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Original Article

NANOPARTICLE PREPARATION OF SIAM CITRUS PEEL EXTRACT (*CITRUS NOBILIS* L. VAR. MICROCARPA) USING SHORT-CHAIN CHITOSAN AND TRIPOLYPHOSPHATE AS CROSS LINKER AND CELLULAR UPTAKE STUDY ON MCF-7 CELL LINE BY *IN VITRO*

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ABSTRACT

Objective: High consumption of oranges causes a lot of orange peel waste. Orange peel contains the compound naringenin, which has a cytotoxic effect on various cancer cells. This research aims to develop a preparation of Siamese orange peel extract nanoparticles with short-chain chitosan and tripolyphosphate carriers as an oral drug delivery system and determine its cytotoxic activity against the Michigan Cancer Foundation-7 (MCF-7) cell line.

Methods: This research uses the micro tetrazolium (MTT) method to see the cytotoxic activity extract of methanol obtained from maceration extraction. The extract was then formulated into nanoparticles using chitosan and tripolyphosphate. Characterization and evaluation of nanoparticles were carried out, including particle size, zeta potential, entrapment efficiency, and stability in the stomach using 0.1 N HCl and in the intestine using Artificial Intestinal Fluid (AIF) *in vitro*. This research was also conducted to assess the ability of nanoparticles to enter MCF-7 cells (cellular uptake).

Results: Nanoparticles were successfully developed from Siamese orange peel extract. The results of the day 0 nanoparticle characterization were spherical, with average particle size 284.3 nm, zeta potential 0.713 mV, entrapment efficiency 96.73%, and stability in 0.1 N HCl at the 0th hours, respectively. 1st, 2nd, and 3rd. 99.16%, 98.70%, 98.47%, 98.31%, stability on AIF at hours 0, 1, 2, 3 and 4 respectively 99.52%, 99.30%, 99.40%, 98.99%, 99.29%. Characterization of nanoparticles on day 25 showed that the average particle size was 196.2 nm, zeta potential 0.476 mV, entrapment efficiency 96.92%, stability in 0.1 N HCl at 0, 1, 2 and 3 h respectively 99.51%, 98.67%, 98.51%, 98.27%, stability in AIF at 0th, 1st, 2nd, 3rd, and 4th hours 99.24 respectively %, 98.76%, 98.46%, 97.93%, 97.58%. Cytotoxic activity of extract Siamese citrus peel against MCF-7 cells with IC50 of 290.58 µg/ml. The result shows that cellular uptake of Siamese citrus peel nanoparticles can penetrate MCF-7 cells.

Conclusion: Stable nanoparticles were successfully developed from Siamese orange peel extract, and their stability was maintained throughout a 30-day storage period. This extract displayed cytotoxic effects and showcased the ability for cellular uptake in MCF-7 cell cultures *in vitro*.

Keywords: Citrus peel extract, Michigan cancer foundation-7, MCF-7, Nanoparticles

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INTRODUCTION

Indonesia has an abundant biodiversity of plant species that are thought to have medicinal properties. One of the exciting plants to study for its anti-cancer activity is the Siamese orange plant, which comes from Sambas Regency, West Kalimantan Province. The part used in this research is Siam orange peel. The high consumption of Siamese oranges causes a lot of orange peel waste. Therefore, Siamese orange peel only becomes agricultural waste and is not used. Siamese orange peel contains the compound naringenin, which has cytotoxic activity. Besides, orange peel waste contains phenols and flavonoids, which have antioxidant activity, too [1].

Cancer stands as a major global cause of mortality, with data indicating its significant impact in the United States and various developing nations. Consequently, research efforts in cancer drugs persist [2]. Michigan Cancer Foundation-7 (MCF-7) cell lines are known to produce bile acids, essential for lipid absorption in the intestines, even though bile acid synthesis typically occurs in the liver. Recent metabolomics research has shown that human breast adenocarcinoma MCF-7 cell lines can synthesizes bile acids. In our current investigation, we utilized MCF-7 cells as a model to explore the potential anti-diabetic, anti-hyperlipidemic, and anti-cancer properties of HWE [3].

Nonetheless, the systemic administration of drugs often leads to unintended side effects due to their influence on unintended targets. Nonspecific drug usage may not yield therapeutic benefits since drug concentrations in tumor cells may fall below the minimum practical level due to limited bioavailability [4]. To address this issue, the use of nanoparticles as drug delivery systems is gaining prominence. Nanoparticles are solid colloidal particles with diameters ranging from 1 to 1000 nm [5]. They are produced through ionic gelation, relying on the attractive forces between the amino groups within chitosan and negatively charged polyanions. These nanoparticles can shield active compounds from degradation, enhance transmucosal transport, and regulate drug release. The utilization of nanoparticles as delivery systems for anticancer drugs is on the rise and holds significant promise in cancer therapy [6]. This study involved extracting Siamese orange peel using the maceration method and methanol solvent. Cytotoxicity of Siamese orange peel extract was tested on the MCF 7 cell line. The active extract was then made into a nanoparticle preparation. Siamese orange peel extract nanoparticles were formulated using the ionic gelation method with the addition of negatively charged tripolyphosphate (TPP). The nanoparticles will be characterized using TEM and PSA to see their morphology and size; then an Entrapment Efficiency test will be carried out. Next, stability tests were carried out in HCl (hydrochloric acid) and AIF (artificial intestinal fluid) to characterize the stability of nanoparticles within the gastrointestinal tract, specifically the stomach and intestines, through *in vitro* testing. *In vitro* testing was conducted to assess the cellular uptake capability of MCF-7 cell cultures as well. Based on literature searches and studies, to the researchers' knowledge, Siamese orange peel nanoparticle preparation has never been done. So, this research needs to be carried out [7].

MATERIALS AND METHODS

Materials

Citrus siam collected from Tebas, Sambas Regency, West Borneo, chitosan (Biotech Surindo, pharmaceutical grade), MCF-7 cells obtained from the Laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University.

Instruments

Scanning Electron Microscopy (SEM) (JOEL-JEM 1400, Japan), DelsaTM Nano Particle Size and Zeta Potential Analyzer (Beckman Coulter, USA), Spectrophotometer UV-Vis (UV Thermo Scientific Genesys 10 UV Scanning), Elisa Reader (Bio-Rad Benchmark Microplate Reader serial no. 11 565, Japan), and Fourier Transform Infrared Spectroscopy (FTIR) (PERKIN ELMER FTIR 100).

Collection and extraction of siam citrus peel

Siamese oranges (*Citrus nobilis L. Var. Microcarpa*) was determined at the Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak, West Kalimantan with number 075/A/lB/FMIPA/UNTAN/2015. The Siamese oranges used were four months old, obtained from Tebas, Sambas Regency, West Kalimantan, Indonesia. The selected Siamese oranges are yellowish green, four months old, and harvested in the morning. We store the Siamese oranges we obtain in a shady and clean place. Next, we select oranges of good quality and protect them from damage. Afterward, we conduct a wet sorting process. Following that, we wash the orange fruit using running water. Subsequently, it is air-dried at room temperature until a consistent weight is achieved [8]. Next, the chopping process reduces the surface area of the Siamese orange peel. Subsequently, the orange rind is processed in a blender until it transforms into a fine powder. After that, the samples were dried in an oven at 40 °C. After drying, the sample is stored in a container [9].

Test cytotoxic methanol extract of siamese orange peel on cell line MCF

Preparation of the test solution

The test solution is made by dissolving the nanoparticles with DMSO and then making a series of test solution concentrations in the culture medium. This work also took place in the LAF.

Examination of cytotoxicity

preparation of MCF7 cells Transfer the MCF 7 cells into a sterile conical tube containing DMEM (Dulbecco's Modified Eagle Medium) and centrifuged at 325 X gravity suspension for 5 min. The supernatant was discarded and replaced with new DMEM medium and then suspended slowly. The cell suspension is centrifuged again at 325 x gravity for 5 min. The supernatant was discarded, then 1 ml of DMEM growth medium containing 20% FBS was added to the pellet and then resuspended slowly until homogeneous. The cells were grown in a few (3-4) pieces in a small tissue culture flask incubated at 37 °C with a 5% CO_2 stream. After 24 h, the medium was replaced, and cells were grown until confluent [3, 10].

Harvest cells

After a sufficient number of cells, the medium was replaced with a new DMEM medium of about 5 ml, and the cells removed from the flask walls, moved into a sterile flask supplemented RPMI medium and centrifuged 325 x gravity for 5 min. Cells were washed using the same medium, and count the number of cells using hemocytometer. The cell suspension plus several media to obtain a cell concentration of 5×10^4 cells/100 ml and ready for research [11].

Test bioesei cytotoxic

This experiment used a 96-well plate. Cells in each well underwent incubation in a CO2 incubator at 37 °C for a duration ranging from 20 to 48 h, followed by an assessment of their densities using a hemacytometer. The wells received an introduction of 100 ml of the test solution, with DMSO as a negative control. Following a 24 h incubation period, neutral red was added to each well, and they underwent an additional 2 h incubation in a CO2 incubator at 37 °C. Subsequently, the cells were washed with PBS, and a 1% SDS solution was applied for a 30-minute incubation, after which absorbance readings were taken at 495 nm and 630 nm using an ELISA Reader. The amount of uptake directly correlates with the number of viable cells. To determine the percentage of cell death at various concentrations with the following formula [12]:

$$\% \text{ Cell death absorbance} = \frac{\text{absorbance of negative control test sample X 100\%}}{\text{Absorbance of negative control}}$$

Preparation of nanoparticle preparations orange peel extract

Preliminary preparation of nanoparticles was made with various concentrations of Siamese orange peel extract chitosan. and TPP on a small scale (8 ml) to obtain the most optimal preparation. Nine formulas were made with different concentrations of chitosan and TPP in each formula. The volume ratio of Siamese orange peel extract: chitosan: TPP, respectively, is 1:6:1. Siam orange peel extract solution was made by dissolving the extract in methanol and produced a brownish-yellow and clear color. The TPP solution is made by dissolving TPP in distilled water and produces a transparent and clear-colored solution. Then, the chitosan solution was made by dissolving the chitosan in acetate buffer pH 4 and producing a transparent and clear-colored solution.

Each solution was mixed by dissolving 1 ml of orange peel methanol extract (5000 µg/ml) into 1 ml of TPP solution in distilled water and stirring using a magnetic stirrer at a 1000 rpm for 10 min. Next, the solution mixture was dropped into 6 ml of chitosan in acetate buffer solution pH 4.0 while stirring with a magnetic stirrer at a 1000 rpm for 1 h. Next, observations are made on the presence of floating physical particles, sedimentation, solution color, sediment, form and turbidity to determine the optimum formula for the next step [13].

Scale up and freeze-drying.

Scale-up aims to increase the batch size to apply the same process in different output volumes. The volume made here is 500 ml. The volume ratio of Siamese orange peel extract: chitosan: TPP respectively is 1:6:1. The scaled-up formula was the optimal formula (formula 1 in table 1) with an extract concentration of 5000 ppm, chitosan concentration of 1% and TPP concentration of 1%, 3 replications (R1, R2 and R3).

Solution was mixed by dissolving 62.5 ml of orange peel methanol extract (5000 µg/ml) into 62.5 ml of TPP solution in distilled water and stirring using a magnetic stirrer at a 1000 rpm for 10 min. Next, the solution mixture was dropped into 375 ml of chitosan in acetate buffer solution pH 4.0 while stirring with a magnetic stirrer at a 1000 rpm for 1 h. The scale-up results are then freeze-dried to produce nanoparticle powder.

Characterization and evaluation of nanoparticles

Nanoparticle morphology was determined using a Scanning Electron Microscope (SEM). The size and distribution of nanoparticles are determined using a Particle Size Analyzer (PSA), surface properties of nanoparticles using a Zetasizer, entrapment efficiency of nanoparticles using spectrophotometry UV-Visible and stability study of nanoparticles in HCl 0,1 N pH 1,0 and artificial intestinal fluid [14].

Cellular uptake

Cellular uptake was seen using the fluorescence microscope. Nanoparticles which are enable to get into the cells will appear fluorescent. This test was carried out by comparing the cellular uptake ability of the test group, namely preparation of Siamese orange peel extract formula number 1 nanoparticles, with naringenin standard, methanol extract of Siamese orange peel and cell control (MCF-7 without treatment).

RESULTS

Collection and extraction of Siamese citrus peel

The stages that play an essential role in determining the quality of raw materials are collecting raw materials, which consists of providing raw materials, collecting raw materials, wet sorting, washing, changing shape, drying, dry sorting, packing, and storage. Siamese Orange fruit requires as much as 35 kg of raw material. The Siamese Oranges we obtained are stored in a shady and clean place, and then we select oranges of good quality and protect them from damage. We carry out a wet sorting process to separate the fruit from adhering dirt, ensuring we obtain clean fruit. We wash citrus fruit using running water. The yield of Siamese oranges obtained after wet sorting was 34.5 kg. The chopping process also makes it easier for solvent penetration into the sample during the extraction process. As a result of processing and changing the shape of the

orange raw material, a sample of 4.6 kg of Siamese orange peel was obtained. The drying process of Siamese orange peel is carried out to remove the remaining water content in the orange peel so that the material is not quickly overgrown by fungi and bacteria, eliminating enzyme activity, which can further break down the active substance content and make storage easier. Drying was carried out using an oven at a temperature of 40 °C. The characteristic that the sample was dry was that it was easily broken when squeezed by hand. After drying, the sample is stored in an airtight, dry and clean container. The results of drying orange peel samples to obtain dried simplicial were 1.96 kg. Dry simplicial is blended to obtain a smoother simplicial with a larger surface area, making it easier to extract the simplicial, expanding the contact area between the simplicial and the solvent, allowing more compounds to be extracted. The simplicial obtained is stored in a tight and dry container and protected from direct sunlight to avoid damage to the simplicial.

Siamese orange peel is extracted by maceration using methanol as a solvent. Maceration is done by extracting simplicial using methanol solvent with several shaking or stirring at room temperature. This method was chosen to avoid compounds being damaged by heating. Siam orange peel extract is obtained by re-maceration, which aims to ensure that the compounds in simplicial can be extracted due to differences in concentration gradients. The macerate is filtered from the dregs filter paper. The maserate obtained is brownish-yellow in color. Next, the macerate is stored using in a dark glass container to prevent damage to the compound components due to oxidation by light. The maserate obtained is then concentrated using an evaporator. The total methanol extract obtained was 46.91 g, so the yield of Siamese orange peel methanol extract was 5.349%.

Test cytotoxic on cell line MCF

Furthermore, the cytotoxic test methanol extract of Siamese orange peel against MCF-7 cells. MCF-7 cells were cultured before the cytotoxic test and cellular uptake test. IC50 for the preparation of nanoparticles is 290.58 µg/ml. The IC50 value of these methanol extract of Siamese orange peel is greater than the IC50 of naringenin standard is 360.52 µg/ml. The IC50 value can indicate the potential of a compound as a cytotoxic agent; the more significant the IC50 value, the less toxic the compound.

Preparation of nanoparticle preparations orange peel extract

Nanoparticles were made using the ionic gelation method. The mechanism for forming chitosan nanoparticles using this method is based on the electrostatic interaction between the amine group of chitosan and the negatively charged polyanion group of TPP. The interaction in preparing of this nanoparticle extract is between the positive charge of the chitosan amine group protonated in an acidic environment and the oxygen atom in TPP. The protonated amine group of chitosan allows chitosan to interact with the negative charge of other compounds; in this case, the extract particles are coated with a primer. This ionic interaction produces nanoparticle sizes, so this technique is called ionic gelation. TPP was chosen as a polyanionic crosslinker because of its nontoxic properties and gel-forming ability when contacted with chitosan. The addition of TPP aims to stabilize the nanoparticles formed through the interaction between the positive charge of chitosan on the surface of the complex and the negative charge of TPP. In addition, increasing TPP concentration can also increase particle size.

Table 1: Design of siam orange peel extract nanoparticle formula, chitosan, TPP

Formula	TPP concentration (%)	Chitosan concentration (%)	Extract concentration (µg/ml)
1	1	1	5000
2	2	1	5000
3	3	1	5000
4	1	2	5000
5	2	2	5000
6	3	2	5000
7	1	3	5000
8	2	3	5000
9	3	3	5000

Table 1 shows the formula plan for Siamese orange peel extract nanoparticles, chitosan, and TPP. Observation results showed that when TPP was added during preparation, a cloudy yellow solution was produced. According to Lima *et al.* 2015, a progressive cloudy appearance occurs when TPP is added to the chitosan environment, which indicates the presence of small-sized particles in the suspension. This cloudy appearance (opalescence) is called the Tyndall effect. The Tyndall effect is a phenomenon of light scattering by colloidal particles. The results of the evaluation of preparations F1, F2, F3, and F4 were that there were few floating particles, no sediment, and the solution had a pale-yellow color and a cloudy appearance. The results of F5 observations, namely on days 0 to day 7, produced a light-yellow solution with a cloudy appearance; there were many floating particles, but no precipitate was formed. The results of F6 observations, namely on day 0 to day 7, produced a light-yellow solution with a cloudy appearance. There were floating particles and no sediment. The results of observations on F7 on days 0 to 7 were that there was no precipitation, many floating particles and a light-yellow solution was produced with a cloudy appearance. The results of observations on F8 on day 0 to day 7 were that it formed a semi-solid mass like a gel. Observation results on F9 on day 0 were in a semi-solid form like a white and yellow ointment, and on days 1 to 7, a mass was formed, which emitted a dark white wax-like appearance.

The optimum formula chosen for scale-up was formula 1 because with the lowest concentration of TPP and chitosan, namely 1%, this formula does not produce precipitation that cannot be re-dispersed, has the smallest number of floating particles, and is an opalescent formula. The results of this research are in line with research conducted by Patil in 2014, which showed that doxorubicin nanoparticles made using 1% chitosan polymer produced a size of 274 nm with a good percent adsorption value (60.13%).

Scale up and freeze-drying.

Scale-up aims to increase the batch size to apply the same process in different output volumes. The scaled-up formula is formula 1 with an extract concentration of 5000 ppm, a chitosan concentration of 1%, and a TPP concentration of 1% three times (R1, R2, and R3). The scale-up results are then freeze-dried to produce nanoparticle powder, which is then characterized to see the characteristics of the resulting nanoparticles.

Characterization of nanoparticles

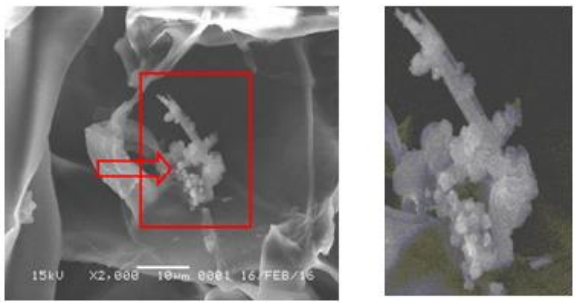

Fig. 1: Nanoparticles morphology using SEM magnification of 2000x

The SEM at 2000x magnification (fig. 1) reveals the visible presence of spherical nanoparticles inside the red box (indicated by arrows). When nanoparticles are viewed from a bar scale (10 μm), it appears that the size of the particles is tiny, already on the nanometer scale.

Table 2: Storage stability of nanoparticles in 25 d

Characteristics	Nanoparticles Day-0				Nanoparticles Day-25			
	R1	R2	R3	x̄±SD, *n=3*	R1	R2	R3	x̄±SD, *n=3*
Particle size (nm)	317.2	380.0	430.0	375.73±56.521	325.7	300.5	252.0	292.73±77,459
Polydispersity Index	0.587	0.51	0.470	0.5223±0.059	0.729	0.653	0.615	0.666±0,058
Zeta Potensial (mV)	0.36	0.28	0.42	0.353±0.070	0.52	0.48	0.24	0.413±0,151
Entrapment Efficiency (%)	96.71	96.72	96.70	96.71±0.010	97.17	97.21	97.19	97.19±0.020

Information: R1: Replication 1, R2: Replication 2, R3: Replication 3, x̄: mean, SD: Standard deviation, (Results are expressed as a mean±SD, n=3), Paired sample t-test (Normal Data) and Wilxocon Test (abnormal data) were carried out to see any differences or changes in nanoparticle preparation samples from day 0 to 25.

Table 3: Normality test

Tests of normality							
	Characteristics	Kolmogorov-smirnov[a]			Shapiro-wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Particle size (nm)	.197	3	.	.996	3	.875
	Polydispersity Index	.249	3	.	.968	3	.655
	Zeta Potential (mV)	.204	3	.	.993	3	.843
	Entrapment Efficiency (%)	.175	3	.	1.000	3	1.000
Day 25	Particle size (nm)	.249	3	.	.968	3	.655
	Polydispersity Index	.253	3	.	.964	3	.637
	Zeta Potential (mV)	.337	3	.	.855	3	.253
	Entrapment Efficiency (%)	.175	3	.	1.000	3	1.000
a. Lilliefors Significance Correction							

Normal distributed test data sig shapiro wilk above 0.05.

Table 4: Paired sample test requirements t-test is only normality test

Paired samples test									
		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
					Lower	Upper			
Pair 1	0 d-25 d	20.5791667	54.7683769	15.8102686	-14.2189999	55.3773332	1.302	11	.220

The significant result obtained is 0.220, which is ≥ 0.05, so it is stated that H0 is accepted and H1 is rejected, which indicates that all parameters have not experienced significant changes. So, it can be concluded that storage of nanoparticle preparations from days 0 to 25 did not experience significant changes in the 4 parameters in table 4.

The results of the measurement of particle size, zeta potential, polydisperse index of powder, and entrapment efficiency of nanoparticles on days 0 and 25 can be seen in table 3. Particle size less than 500 nm can avoid the reticuloendothelial system, which causes a drug to circulate longer [15]. The size of the nanoparticles obtained following the requirements in the drug delivery system is less than 300 nm [16]. The nano-sized active substance will be easier to absorb because it can increase the solubility of particles and the surface area so that the bioavailability of the active substance in the body will also increase [17]. The polydispersity index indicates the particle size distribution in which the polydispersity index range is between 0 and 1. A polydispersity index that values more than 0.5 indicates high heterogenicity [18]. Zeta potential value due to the effect of counter ions between the positive charge of the chitosan with the negative charge of the TPP. If the zeta potential values are close to zero, then the system is increasingly unstable [19, 20]. The results show the value of the zeta potential of less than±30 mV (0,713 mV). These results indicate the powder nanoparticles formed are unstable in a suspended dosage form, making them prone to aggregation. Entrapment efficiency is a parameter that describes the ability of polymers (chitosan) to trap the active ingredient in the formation of nanoparticles [21]. The results of adsorption efficiency show that the preparation of nanoparticles using polymer chitosan and NaTPP has a capacity for entrapment of active substances, which is very good for more than 90% and approaching a value of 100%.

Chitosan complex with sodium tripolyphosphate, which adsorbs flavonoids, was confirmed using an FTIR spectrophotometer instrument. The formation of crosslink chitosan-sodium tripolyphosphate can be seen from the IR absorption freeze-dry solid nanoparticles. Determining the degree of deacetylation carried out by FTIR analysis, the FTIR analysis will detect groups contained in the functional group of chitosan, the functional group NH, OH, CC, CH, and C = O to chitin. Wave number 1583 cm-1 indicates the N-H groups to primary amines, 1747 cm-1 for C = O amide group, and 1071, 1132 cm^{-1} CN aliphatic. Group NH, C = O, and CN derived from chitosan. In the 3000-3400 cm^{-1} wave numbers are peak absorption area indicating the-OH group in the structure of glucosamine. The top of the catchment area is a characteristic of the structure of the polysaccharide chitosan [22]. Crosslink of chitosan marked by the peak of C = O at 1132 cm-1. Range of peak to a phosphate group (P = O), namely from 1100 to 1200 cm^{-1}.

The significant result obtained is 0.325, which is more than ≥ 0.05, so it is stated that H0 is accepted and H1 is rejected, which indicates that the data has not experienced a significant change. So, it can be concluded that storage of nanoparticle preparations in 0.01 HCL from days 0 to 25 did not experience significant changes in the % of separated flavonoids in table 5.

Table 5. Nanoparticles stability in 0.1n HCL pH 1

Hours	% In separated flavonoids							
	Day-0				Day-25			
	R1	R2	R3	x̄±SD, *n=3*	R1	R2	R3	x̄±SD, *n=3*
0	99.15	99.18	99.15	99.16±0,017	99.50	99.49	99.51	99.51±0,010
1	98.70	98.71	98.69	98.70±0,010	98.66	98.68	98.67	98.67±0,010
2	98.47	98.46	98.48	98.47±0,010	98.52	98.51	98.50	98.51±0,010
3	98.30	98.30	98.32	98.31±0,012	98.26	98.27	98.28	98.27±0,010

Information: R1: Replication 1, R2: Replication 2, R3: Replication 3, x̄: Mean, SD: Standard deviation, (Results are expressed as a mean±SD, n=3), A normality test was carried out first to see whether the data was normally distributed.

Table 6: Normality test

Tests of normality							
	Hours	Kolmogorov-smirnov[a]			Shapiro-wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Day 0	1	.385	3	.	.750	3	.000
	2	.175	3	.	1.000	3	1.000
	3	.175	3	.	1.000	3	1.000
	4	.385	3	.	.750	3	.000
Day 25	1	.175	3	.	1.000	3	1.000
	2	.175	3	.	1.000	3	1.000
	3	.175	3	.	1.000	3	1.000
	4	.175	3	.	1.000	3	1.000

a. Lilliefors significance correction

Table 7: Wilcoxon test

Test statistics[a]	
	Day 25–Day 0
Z	-.983[b]
Asymp. Sig. (2-tailed)	.325
a. Wilcoxon Signed Ranks Test	
b. Based on negative ranks.	

Table 5 shows the results of the nanoparticles stability test in 0.1N HCl pH 1. The %RSD value from this test meets the AOAC standard, namely<2%, which shows that the method used has good repeatability (precision). Nanoparticle stability testing was conducted using conditioned media HCl as the acidic conditions of the stomach. Tests was conducted to determine the stability of the nanoparticle's bond in stomach acid. The results of stability testing of nanoparticles in 0.1N HCl pH 1 can be seen in table 7. The results obtained show that the % of active substances that cannot be separated from the nanoparticles is close to 100%,, which means that the resulting nanoparticles have good stability in 0.1 N HCl pH 1 so that they can protect the active substances contained therein. According to Liu in 2004, this could be caused by the addition of TPP as a crosslinker agent, which is effective in increasing the chemical stability of chitosan membranes.

Table 8: Nanoparticles stability in AIF

Hours	% Unseparated flavonoids							
	Day 0				Day 25			
	R1	R2	R3	x̄±SD, *n=3*	R1	R2	R3	x̄±SD, *n=3*
0	99.52	99.51	99.53	99.52±0.010	99.23	99.25	99.24	99.24±0.010
1	99.31	99.30	99.29	99.30±0.010	98.77	98.76	98.75	98.76±0.010
2	99.39	99.41	99.40	99.40±0.010	98.46	98.45	98.47	98.46±0.010
3	98.98	98.99	99.00	98.99±0.010	97.92	97.93	97.94	97.93±0.010
4	99.28	99.30	99.29	99.29±0.010	97.57	97.59	97.58	97.58±0.010

Information: R1: Replication 1, R2: Replication 2, R3: Replication 3, x̄: Mean, SD: Standard deviation, (Results are expressed as a mean±SD, n=3)

Table 9: Normality test

Tests of normality							
	Hours	Kolmogorov-smirnov[a]			Shapiro-wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Day 0	1	.175	3	.	1.000	3	1.000
	2	.175	3	.	1.000	3	1.000
	3	.175	3	.	1.000	3	1.000
	4	.175	3	.	1.000	3	1.000
	0	.175	3	.	1.000	3	1.000
Day 25	1	.175	3	.	1.000	3	1.000
	2	.175	3	.	1.000	3	1.000
	3	.175	3	.	1.000	3	1.000
	4	.175	3	.	1.000	3	1.000
	0	.175	3	.	1.000	3	1.000
a. Lilliefors Significance Correction							

The test data is normally distributed; Sig Shapiro Wilk is above 0.05

Table 10: Paired sample test

Paired samples test									
		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. deviation	Std. error mean	95% Confidence interval of the difference				
					Lower	Upper			
Pair 1	0 d-25 d	.9060000	.5065824	.1307990	.6254640	1.1865360	6.927	14	.000

The significant result obtained is 0.000, which is<0.05, so it is stated that H1 is accepted and H0 is rejected, which indicates that the parameters have experienced significant changes. So it can be concluded that storage of nanoparticle preparations in AIF from days 0 to 25 experienced significant changes in the % unseparated flavonoids in table 10 table 8 below shows the results of the stability of the nanoparticles in AIF test. The %RSD value from this test meets the AOAC standard, namely<2%, which shows that the method used has good repeatability (precision). Nanoparticle stability testing was conducted using conditioned media such as AIF fluid conditions in the gut. Media AIF consists of a mixture of electrolyte salts at pH 7. The results of stability tests of nanoparticles in the AIF can be seen in table 8. The results obtained show that the % of active substance that is not released from the nanoparticles is close to 100%, which means that the resulting nanoparticles have good stability in AIF, which is interpreted as fluid in the intestine.

Cellular uptake

Cellular uptake testing is carried out to see the ability of the nanoparticle preparation to penetrate the membrane and enter the cell. The size and shape of nanoparticles, zeta potential, and stability of nanoparticle bonds play an important role in cellular uptake of a drug substance. When they reach the target cell, the positively charged nanoparticles will bind to the negatively charged cell membrane and trigger endocytosis so that the nanoparticles can enter the cell. Once inside the cells, chitosan will be degraded by the lysozyme enzyme so that the extract and bioactive compounds contained therein are released and cause a therapeutic effect. The greater the cellular uptake, the greater the bioavailability and the effectiveness of therapy will also increase [23]. The results of the cellular uptake research can be seen in fig. 2, which shows that Siam Orange Peel extract nanoparticles can enter MCF-7 cells *in vitro*. The results of comparing the test group with the control group showed that Siamese orange peel extract, chitosan, and TPP prepared into nanoparticles were able to increase the penetration power of Siamese orange peel extract so that more bioactive compounds in the extract were able to enter cells compared to extracts that were not made into nanoparticles.

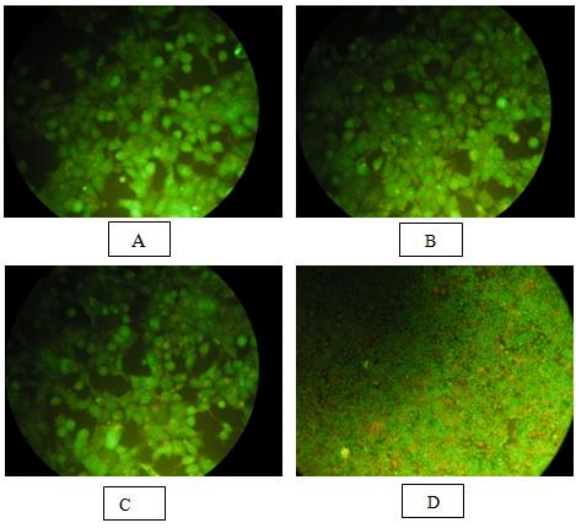

Fig. 2: Cellular uptake on MCF-7 cell using fluorescence microscope. (a) nanoparticle, (b) naringenin standard, (c) methanol extract of Siamese orange peel, (d) cell control negative (MCF-7 without treatment

CONCLUSION

Stable nanoparticles were successfully developed from Siamese orange peel extract. Siam citrus peel extract nanoparticles have a spherical shape, the average size of nanoparticles was 284.3 nm, zeta potential was 0,713 mV, and entrapment efficiency was 96.73%. The stability of nanoparticles bond in 0.1N HCl pH 1 at 0, 1, 2, and 3 h, respectively, was 99.16%, 98.70%, 98.47%, and 98.31%. Nanoparticle stability in AIF at 0, 1, 2, 3 and 4 h, respectively, was 99.52%, 99.30%, 99.40%, 98.99%, and 99.29%. The result shows that cellular uptake of Siam citrus peel nanoparticles can enter into MCF-7 cells.

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AUTHORS CONTRIBUTIONS

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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