Stability, permeation, and cytotoxicity reduction of capsicum extract nanoparticles loaded hydrogel containing wax gourd extract

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Abstract

The aim of this study was to develop hydrogel loaded with capsicum extract nanoparticles and wax gourd extract for transdermal delivery of capsaicin. The addition of wax gourd extract was supposed to reduce cytotoxicity of capsaicin in capsicum extract against HaCaT keratinocyte cell line. Capsicum extract nanoparticles were prepared by solvent displacement method using hyaluronic acid as a stabilizer. The physical and chemical stability of capsicum extract nanoparticles were investigated by dynamic light scattering technique and UV-Visible spectrophotometry, respectively. Hydrogel loaded with capsicum extract nanoparticles and wax gourd fruit extract was then formulated by using Carbopol 940\textsuperscript{a} as a gel-forming agent for transdermal delivery. The skin permeability of capsaicin from the hydrogel was evaluated by Franz diffusion cell approach. The cytotoxicity reduction of capsicum extract nanoparticles and capsaicin extract nanoparticles by mixing with wax gourd extract was determined by MTT assay. The results showed that capsicum extract nanoparticles exhibited an average diameter of 168.4 ± 5.3 nm with a polydispersity index and zeta potential value of 0.26 ± 0.01 and 45.7 ± 7.1 mV, respectively. After two month-storage, particle size, polydispersity index, and zeta potential values of capsicum extract nanoparticles stored at 4°C, 30°C, and 45°C did not significantly change. The capsaicin content decreased to 78%, 71%, and 72% when stored at 4°C, 30°C, and 45°C for three months, respectively. The pH values of hydrogel containing capsicum extract nanoparticles were found to be in the range of 5.58–6.05 indicating good stability. The hydrogel exhibited a pseudoplastic character. The rate of permeation flux of capsaicin from hydrogel was 7.96 μg/cm²/h. A significant increase in cell viability was observed when the cells were incubated with capsicum extract nanoparticles mixed with wax gourd, compared to capsicum extract nanoparticles alone. The wax gourd extract in the hydrogel protected HaCaT cells from capsaicin cytotoxicity, thus may provide a new approach for delivery of capsaicin to reduce cytotoxicity to skin cells.

1. Introduction

Capsaicin, a main active substance of capsaicinoids, has been used topically to control peripheral nerve pain (Fattori et al., 2016). Capsaicin at low concentration (0.025–0.1%) included in the topical dosage forms such as cream, gel, ointment, lotion, and liquid is indicated for relieving pain from arthritis and musculoskeletal pain. High concentration of capsaicin (8% patch) is FDA approved for the treatment of postherpetic neuralgia, neuropathic pain, and neuropathy postoperative complications (Rollyson et al., 2014; Lakhoud and Baranidharan, 2016). The high or repeated doses of capsaicin administered to the patients stimulated pain sensation followed by analgesia (Fattori et al., 2016; Anand and Bley, 2011). The main mechanism underlying pain relieving effect of capsaicin is attributed to the reversibly depletion of substance P from nociceptive sensory fibers expressing the transient receptor potential vanilloid receptor type 1 (TRPV1) (Jara-Oseguera et al., 2008; Caterina et al., 1997). Capsaicin is a selective agonist of the TRPV1,

Original article

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an ion channel receptor highly expressed on nociceptive nerve fibers. Exposure to high or repeated dose of capsaicin desensitized TRPV1 receptors to a refractory state, resulting in the inhibition of receptor function (Smutzer and Devassy, 2016; Basith et al., 2016; Moran and Szallasi, 2018). The desensitization of TRPV1 leads to the depletion of neuropeptides including substance P in the nerve fibers, a neuropeptide acting as a pain neurotransmitter and neuromodulator. Other mechanism includes degeneration of sensory fibers (Gibbons et al., 2010; Lynn, 1990; Haanpaa and Treede, 2012).

Pain relieving products containing capsaicin generally cause skin irritation after application (Knotkova et al., 2008). The skin irritation may be related to cytotoxicity of capsaicin to skin cells. Ko et al reported that 0.025 to 0.20 %w/v capsaicin reduced growth of keratinocytes to 21% to 31% in a concentration-dependent manner in 6 h after exposure to capsaicin and complete cell death was observed at all concentrations of capsaicin at 48 h (Ko et al., 1998). Kim et al have shown that capsaicin was toxic to human skin fibroblast by inducing chromatin condensation and reduced survival rate to 25–50% at 24 h and antioxidants including 100 mM Trolox and 0.1 and 0.3 mM ascorbic acid reduced capsaicin cytotoxicity. (Kim et al., 2004). The authors concluded that capsaicin induced cytotoxicity on human skin fibroblast by apoptotic pathway, where antioxidants can prevent it. Skin irritation involved reactions of cells in epidermis and dermis, particularly keratinocytes, which are firstly affected. Therefore, keratinocytes are the most appropriate target cells for evaluating skin irritancy of substances (Hoh and Maier, 1993). The in vitro cytotoxicity assay against keratinocytes has been developed to detect toxicity of compounds at the cellular level (Hoh and Maier, 1993; Rosen and Goldberg, 1985). The use of primary human keratinocytes has limitation since the interpretation of experimental data obtained from primary cells are not reliable due to donor to donor variability, short culture lifetime, and variations between passages (Colombo et al., 2017). Several studies compared using primary keratinocytes and immortalized HaCaT cell line to evaluate their potential as models for skin irritancy testing. Hoh and Maier revealed that HaCaT immortalized cell line was significantly more sensitive than primary keratinocytes, while the viability of both cell types correlate with results of in vivo Draize irritation test in animals (Hoh and Maier, 1993). This result was confirmed by Olschlager et al reporting that immortalized HaCaT cell line was sensitive to sodium dodecyl sulfate three times higher than the primary keratinocytes (Olschlager et al., 2009). HaCaT cell line was previously used to test the cytotoxicity of capsaicin in several studies (Wang et al., 2017; Huang et al., 2012). In addition, HaCaT cells were used as a model to study the effects of capsaicin on inflammatory and nociceptive responses to acute keratinocyte damage (Huang et al., 2012; Southall et al., 2003; Pecze et al., 2008; Tóth et al., 2009).

Benincasa hispida or wax gourd is a plant in the family Cucurbitaceae. The fruit of Benincasa hispida has shown many pharmacological properties including anti-ulcer, anti-oxidant, and anti-inflammation (Rachchh and Jain, 2008; Shetty et al., 2008; Rachchh et al., 2011). The anti-ulcer activity of wax gourd fruit was examined in vivo. Grover and Rath reported the anti-inflammatory effect of wax gourd fruit aqueous extract (Gover and Rathi, 1994). The anti-inflammatory property might be related to the free radical scavenging activity (Zaini et al., 2011).

Topical or transdermal capsaicin as a single therapy or in combination with other analgesics has a promising effect on adjuvant therapy for neuropathic pain. However, capsaicin can cause a burning sensation on the applied skin, resulting in a skin irritation and redness lasting for several hours. Therefore, most patients cannot tolerate the drug and have low compliance. In an attempt to minimize local adverse effects and increase capsaicin product tolerability, nanotechnology and a combination of wax gourd extract with capsaicin in transdermal pain killer formulation have been introduced in this study to improve capsaicin delivery and reduce the adverse effects.

2. Materials and methods

2.1. Materials

Capsicum annuum and Benincasa hispida fruits were purchased from the local market in Nakhonnyak, Thailand. Capsaicin and Hyaluronic acid sodium salt, MW of 8,000–15,000 were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Carbopol 940® and triethanolamine were bought from MySkinRecipes (Bangkok, Thailand). Glycerin and 1,3-Dimethylol-5,5-dimethylhydantoin (DMDM hydantoin) were purchased from P.C. Drugs (Bangkok, Thailand). HaCaT cell line was purchased from Cell Lines Service GmbH (Eppelheim, Germany). Dulbecco’s Modified Eagle’s Medium with 4,500 mg/L glucose and sodium bicarbonate, fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco (Massachusetts, USA). 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 95% ethyl alcohol were purchased from Amresco Inc. (Ohio, USA).

2.2. Methods

2.2.1. Extraction of capsaicin from capsicum annuum

The dried chili fruits without stalks were powdered and sieved through a sieve number 40 to get fine powder. The fine powder (150 g) was macerated with 1.5 L of 95% ethanol at room temperature for 7 days. The extract was filtered using a vacuum pump and evaporated at 50 °C under vacuum in a rotary evaporator (Rotavapor® R-300, Büchi, Switzerland) to remove ethanol until the red-brown soft crude extract was obtained. The extract was analyzed to determine %yield expressed as a percentage of the weight of chili extract relative to the weight of dry sample used for extraction. The extract was stored at room temperature until use.

2.2.2. Extraction of wax gourd, Benincasa hispida, fruit and determination of total phenolic content in the extract

The fruits were washed with tap water and wiped. Each fruit was cut and peeled. Then the flesh was processed by using a blending mixer, yielding a fruit juice. The juice was filtered through a white clean cloth three times. The supernatant was then spray dried using a spray dryer (Büchi Mini Spray Dryer B-290, Flawil, Switzerland) at inlet temperature of 140 °C, outlet temperature of 90 °C, and 100% aspirator until powders were obtained. The yield of extract was determined and expressed as a percentage of the weight of spray dried wax gourd extract relative to the weight of fresh sample used for extraction. The dried powder was stored in a tightly closed container at room temperature until use.
The total phenolic content of wax gourd fruit extract was determined by using Folin-Ciocalteu reagent, which was a colorimetric assay of phenolic and polyphenolic antioxidants. The wax gourd extract was prepared by weighing 10 g of the spray dried extract and dissolved in 100 mL of deionized water to obtain 100 mg/mL. The wax gourd extract 100 μL was added to 100 μL Folin-Ciocalteu, 100 μL 7.5% v/v Na₂CO₃, and 700 μL of deionized water and mixed thoroughly. The mixture was incubated at room temperature in the dark for 90 min before the absorbance was read at the wavelength of 765 nm. The amount of the total phenolic content was determined as milligrams of gallic acid equivalent (GAE) using a standard curve of gallic acid in ethanol at the concentration range of 15–1000 μg/mL.

2.2.3. Preparation of capsicum extract nanoparticles

Capsicum extract enriched nanoparticles coated with hyaluronic acid were formulated by using a solvent displacement technique (Burasanukhon et al., 2017). First, Capsicum annuum extract (chili extract) was weighed for 100 mg and dispersed in 5 mL of 95% ethanol, a water miscible solvent. The dispersion was sonicated in a sonicator bath for 3 min to enhance the solubility of capsicum extract in 95% ethanol. The dispersion was then filtered through a nylon syringe filter with a pore size of 0.22 μm. Then 2 mL of capsicum extract solution was introduced (8 mL/h) into 15 mL aqueous phase containing 0.05% hyaluronic acid as a stabilizer, under magnetic stirring rate of 635 rpm. The dispersion of capsicum extract nanoparticles were stirred for 2 h to remove ethanol from the colloidal dispersion.

2.2.4. Determination of particle size, size distribution, zeta potential and physical stability of capsicum extract nanoparticles

The particle size, polydispersity index (PDI), and zeta potential values were carried out using a dynamic light scattering technique (Zetasizer ZS, Malvern, UK). Stability testing involving particle size, size distribution, and charge of nanoparticles were conducted at given time intervals over 2 month-storage at 4 °C, 30 °C, and 45 °C. The effects of temperature and time on physical stability of capsicum extract nanoparticles were investigated. 5 mL of capsicum extract nanoparticles in deionized water (2.7 mg/mL) were filled in glass vials and stored at 4 °C, 30 °C, and 45 °C for 2 months. At certain time interval (1, 2, 4, and 8 weeks), dynamic light scattering technique was conducted to monitor the effect of the storage condition on the size, polydispersity index, and zeta potential of the nanoparticles.

2.2.5. Determination of capsaicin concentration in nanoparticles and chemical stability of capsicum extract nanoparticles

Capsaicin in capsicum extract nanoparticles were quantified by UV–Vis spectrophotometry (Shimadzu, Japan). Capsaicin was dissolved in dimethyl sulfoxide (DMSO) and was diluted with DMSO to obtain 20–200 μg/mL capsaicin standard solution. The capsaicin content in the nanoparticles was measured at 280 nm wavelength and was calculated based on the absorbance values of standard solution (Shrivastava and Saxena, 2011). To determine the concentration of capsaicin in nanoparticles, capsicum extract nanoparticles were dissolved in DMSO and the absorbance at 280 nm wavelength was measured by a UV–Vis spectrophotometer. Hyaluronic acid solution diluted in DMSO was used as a blank for the measurement.

Capsaicin extract nanoparticles suspended in deionized water (2.7 mg/mL) were stored at 4 °C, 30 °C, and 45 °C for 3 months. At storage time interval, i.e. 1, 2, 4, 8, and 12 weeks, samples were collected and dissolved in DMSO. The amount of capsaicin in the nanoparticles was determined by UV–Vis spectrophotometer at 280 nm. The amount of capsaicin remaining in the nanoparticles were presented as percentage of capsaicin remaining.

\[
\%\text{Capsaicin remaining in NPs} = \frac{\text{Amount of capsaicin in NPs}}{\text{Amount of capsaicin in freshly prepared NPs}} \times 100
\]

2.2.6. Preparation of hydrogel containing capsicum extract nanoparticles and wax gourd extract

The spray dried wax gourd powders were weighed out 5 g and dispersed in 500 mL of deionized water to obtain concentration of 1%w/v wax gourd. The dispersion was stirred for 2 h to ensure the homogeneity of wax gourd in water and was filtered through filter paper, resulting a clear solution. The hydrogel was prepared by adding 5 g of Carbopol 940® into 500 g of wax gourd solution. The dispersion was stirred until it was homogenous. Glycerin (7.5 g) and DMDM hydantoin (0.5 g) were added into above solution. The obtained solution was weighed for 325 g and mixed with 175.5 g of capsicum extracts nanoparticle dispersion containing 0.5 g capsicum extracts. Then, triethanolamine (4 g), a neutralizer, was slowly added into the mixture to adjust pH of solution and allow Carbopol 940® to form hydrogel.

2.2.7. pH measurement of hydrogel containing capsicum extract nanoparticles and wax gourd extract

The hydrogel containing capsicum extract nanoparticles was stored at 4 °C, 30 °C, 45 °C and pH of the preparations was measured in triplicates at 25 °C after storage for 5 months and after heating–cooling cycle stability tests using a pH meter (model OR-3005, Orion, USA).

2.2.8. Rheological measurement

Measurement of hydrogel containing capsicum extract nanoparticle rheology was performed using Thermo Scientific HAAKE RheoStress 1 rheometer equipped with a plate and plate geometry (1.0 mm gap, 60 mm diameter). The temperature of the hydrogel was set at 25 °C. The ranging of shear rate was from 0.01 to 200 s⁻¹ with the frequency of 1 Hz. The stability of hydrogel containing capsicum extract nanoparticles was investigated after storage for six cycles between refrigerator temperature (4 °C) for 24 h and 40 °C for 24 h. The storage temperature effect on viscosity of hydrogel was also investigated by incubating hydrogel samples at 4 °C, 30 °C, and 45 °C for 5 months. Samples were subjected to rheological measurement after freshly preparation and at the end of stability testing.

2.2.9. Permeation studies using Franz diffusion cells

Capsaicin diffusion from the hydrogel across excised newborn pig skin was investigated using vertical Franz cells and equipment (PermeGear, Inc., Hellertown, PA). Franz diffusion cell is widely used and accepted by the European Medicines Agency Guideline (EMA) to determine the drug permeation across the skin layers (Neupane et al., 2020). It is considered as a gold standard for ex vivo skin permeation study (Zsikos et al., 2019). The skin isolated from newborn porcine is considered as an alternative to human skin. The new born pig skin is normally preferred due to the similarity to human skin structure (Neupane et al., 2020). One dead newborn pig skin was obtained and frozen within 24 h after death in the farm. After the newborn pig was unfrozen, the skin was separated easily with the aid of a razor blade. The newborn pig did not have so much fat under the skin. The remaining fat was removed by the razor blade. The skin was observed visually for skin damage and the standard deviation of results were taken into consideration. The receptor cells were filled with phosphate buffered saline (PBS), pH 7.4 and allowed to equilibrate at 37.0 °C in the heated magnetic block for 15 min. The skin was cut to give the available diffusion area of the vertical diffusion cell of 1.77 cm². The skin was
cells were seeded in 96-well plates at a density of 10^4 cells/well. The cell viability was assessed by using MTT assay. The zeta potential value of the capsicum extract nanoparticles was determined by UV–Vis spectrophotometry at the maximum wavelength of 270 nm (Cirino et al., 2016). The standard curve was plotted between the absorbance at 270 nm and capsaicin concentrations in a range of 20–200 µg/mL. The amount of capsaicin in nanoparticles was calculated using the standard curve. The cumulative amount of capsaicin permeated through the skin was calculated and plotted as a function of time. The capsaicin flux was calculated by the slope of the linear portion of the permeation curve and expressed as the mass passing across 1 cm² of the skin over time.

2.2.10. MTT cytotoxicity assay and cell proliferation assay

The human skin HaCaT cell line was cultured in DMEM high glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Cell cultures were maintained in a humidified incubator with 5% CO₂ at 37°C under magnetic stirring. The concentration of capsaicin in the receiver medium were determined by UV–Vis spectrophotometry after 2 months. Fig. 1 shows that from 0 to 8 weeks, particle size, PDI, and zeta potential values of the capsicum extract nanoparticles stored at 4°C, 30°C, and 45°C did not significantly change.

2.2.11. Statistical analysis

Data were presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) and Newman–Keuls post hoc test were performed for statistical analysis. To compare the significance of the difference between the means of two groups, a t-test was performed. A p value<0.05 was considered statistically significant.

3. Results

3.1. The yield of C. Annuum and B. Hispida fruit extractions and total phenolic content in the extract

The capsaicin content in freshly prepared nanoparticles was 291.53 ± 1.49 µg/mL according to the standard curve (Supplementary Fig. 1). The stability of capsaicin in the nanoparticles was observed by tracking the capsaicin content over time (Fig. 2). The remaining of capsaicin in nanoparticles stored at 4°C, 30°C, and 45°C for 3 months significantly decreased to 30.4 ± 7.2 µg/mL. The physicochemical characteristics of the hydrogel containing capsicum extract nanoparticles remained unchanged for five months when it was kept at 4°C, 30°C, and 45°C in a sealed container.

3.2. Characterization and stability of capsicum extract nanoparticles

The pH values of hydrogel containing capsicum extract nanoparticles were found to be in the range of 5.58–6.05. A plot of viscosity versus shear rate for the hydrogel containing capsicum extract nanoparticles is shown in Fig. 4. The results for the hydrogel containing capsicum extract nanoparticles were compared to those of the freshly prepared hydrogel.
clearly indicated that the apparent viscosity of the hydrogel containing capsicum extract nanoparticles decreased significantly with increasing shear rate i.e. from around 100,000 cP at shear rate of 0.9 (1/s) to 1,000 cP at shear rate of 200 (1/s), indicating that the hydrogel exhibited a pseudoplastic character. The rheograms of the hydrogels after heating–cooling stability study and long-term sta-

Fig. 1. Effect of incubation time and temperature on (A) particle size, (B) PDI, and (C) zeta potential of capsicum extract nanoparticles. Data were presented as the mean ± SD.
bility study (5 months) suggested pseudoplastic flow of the hydrogel. The viscosity of hydrogel containing capsicum extract nanoparticles was reduced after storage at 45°C for 5 months i.e. from about 100,000 cP at shear rate of 0.9 (1/s) to around 50,000 cP at the same shear rate.

3.7. Skin permeation of capsaicin from hydrogel

The cumulative amount of capsaicin permeated through the newborn pig skin is presented in Fig. 5. The amount of capsaicin permeated increased with increasing incubation time. The greatest permeation was observed where 42.64 ± 9.70% capsaicin permeated from the donor chamber through the newborn pig skin into the receptor medium within 24 h. The amount of permeated capsaicin was calculated from linear regression analysis of standard curve (Supplementary Fig. 2).

3.8. Protective effect of wax gourd against cytotoxicity of capsicum extract nanoparticles

To determine the protective effect of wax gourd on capsicum extract nanoparticles induced cytotoxicity, 0.5% of wax gourd was mixed with capsicum extract nanoparticles. The viability of HaCaT cells exposing to capsicum extract nanoparticles and capsicum extract nanoparticles mixed with wax gourd extract was expressed as percentage compared to the control as shown in Fig. 6. Wax gourd extract was able to protect HaCaT cells from the cytotoxicity effect of capsicum extract nanoparticles. A significant increase in cell viability was observed when the cells were incubated with capsicum extract nanoparticles (0.68 and 1.35 mg/mL) mixed with wax gourd after the treatment for 24, 48, and 72 h. The viability of HaCaT cells treated with the 0.68 and 1.35 mg/mL of capsicum extract nanoparticles for 24 h were 49.0 ± 13.0 and 32.1 ± 1.9% of control, respectively, whereas the cell viability of cells treated with 0.68 and 1.35 mg/mL of capsicum extract nanoparticles plus 0.5% wax gourd were 81.1 ± 8.1 and 87.6 ± 11.7% of control, respectively. After 48 h of incubation, %cell viability of HaCaT treated with 0.68 and 1.35 mg/mL of capsicum extract nanoparticles decreased to 27.4 ± 2.7 and 24.1 ± 0.7%, respectively, while that of cells treated with nanoparticles at the same concentration plus 0.5% wax gourd were 60.6 ± 5.0 and 53.5 ± 5.8%, respectively. At 72 h, the viability of cells treated with the 0.68 and 1.35 mg/mL of capsicum extract nanoparticles were reduced to 8.8 ± 1.3 and 8.1 ± 0.1%, respectively, whereas that of cells treated with the same concentrations of nanoparticles mixed with wax gourd were 38.1 ± 7.8 and 26.5 ± 2.1%, respectively. The IC50 values of capsicum extract nanoparticles and capsicum extract nanoparticles mixed with wax gourd are presented in Table 1. The IC50 values of both samples decreased with incubation time. The IC50 values of capsicum extract nanoparticles without wax gourd were 638.3, 401.4, and 213.6 mg/mL, respectively. In the presence of wax gourd extract, the IC50 values of capsicum extract nanoparticles increased about 48.6, 3.0, and 1.7 times after incubation with cells for 24, 48, and 72 h.

4. Discussion

The formation of capsicum extract nanoparticles by a solvent displacement method was driven by the rapid diffusion of ethanol
to an aqueous hyaluronic acid solution. The viscosity of the organic phase and the velocity of solvent diffusion affected the size of the nanoparticles (Beck-Broichsitter et al., 2010). In this study, 20 mg/mL of capsicum extracts in ethanol solution and the stirring speed of 635 rpm was considered optimal for generating <200 nm size of the nanoparticles. The significance of using hyaluronic acid as a steric stabilizer was reported in our previous studies (Chittasupho et al., 2018; Chittasupho et al., 2020). In addition, the negative charge of hyaluronic acid adsorbed on the nanoparticle surface increased physical stability of the nanoparticles by electrostatic repulsion. The capsicum extract nanoparticles exhibited physical stability when stored at 4 °C, 30 °C, and 45 °C for 2 months. The adsorption of hyaluronic acid provided the negative zeta potential of capsicum extract nanoparticles which may help prevent nanoparticles from agglomeration and sedimentation (Almalik et al., 2017).

The %remaining of capsaicin in the nanoparticles decreased to 78%, 71%, and 72% when stored at 4 °C, 30 °C, and 45 °C for 3 months, respectively. The decrease in capsaicin concentrations at different temperatures (4 °C, 30 °C and 45 °C) were not significantly different. These results agreed with the stability study of 0.1% capsaicin solution, showing that the concentrations of capsaicin in the solution were not significantly different when samples were stored at 5 °C, 25 °C, and 30 °C for 3 months (Balabathula et al., 2013;4:142). Giuffrida et al reported that capsaicin content in chili powder after storage for 6 months at room temperature (20–24 °C) remained 84%. The decrease in capsaicin in nanoparticles might be a result of both chemical degradation and release of capsaicin to aqueous medium (Giuffrida et al., 2014). The decrease in capsaicin contents compared to the freshly prepared samples may be a result of thermal and oxidative degradation (Arifin and Djaeni, 2018). The chemical stability of capsaicin at elevated temperature was extensively studied by Si et al (Si et al., 2014). It was suggested that the influence of temperature below 65 °C on the reduction of capsaicin content was minimal. However, as the temperature increased to 65 °C and further to 100 °C, the reduction of capsaicin content was more prominent (Si et al., 2014). In addition, the degradation of capsaicin mainly depended on the pH of the formulation. The capsaicin degradation rate was higher at both acidic and alkaline conditions (Si et al., 2014). It was reported that capsaicin was relatively stable under neutral condition (Si et al., 2014).

The hydrogel containing capsicum extract nanoparticles and wax gourd extract was stable at least for 5 months at neutral pH which was sufficient to obtain a good viscosity, clarity of the hydrogel, and good stability of the active compound. In addition, the pH of the hydrogel was close to the skin pH which may reduce skin irritation. Hence, the prepared hydrogel was suitable for transdermal application. The rheograms of the hydrogels after heating–cooling stability study and long-term stability study (5 months) suggested pseudoplastic flow of the hydrogel. The hydrogel flew instantaneously upon application of stress and displayed shear thinning behavior without a yield stress. Therefore, when the hydrogel was applied to the skin, the increase in shear rate may result in the less resistance to flow and the release of the entrapped capsicum extract nanoparticles. Fig. 4 shows a decrease in hydrogel viscosity when hydrogel was stored at 45 °C for 5 months. Barry and Meyer reported that increased temperature gradually decreased the apparent viscosity of Carbopol 940® hydrogel (Barry and Meyer, 1979). Increase in temperature was associated with increase in intermolecular distance of polymer molecules.

Fig. 4. Effect of storage temperature on the viscosity and rheology of capsicum extract nanoparticle loaded hydrogel as a function of shear rate after storage at 4 °C, 30 °C, and 45 °C for 5 months and six heating–cooling cycles.

Fig. 5. In vitro skin permeation of capsaicin from capsicum extract nanoparticles loaded hydrogel in phosphate buffer (pH 7.4).
resulting in the lower resistance of the hydrogel to flow, hence 
decreasing the viscosity (Lyapunov et al., 2015). Carbopol 940®
is a gelling agent used to facilitate transdermal application. It is safe 
as a cosmetic ingredient. Clinical studies showed that Carbopol 
940® has low potential for skin irritation and sensitization at con-
centrations up to 100% ([52]).

The permeation of capsaicin through the skin could be attribu-
ted to the diffusion of capsaicin extract nanoparticles coated with 
hyaluronic acid through stratum corneum and drug release (Prow 
et al., 2011). The rate of permeation flux was 7.96 μg/cm²/h which 
was higher compared to the previous reports. The type of formula-
tion base influenced the permeation rate of capsaicin. Wang et al.
reported that the flux of 0.075% Zostrix® and Capsaicin-c® cream containing capsaicin through nude mouse skin were 3.56 and 2.52 μg/cm²/h, respectively (Wang et al., 2001). It was found that <1 μg/cm²/h of capsaicin penetrated from hydrophilic ointment base through pig skin (Fang et al., 1995). The flux of capsaicin penetrated through synthetic water soluble cellulose from sodium carboxymethyl cellulose was 4.63 μg/cm²/h (Wang et al., 2001). According to the results, capsaicin permeation through skin was enhanced by loading capsaicin in nanoparticulate formulations (Wang et al., 2017). In our study, the hydrogel showed higher permeation rate, suggesting that the rate of drug diffusion from the carrier did not limit the skin contact. The capsaicin rapidly released from the nanoparticles leading to an increase in flux across the skin. These results suggested that nanoparticles enhanced water solubility of capsaicin and did not retard the release of the drug to the skin. Low viscosity of the hydrogel facilitated release of capsaicin from the product and favored the degree of skin penetration. The lowest viscosity of the hydrogel was about 1000 cPs which could facilitate permeation of the capsaicin. Since the capsaicin permeated across the skin barrier, it was suggested that analgesic effect may occur rapidly and patients could achieve a fast pain relief. After 6 h, skin permeation occurred at the lower rate suggesting a sustained permeation. The smaller amount of capsaicin achieved the skin may reduce the sensation of irritation. In this study, hydrogel containing capsaicin extract nanoparticles was formulated to avoid using solvents or solubilizing agents such as ethanol to solubilize lipid on the stratum corneum. Due to the low flux and long lag time, adding ethanol in the gel formulation as a skin permeation enhancer to increase capsaicin skin permeation rate was reported in several studies (Lee et al., 1993; Hatanaka et al., 1995). Ethanol in the formulation was known to be associated with skin irritation or contact dermatitis, especially in humans with an aldehyde dehydrogenase (ALDH) deficiency (Lachenmeier, 2008). Therefore, formulating hydrogel by applying nanotechnology could help reducing the use of organic solvents in the system.

Pure capsaicin has been shown to be toxic to keratinocytes and fibroblasts (Ko et al., 1998; Pecze et al., 2008). Ko et al reported that application of 0.025 to 0.2%w/v capsaicin reduced keratinocyte growth by 21–31% in the first 6 h (Ko et al., 1998). Pecze et al also showed the cytotoxicity of capsaicin against HaCaT cell line at the concentration of 100 μM and higher (Pecze et al., 2008). Hyaluronic acid is a natural skin component and it is non-toxic in acute animal toxicity studies of several species given by different routes of administration (Zerbinati et al., 2018). Therefore, it might be implied that the cytotoxicity of capsaicin extract nanoparticles was resulted from capsaicin. Compared with untreated cells, treatment with wax gourd caused a significant increase in HaCaT cell viability in response to capsaicin extract nanoparticles. These results demonstrated that 0.5% wax gourd was able to protect HaCaT cells from capsaicin extract nanoparticle induced cytotoxicity at high concentrations. The results of MTT assay suggested that HaCaT cell viability was about 80% or more when treated with 96.3 μM of capsaicin equivalent capsicum extract nanoparticles. Capsaicin was shown to induce oxidative stress and DNA damage resulting in cell death (Lee et al., 2004; Zhang et al., 2008). Wax gourd fruit extract has shown to be good sources of antioxidants including phenolic compounds (Deng et al., 2013; Abdullah et al., 2012). It was reported that phenolic compounds in wax gourd extract can protect cells from oxidative damage and decrease generation oxygen reactive species (Abdullah et al., 2012). Therefore, the cytotoxicity reduction from wax gourd fruit extract may be related to the phenolic compounds in the extract.

### 5. Conclusion

In the present study, capsicum extract nanoparticles loaded hydrogel was successfully developed. The physical and chemical stability study of capsicum extract nanoparticles were temperature independent. The hydrogel containing capsicum extract nanoparticles provides controlled release, and enhanced permeation through newborn pig skin. The wax gourd extract in the hydrogel protected skin cells from capsicum extract cytotoxicity, thus may provide a new approach for delivery of capsaicin with reduced cytotoxicity to skin cells.

### Declaration of Competing Interest

We declare that we have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this article.

### Acknowledgements

The authors gratefully appreciate Assistant Professor Dr. Veera-at Teeranachaideekul, Faculty of Pharmacy, Mahidol University for providing HaCaT cell line used in this study. We also acknowledge the use of the facilities of the Research Center for Drug Discovery and Development, Srinakharinwirot University.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.10.001.

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