was decreased by 20%. In case of rosmarinic acid was not decrease about 20% in the time interval, the experiment was continued to the following days.

1.3 Preparation of encapsulated rosmarinic acid microparticles

The formulations of encapsulated RA microparticles prepared by spray drying and freeze drying process as following table;

Table 4 Processes and formulations of encapsulated RA microparticles

Spray drying			Freeze drying	Polymer concentration and ratio
Blank (without RA)	Non-crosslinked	Crosslinked	Crosslinked	
SNB-1AG	SN-1AG	SC-1AG	FC-1AG	AG 1%
SNB-1DG	SN-1DG	SC-1DG	FC-1DG	DG 1%
SNB-1DGAG1:1	SN-1DGAG1:1	SC-1DGAG1:1	FC-1DGAG1:1	DG+AG 1%, 1:1
SNB-1DGAG2:1	SN-1DGAG2:1	SC-1DGAG2:1	FC-1DGAG2:1	DG+AG 1%, 2:1
SNB-2AG	SN-2AG	SC-2AG	FC-2AG	AG 2%
SNB-2DG	SN-2DG	SC-2DG	FC-2DG	DG 2%
SNB-2DGAG1:1	SN-2DGAG1:1	SC-2DGAG1:1	FC-2DGAG1:1	DG+AG 2%, 1:1
SNB-2DGAG2:1	SN-2DGAG2:1	SC-2DGAG2:1	FC-2DGAG2:1	DG+AG 2%, 2:1

1.3.1 Spray drying process

Polymer solution were prepared by dispersing the polymer 0.3 or 0.6 g in purified water 30 ml to make 1% and 2% polymer concentration respectively and stirred for 12 hours for completely swelling. Noncrosslinked-spray dried microparticles were prepared by adding 75 mg of rosmarinic acid into the polymer solution to make the RA: polymer ratio of 1:4 by weight for the 1% polymer formula and the ratio of 1:8 for the 2% polymer formula respectively. The obtained solution were then vigorously stirred by magnetic stirrer and sprayed into the chamber at the temperature of 130°C and the spray rate of 2 ml/min. The outlet temperature was kept at 70-75 °C. Then crosslinked-spray dried microparticles were prepared by dispersing the obtained noncrosslinked microparticles into 30 ml of 5%w/v calcium chloride solution for 5 min then washed 3 times with methanol and 2 times with purified water and dried by freeze drying with condition as Table 5.

1.3.2 Freeze drying process

Samples of 30 ml of polymer and rosmarinic acid were prepared at the same concentrations and ratios as indicated in the spray drying process and added 0.9 g of mannitol into the solution to make 3%w/v concentration, then added 500 µl of 5%w/v calcium chloride as crosslinking agent. After crosslinking, freeze drying process condition as shown in Table 5 was used to obtain the matrix of RA-loaded freeze dried product and the resulting cake product was sieved by using mesh no.30 to make fine particles.

Table 5 Freeze drying step for encapsulated RA microparticles

Processing	Temperature (°C)	Time (mins)	Vacuum (mT)
Freezing	0	0	600
Freezing	-5	0	600
Freezing	-10	0	600
Freezing	-15	0	600
Freezing	-20	0	600
Extra freezing	-20	60	600
Primary drying	-20	0	600
Primary drying	-20	120	600

Primary drying	-10	0	600
Primary drying	-10	120	600
Primary drying	0	0	600
Primary drying	0	120	600
Primary drying	10	0	600
Primary drying	10	120	600
Primary drying	20	0	600
Primary drying	20	120	600
Primary drying	30	0	600
Primary drying	30	60	600
Secondary drying	20	60	600

1.4 Characterization of encapsulated rosmarinic acid microparticles

1.4.1 Morphological characterization

1.4.1.1 Scanning electron microscope

Electron photomicrographs of spray dried microparticles and freeze dried particles were taken by using a scanning electron microscope (JEOL model JSM-5410W SEM machine). The samples were placed on aluminium stud and coated under an argon atmosphere with a fine gold layer then the particle morphology of the products were observed at 15 kV.

1.4.1.2 Dynamic Light scattering

Particle size was measured by light scattering technique using Malvern® Mastersizer. Ethanol was used as solvent to avoid polymer swelling. The microparticles were dispersed in ethanol in a 250 ml beaker. The particle size was measured three times and reported as volume-mean diameter D[4,3].

1.4.2 Differential Scanning Calorimetry

Thermal analysis by DSC was carried out to determine the transition energy of the encapsulated samples using Mettler Toledo DSC822^e. The samples of 3-5 mg were run at a scanning rate of 10° C per min under N_2 atmosphere (60 ml/min) and within a temperature range of 25-250°C. Rosmarinic acid, an encapsulated RA microparticles, polymer and physical mixture were tested.

1.4.3 Fourier Transform Infrared Spectrometry

FTIR spectra of encapsulated RA microparticles were measured by a Thermo scientific Nicolet iS10 FTIR spectrometer with an Attenuated Total Reflectance (ATR) accessory (Thermo Fischer Scientific, USA). Rosmarinic acid, polymer and encapsulated RA microparticles were recorded. The spectra were analyzed by OMNIC software.

1.4.4 X-Ray diffraction analysis

X-ray powder diffraction (XRD) was carried out to determine the solid state of active substance which incorporate in spray dried microparticles and freeze dried powders. The XRD patterns were recorded using MiniFlex II (Rigaku, Japan) equipped with CuKα anode (λ= 1.5406Å), 15.0 mA, 30.0 kV and slit path 1.25°. The data were collected at 1° 2θ/min with an angular step size 0.01° 2θ. Scanning range was from 5° - 40° 2θ/min. All data were processed using PDXL® software (version 1.8.1.0)

1.4.5 Determination of entrapment efficiency

High Pressure Liquid Chromatography (HPLC) was used to analyze the entrapment efficiency (% EE) of the encapsulated RA by using Cosmosil®C18 column. The mobile phase consisted of methanol and water at the ratio 35:65 with 0.1% v/v of acetic acid. The isocratic flow rate of mobile phase was controlled to be at 1 ml/min and at 35°C. The sample for analysis was prepared by dispersing 30 mg of the encapsulated RA in 5 ml of 3% w/v trisodium citrate solution for 12 hours. The solution was then diluted with methanol to 50 ml and centrifuged at 3,200 rpm for 15 minutes. The obtained solution was filtered through 0.22µm nylon syringe filter. 10 µl of the filtered solution was injected into the column and detected with UV detector at the wavelength of 320 nm and then analyzed by HPLC. The freeze dried RA microparticles were usually investigated in term of %assay of rosmarinic acid. Since rosmarinic acid was incorporated into the polymer solution and there was no lost step in the freeze drying process, so term of entrapment efficiency were reported in this study in order to compare with the performance of spray drying process. Entrapment efficiency were acquired from following equation:

$$\%EE = \frac{\textit{Actual amount of rosmarinic acid in microparticles}}{\textit{Theoretical amount of rosmarinic acid in microparticles}} \times 100$$

1.4.6 *In vitro* rosmarinic acid release study

In vitro release study of the encapsulated RA microparticles was performed using modified Franz diffusion cell with cellulose acetate membrane. Phosphate buffer pH 5.5 and phosphate buffer pH 7.4 with ethanol added at the concentration of 5%v/v were used as donor and receptor medium, respectively. The temperature of receiver medium was maintained constant at 32±0.5°C. The amount of 10 mg of encapsulated RA particles was dispersed onto the donor compartment and 2 ml of receptor fluid was periodically withdrawn from the receptor compartment, which was replaced with the same amount of fresh medium, and assayed by HPLC.

1.4.7 Stability study of encapsulated RA microparticles

Stability of encapsulated RA microparticles was observed for 3 months by using accelerating condition with temperature controlled at 40°C in hot air oven and humidity controlled at 75%RH by saturated sodium chloride in desiccator. The sample was taken for RA remaining analysis at 0, 1 and 3 months after storage.

2. *Thunbergia laurifolia* callus extract microparticles

2.1 Callus induction and proliferation from T. laurifolia leaves

T. laurifolia leaves, collected at faculty of pharmaceutical sciences, Chulalongkorn University, were induced to create callus formation which was supplied by department of Pharmacognosy and Pharmaceutical Botany. The TL callus was cultured in 4.4 g/L of MS media with NAA 1 mg/L and BA 2 mg/L as plant hormones and 30g/L of sucrose as carbon source. The ingredients of media were added into purified water and stirred and then adjusted pH to 5.7-5.8 with hydrochloric acid or sodium hydroxide. Then agar was added at the concentration of 8 g/L. The medium was heated in the microwave oven until completely dissolved, then each of 25 ml medium was pour into the glass bottle and cooled down at room temperature, steriled by autoclaving for 20 min. The obtained medium was used for callus subculture.

The callus was subcultured every months and then harvested and dried by lyophilization. Ethanol was used as a solvent for extraction. The extraction process of the dried callus was performed by using 30 ml of ethanol to macerate 1g of dried callus, then ethanol was removed by rotary evaporation at 42°C and analyzed for RA amount as chemical marker by HPLC.

2.2 Preparation of encapsulated TC microparticles

The formulations of encapsulated-TC microparticles were prepared by spray drying and freeze drying process as shown in Table 6.

Table 6 Formulations of encapsulated-TC microparticles

Formulation	Polymer concentration and ratio (DG:AG)
TC-SN-1AG	AG 1%
TC-FC-1DG	DG 1%
TC-FC-2AG	AG 2%
TC-FC-2DG	DG 2%
TC-FC-2DGAG1:1	DG+AG 2%, 1:1

2.3 Characterizations of encapsulated TC microparticles

2.3.1 Morphological characterization

2.3.1.1 Scanning Electron Microscope

Electron photomicrographs of encapsulated TC microparticles were taken with a scanning electron microscope (JEOL model JSM-5410W SEM machine). The samples were placed on aluminium stud and coated under an argon atmosphere with a fine gold layer then the particle morphology of microparticles were observed at 15 kV.

2.3.1.2 Dynamic Light scattering

Particle size was measured by light scattering using Malvern mastersizer2000. Ethanol was used as solvent to avoid polymer swelling. The encapsulated TC microparticles were dispersed in ethanol in a 250 ml beaker. The particle size was measured three times and volume-mean diameter, D[4,3] was reported.

2.3.2 Differential Scanning Calorimetry

The samples of 3-5 mg were run at a scanning rate of 10°C per min under N_2 atmosphere (60 ml/min), temperature range of 25-250°C. Callus extract and encapsulated TL callus extract were tested.

2.3.3 Fourier Transform Infrared Spectrometry

FTIR spectra of encapsulated TC microparticles were measured by a Thermo scientific Nicolet iS10 FTIR spectrometer with an Attenuated Total Reflectance (ATR) accessory (Thermo Fischer Scientific, USA). TC and encapsulated TC microparticles were recorded. The spectra were analyzed by OMNIC software.

2.3.4 X-Ray diffraction analysis

X-ray powder diffraction (XRD) was used to determine about the solid state of TL callus extract incorporated into the microparticles. The XRD patterns were recorded using MiniFlex II (Rigaku, Japan) equipped with CuKα anode (λ= 1.5406Å), 15.0 mA, 30.0 kV and slit path 1.25°. The data were collected at 1 ° 2θ/min with an angular step size 0.01 ° 2θ. Scanning range was from 5 °- 40 ° 2θ/min. All data were processed using PDXL® software (version 1.8.1.0).

2.3.5 Determination of entrapment efficiency

High Pressure Liquid Chromatography (HPLC) was used to analyze the entrapment efficiency (% EE) of the encapsulated TC by measuring the RA contents, using Cosmosil® C18 column. The mobile phase solution was prepared by using methanol and water at the ratio 35:65 in 0.1% v/v of acetic acid. The flow rate of mobile phase was controlled to be at 1 ml/min and at 35°C. The 10 µl of the filtered solution was injected into HPLC and equipped with UV detector at the wavelength of 320 nm. The sample for analyzing was prepared by dispersing 50 mg of the encapsulated TC in 5 ml of 3% w/v sodium citrate solution for 12 hours. The solution was then diluted with methanol to 50 ml and centrifuged at 3,200 rpm for 15 minutes. The obtained solution was filtered through 0.22µm nylon syringe filter and analyzed for RA concentrations by HPLC.

2.3.6 *In vitro* rosmarinic acid release study of encapsulated TC

In vitro release study of the encapsulated TC were performed using modified Franz diffusion cell with cellulose acetate membrane. Phosphate buffer pH 7.4 and pH 7.4 added with ethanol at the concentration of 5%v/v were used as donor and receptor medium, respectively. The temperature of receiver medium was maintained constant at 32±0.5°C. The amount of 30 mg of encapsulated TC particles was dispersed onto the donor compartment and 2 ml of dissolution fluid was periodically withdrawn from the receptor compartment which replaced with the

same amount of fresh medium and assayed by HPLC. The release study were tested for RA for 12 hours with the sampling time at 0, 1, 3, 6, 9 and 12 hrs, respectively.

2.3.7 Stability study of encapsulated TC microparticles

TC-loaded microparticles and powders were stability observed for 3 months. The three storage conditions were controlled at 40°C 75%RH, 30°C 75%RH and 4°C. The sample was taken for RA remaining analysis after storage for 1, 2 and 3 months respectively. The samples were diluted to the final concentration of 100 µg/ml before analysis by HPLC.

3. TC MPs-loaded freeze-dried patch

3.1 Preparation of TC MPs-loaded freeze-dried patch

DG solution was prepared by dispersed 1g of DG into 100 ml of purified water to make 1% DG solution for creating DG freeze-dried patch and TC MPs from the formulations in Table 7. The mixture of 13.7 ml for each formulation was then pour into the 14x3cm^2 size aluminium mold which was used to form a patch and the mold was put into freeze dryer to produce TC MPs-loaded freeze dried patch. The two control groups were PTC and PTCexc which added TC and TC with excipients, respectively. The powders of excipients were DG, AG, mannitol and calcium chloride which placed as the same ratio as in TC MPs formulations.

Table 7 Formulations of TC MPs-loaded freeze dried patch

Formulation	Polymer concentration and ratio (DG:AG)
PTC	no polymer added
PTCexc (for stability study only)	Polymer and excipients added as powders
PTC-SN-1AG	AG 1%
PTC-FC-1DG	DG 1%
PTC-FC-2AG	AG 2%
PTC-FC-2DG	DG 2%
PTC-FC-2DGAG1:1	DG+AG 2%, 1:1

Antioxidant activity of rosmarinic acid, a chemical marker, was above 2.71 µg/ml so the concentration in receiver medium at 5 µg/ml was used as amount of maximum release. Due to Franz diffusion cell receiver volume was 13 ml with the area of release was 2.27 cm^2, total rosmarinic acid amount in receiver medium was 65 µg and area of freeze dried patch was 42 cm^2 so the total amount of added TC MPs in the patch were calculated by rosmarinic acid content.

3.2 Characterization of TC MPs-loaded freeze dried patch

3.2.1 *In vitrc* release study of TC-MPs-loaded freeze dried patch

In vitro release study of the TC MPs-loaded freeze dried patch were performed using modified Franz diffusion cell with cellulose acetate membrane. Phosphate buffer pH 7.4 and pH 7.4 added 5%v/v ethanol were used as donor and receptor medium, respectively. The temperature of receiver medium was maintained constant at 32±0.5°C. For 12 hours at time intervals, 2 ml of dissolution fluid was periodically withdrawn from the receptor compartment which replaced with the same amount of fresh medium and assayed by HPLC. The control group was TC-loaded in freeze dried patch (PTC).

3.2.2 Stability study of TC MPs-loaded freeze dried patch

TC MPs-loaded freeze dried patch and control groups were stability observed for 3 months. The three storage conditions were controlled at 40°C/75%RH, 30°C/75%RH and 4°C. The sample was taken for RA remaining analysis after storage for 1, 2 and 3 months respectively. The control groups of freeze dried patch were TC loaded in freeze dried patch (PTC) and TC loaded in freeze dried patch containing mannitol and calcium chloride as in freeze dried excipients (PTCexc).

4. Statistical analysis

The results were analyzed by One-way ANOVA. Test of homogeneity of variances were performed. For Post-Hoc analysis, Tukey was performed for the results with homogeneity of variances and Dunnett T3 for the results with unhomogeneity of variances.