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Influence of Hesperetin Concentration in Poloxamer P84 and TPGS Mixed Micelles on Physical Characteristics and Cytotoxicity in T47D Cell Line

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Abstract

Background*: Hesperetin is a natural compound that has several properties including anticancer, but has limitation on low solubility in water. In this case, the development of a hesperetin delivery system using the micellar system is carried out.* ***Objective:*** *The current study aims to determine the effect of drug concentration on the physical characteristics and cytotoxicity of the mixed micelle.* ***Methods:*** *In this study, mixed micelles were formulated with D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and poloxamer P84 as polymers through the thin film method, with hesperetin loaded at four different concentrations, i.e., 5 mg (F1), 10 mg (F2), 20 mg (F3), and 40 mg (F4). The mixed micelles were formulated using thin film hydration method. The evaluation of micelle's physical characteristics was the measurement of particle size, Critical Micelle Concentration (CMC) value, drug loading, and drug entrapment efficiency. The evaluation of cytotoxicity used the T47D cell line and Micro Tetrazolium (MTT) method.* ***Results:*** *The CMC value of the mixed micelle was 0.0029% w/v, which was lower than the CMC of TPGS and poloxamer P84 only. The particle size of the micelles produced was between 17.07–20.37 nm. Among the various formulations, F3 showed relatively small particle size and has homogeneous particle size, high drug loading and encapsulation efficiency, and low IC_{50}. Based on the study, particle size of F3 was 17.93± 0.32 nm with polydispersity index (PDI) of 0.256±0.034. The drug loading percentage of F3 was 4.0092 ± 0.0048% with an encapsulation efficiency of 94.5492±0.0013%. Based on cytotoxicity test using MTT method, F3 has low IC_{50}, there was 4.036 ppm.* ***Conclusion:*** *Hesperetin-loaded mixed micelles offer potential as anticancer drugs that improve hesperetin efficacy. The results showed that F3 was the most potent anticancer formulation based on the physical characteristics and cytotoxicity tests.*

Keywords: *cytotoxicity, hesperetin, mixed micelles, physical characteristics*

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INTRODUCTION

Cancer is one of the diseases characterized by abnormal, uncontrolled, fast, and continuous cell growth caused by mutation of genes that control proliferation (Kumbhar at el., 2017). Cancer cells can cause damage to surrounding tissues and can metastasize, which spreads to other tissues. Cancer cells grow from the body tissue cells that grow abnormally and can be malignant (Arafah & Notobroto, 2017). Chemotherapy is one of the most commonly used cancer treatments (Kumbhar at el., 2017). Chemotherapy is a cancer treatment using a chemical compound but has adverse side effects, besides killing cancer cells, it can also kill normal cells in the body especially those that have rapid cell division (Setiawan, 2015). To overcome this, an anticancer was developed with a drug delivery system to deliver drugs to cancer cells without affecting normal cells. Hesperetin is one of the chemical compounds that can be used as an anticancer. Hesperetin is an aglycone of hesperidin which is included in flavonoid compounds (Stanisic, 2018). Based on research by Choi (2007), hesperetin can inhibit cell proliferation, induce cell rest in the G1 phase, and induce cell apoptosis (Choi, 2007). Hesperetin is classified under BCS class 2 due to it is high permeability but low water solubility, which leads to reduced bioavailability in the body when taken orally (Shete et al., 2015). A micelle system can be utilized to enhance solubility (Choi, 2007).

Micelles are self-assembling microstructures of surfactants in water and generally have small particles sizes of < 50 nm in diameter. Micelles can protect drugs that have low water solubility or hydrophobic drugs and can be carriers to deliver drugs to target cells (Lu et al., 2019). Micelles have a relatively small particle size, namely nanoparticle size, this causes the micelles system to avoid detection and destruction by the endoplasmic reticulum so that can have longer circulation time in systemic. However, micelles should not be too small as they can be easily filtered by the kidneys and excreted in the urine (Saxena & Hussain, 2013). Generally, the micelle structure of block copolymers is spherical and block copolymers have larger hydrophilic blocks than hydrophobic blocks with the core encapsulating the active compounds (Croy & Known, 2006; Lombardo et al., 2015). The spherical shape has low free energy so it can reduce the hydrophobic block to interact with the aqueous environment. This can increase the stability of the micelles system in water (Croy & Known, 2006; Lombardo et al., 2015).

Poloxamer is a polymer that is commonly used in micellar systems. Poloxamer is a block copolymer composed of ethylene oxide (EO) and propylene oxide (PO). Generally, the structure is EO_x – PO_y – EO_x. Poloxamer is a self-assembly system with PO as the hydrophobic core and EO as the hydrophilic tail. The drugs will be encapsulated within the hydrophobic core, while the hydrophilic tail will help maintain the stability of micelles (Gao et al., 2008; Saxena & Hussain, 2012). One of the poloxamers that can be used is the poloxamer P84. Poloxamer can be used as a carrier of cancer drugs but it has low encapsulation capacity and high CMC value (Zarrintaj et al., 2020). To improve the low encapsulation capacity and high CMC value, poloxamer P84 will be combined with other micelle-forming compounds, which can be D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) (Zhang et al., 2014). TPGS is a natural compound that is a derivative of vitamin C (α-tocopheryl) and polyethylene glycol 1000 succinate which can improve solubility and absorption. It can be a carrier form of a lipid-based drug delivery system. The combination of poloxamer and TPGS will produce a mixed micelle system (Gao et al., 2008; Saxena & Hussain, 2012). TPGS and poloxamer will interact at the hydrophobic block by hydrogen bonding. The aromatic ring on hesperetin will interact with the hydrocarbon group of poloxamer P84 and the aromatic ring of TPGS. The phenolic hydroxyl of hesperetin will make hydrogen bonds with TPGS and poloxamer P84 (Liu et al., 2019). TPGS and poloxamer P84 mixed micelles have low CMC value, small particle size, and high encapsulation efficiency. It can be used to entrap anticancer drugs that have low water solubility. The drug release will be maintained so that the bioavailability will be maintained for some time. Mixed micelles of poloxamer P84 and TPGS have a synergistic effect to inhibit p-glycoprotein (P-gp) thus increasing the effectiveness of hesperetin and can deliver high concentrations of the drug to cancer cells (Saxena & Hussain, 2012).

Based on research by Arifah (2019), the mixed micelles of TPGS and poloxamer P84 in the ratio 1:4 had good stability and physical characteristics but the formulation had a low concentration of hesperetin about 5 mg (Arifah, 2019). Further research is needed to increase the concentration of hesperetin by considering it stability and physical characteristics. Changes in the level of drug encapsulated in mixed micelles can change the physical characteristics but have the same CMC value (Mandal et al., 2017). An increase in the loading

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capacity can increase the levels of hesperetin that can be encapsulated in the micelle system. An increase in the hesperetin can enhance the interaction of drugs with micelle-forming polymers and can improve the kinetic stability of micelles (Zhou et al., 2016).

The method of micelle preparation is based on the physicochemical properties of micelle-forming polymer which have an effect on the physicochemical properties of micelles. In addition, the amount of polymer can also influence to physical properties of micelles such as particle size, homogeneity, drug loading capacity, encapsulation efficiency, and micelle stability, so it is necessary to optimize the micelle preparation with good physicochemical properties. Generally, micelle systems are fabricated using physical methods such as solvent evaporation, dialysis, direct dissolution, and thin film hydration. Thin film hydration is a commonly used method because it is easy, simple, and produces small and uniform particle sizes (Ai et al., 2014).

One evaluation of the effectiveness of the micelle system is cytotoxicity testing. The size, charge, shape, and structure of micelles can affect the interaction of micelles and the biological environment (Adjei & Sharma, 2014). The cytotoxicity test is a method to determine the potential of a compound that can induce cell damage (Damiani et al., 2009). Micro tetrazolium (MTT) is a commonly used method in cytotoxicity testing (Cancer Chemoprevention Research Center, 2012). The micro tetrazolium assay has a colorimetry principle, namely tetrazolium salt (3 - (4,5 – dimethyltiazole – 2 – il) – 2,5 – diphenytetrazolium bromide) which is yellow salt will dissolve and turn into purple formazan which cannot be dissolved by membrane reductase and cell plasma. In this study, an ELISA reader was used (Cancer Chemoprevention Research Center, 2012; Nga et al., 2020; Raveendran, 2012). IC_{50} is a cytotoxicity outcome parameter. A low IC_{50} value correlates with high cytotoxicity (Damiani et al., 2009). According to in vitro studies, hesperetin is believed to have a higher IC_{50} value compared to hesperetin loaded in mixed micelles. This is because mixed micelles enhance the delivery of hesperetin to cancer cells, leading to increased accumulation within the cells and correlates with increased efficacy as an anticancer (Zarrintaj et al., 2020). However, increasing the content of the drug loaded in the micelle system cannot always increase it is cytotoxicity effect because micelles have a maximum capacity to load drugs (Callari et al., 2017). This research aims to determine the effect of increasing the level of hesperetin loaded in mixed micelles of poloxamer P84 and TPGS on the physical characteristics and effectiveness of mixed micelles as carriers by cytotoxicity test.

MATERIALS AND METHODS

Materials

Hesperetin was purchased from Xi'an Xiaocao Botanical Development, China. D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was purchased from Sigma Aldrich, Singapore. Poloxamer P84 was purchased from BASF, Germany. Potassium Iodide (KI) was purchased from Merck, Germany. Iodine (I_2) was purchased from Merck, Germany. Ethanol 96% was purchased from Merck, Germany. 3-(4,5-Dimethylthiazole-2-il)-2,5-diphenyltetrazolium bromide was purchased from Biobasic Inc. Dimethyl Sulfoxide (DMSO) and Phosphate Buffer Saline (PBS) was purchased from Invitrogen, USA. SDS 10% in 0,01 N HCl, Culture Medium (CM) (1% penicillin – streptomycin, 10% FBS (*Fetal Bovine Serum*), Trypsin-EDTA, and ad 100% *Gibco Roswell Park Memorial Institute* (RPMI) 1640 Medium) was purchased from Gibco, USA. Sodium Bicarbonate was purchased from Nacalai, Tesque.

Method

Micelle preparation by thin film hydration method

Micelle preparation with this method was done by dissolving the polymers and active compounds into an organic solvent, such as 96% ethanol, then followed by the solvent's evaporation and reconstitution to make the formation of the micelles (Bodratti & Alexandridis, 2018). Hesperetin, TPGS, and poloxamer P84 were dissolved in 5.0 mL 96% ethanol in a round bottom flask and mixed. Then 96% ethanol was removed using rotary vacuum evaporation (Buchi R-100) at 50˚C for 1 hour to form a drug-containing thin film and further dried in a vacuum desiccator for 24 hours to remove the residue. The 10.0 mL distilled water was added to the drug-containing thin film and the solution was mixed by rotary evaporation at 50˚C for 30 min without vacuum and allowed to reach room temperature. The micelle solution was prepared by passing it through a 0.2 µm filter to distribute the micelles structures and remove free hesperetin. The micelles form flowed through the membrane filter. Micelles were prepared in four different formulas as in Table 1:

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Table 1. Mixed micelles of TPGS and poloxamer P84 with hesperetin loading at various concentrations

Materials	Function	Amount (mg)			
		F1	F2	F3	F4
Hesperetin	Active compound	5.0	10.0	20.0	40.0
TPGS (2,5 mM)	Polymer	37.825	37.825	37.825	37.825
Poloxamer P84 (10 mM)	Polymer	420.0	420.0	420.0	420.0
Distilled water	Hydration media	10.0 ml	10.0 ml	10.0 ml	10.0 ml

Particle size and polydispersity index (PDI)

Dynamic Light Scattering (Delsa™ Nano Beckman Coulter) was used to measure the particle size and polydispersity index. Each sample was taken as much as 2.5 ml. The measurements were repeated three times. The reported experimental result of each sample was expressed as a mean size ± SD and PDI ± SD.

Critical micelles concentration

CMC value test used iodine as the hydrophobic probe. Iodine storage was carried out in a dark place and protected from direct sunlight because iodine can decompose if exposed to light (Fallah et al., 2020). First, the standard solution of KI/I_2 was made from 0.5 g of I_2 and 1 g of KI in 50.0 ml of distilled water. The polymer solution was made with a concentration of 0.0001% w/v - 0.0055 % w/v (1 – 55 ppm). Then standard KI / I_2 was added as much as 25 µL. Incubation was carried out for 12 hours with room temperature conditions and a dark environment before measurement. Before absorbance measurement was carried out, the maximum wavelength of absorbance was determined using a UV–Vis Spectrophotometer (Double Beam Spectrophotometer HITACHI UH5300). The CMC value corresponds to the concentration that undergoes a sharp increase in the observed absorbance (Gao et al., 2008).

Drug loading and encapsulation efficiency

UV-Vis spectrophotometry (Double Beam Spectrophotometer HITACHI UH5300) was used to measure the percent of drug loading and encapsulation efficiency at a measurement wavelength of 288.5 nm. A linear regression was established before conducting the formula analysis, which then served as the basis for determining the hesperetin content loaded in the micelle system. First, each sample was diluted with 96% ethanol to disrupt the micelle system, allowing the measurement of hesperetin content within the micelles. The drug loading and encapsulation efficiency percentage were determined using the following equations.

$$\%\ \text{Drug Loading} = \frac{\text{Weigh of hesperetin in micelle}}{\text{Weigh of the feeding copolymer and hesperetin}} \text{x} 100\%$$

$$\%\ \text{Encapsulation Efficiency} = \frac{\text{Weigh of hesperetin in micelle}}{\text{Weigh of the feeding hesperetin}} \text{x} 100\%$$

In vitro cytotoxicity study

In vitro cytotoxicity of hesperetin-loaded mixed micelles was evaluated using the MTT assay. Briefly, T47D cells were seeded to 96-well culture plates at a density of $5x10^3$ cells/well with 100 µL/well each formulation, then incubated for 24 h at 37˚C under 5% CO_2. The medium was replaced with fresh medium, and 100 µL of samples containing free hesperetin, blank micelles, and hesperetin-loaded micelles at different concentrations (20 ppm, 30 ppm, 40 ppm, 60 ppm, 100 ppm, 150 ppm, and 200 ppm) were added. The cells were then incubated for an additional 24 h at 37˚C under 5% CO_2. After the incubation, the media was removed and the cells were washed twice with 100 µL PBS. Then, 100 µL MTT solution was added, and the cells were incubated for 4 h at 37˚C under 5% CO_2. To stop reaction, 10% SDS in 0,01 N HCl was added to each well, followed by incubation for 24 hours in a dark. Cell viability was assessed by measuring absorbance at 570 nm using an ELISA reader (Thermo Fisher), and concentration at which 50% cell viability is inhibited (IC_{50}) was calculated using the following equations then the IC_{50} value was obtained using Probit analysis.

$$\%\ \text{Viability cells} = \frac{\text{Absorbance of the formulation} - \text{Absorbance of control medium}}{\text{Absorbance of control cells} - \text{Absorbance of control medium}} \text{x} 100\%$$

RESULTS AND DISCUSSION

The mixed micelles result in a liquid formulation with clear organoleptic characteristics, being transparent, odorless, colorless, and tasteless. The occurrence of turbidity during storage indicates instability of the formulation, which is observed in F4.

Particle size and polydispersity index (PDI)

The particle size of micelles must be controlled because can affect the solubility. It can be determined by dynamic light scattering (DLS), also referred to as photon correlation spectroscopy or quasi-elastic light scattering. DLS can detect the Brownian motion of the particles which can be correlated with particle size. Brownian motion is based on random particles scattering due to collisions between molecules so that translation occurs and can be detected by DLS. Larger

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molecules have a slower Brownian motion. The result of particle size and PDI value can be seen in Table 2.

Table 2. Particle size and PDI value of micelles

Formulations	Particle Size (nm)	PDI
F1	17.07 ± 0.47	0.262 ± 0.034
F2	20.37 ± 0.25	0.266 ± 0.011
F3	17.93 ± 0.32	0.256 ± 0.034
F4	18.20 ± 0.36	0.170 ± 0.019

Based on the study, all the formulations have small particles and uniform size. The uniform particle size can be described by the polydispersity index (PDI), in which the uniform particle has a range between 0.170 – 0.266. PDI values smaller than 0.7 indicate the particles has a narrow size distribution. In drug delivery system applications, a PDI of 0.3 and below indicates a homogenous population of the particles (Danaei et al., 2018). F2 has the biggest particle size, it is 20.37 ± 0.25 nm and the smallest particle size was F1, which is 17.07 ± 0.47 nm. Based on analytical studies of one way ANOVA, it can be known increasing hesperetin concentration has an impact on particle size of all formulations ($P < 0.005$).

In this study, the decreasing particle size of F3 caused increasing the interaction of the drugs and micelle-forming compounds. It has been reported that increasing the hydrophobic drug into micelles enhances the hydrophobic forces during micellization, strengthening in the interaction between the drug and micelle-forming compounds while also improving kinetic stability (Zhou et al., 2016). On the other hand, F4 precipitation caused by the level of hesperetin loaded in micelle has exceeded the capacity. Based on research by Liu et al. (2006), if the drug concentration is increased to a level that surpasses the solubilization capacity of the micelles, it can affect to precipitation (Liu et al., 2006).

Critical micelle concentration

Based on the study the maximum wavelength obtained is 351.5 nm. The results of the I_2 absorption measurement can be seen in Table 3.

Based on the results, a log curve of polymer concentration and I_2 absorption is then carried out as shown in Figure 1**.** A sharp spike in absorption was obtained in polymer solutions at 30.041 ppm, which is 0.378. Then calculations were carried out to obtain the intersection of the two regression equations before and after the absorbance with the substitution method so that the critical concentration of micelles was 28.99 µg/mL or equal to 0.0029% w/v.

Table 3. I_2 absorbance results at a maximum wavelength of 351.5 nm

Polymer concentration (ppm)	Log of polymer concentration (Log C)	Absorbance I_2
1.001	0.000	0.261
5.007	0.700	0.298
7.510	0.876	0.323
10.014	1.001	0.325
15.020	1.177	0.339
20.027	1.302	0.337
25.034	1.398	0.327
30.041	1.478	0.378
35.048	1.545	0.343
40.054	1.603	0.358
45.061	1.654	0.372
50.068	1.700	0.384
55.075	1.741	0.397

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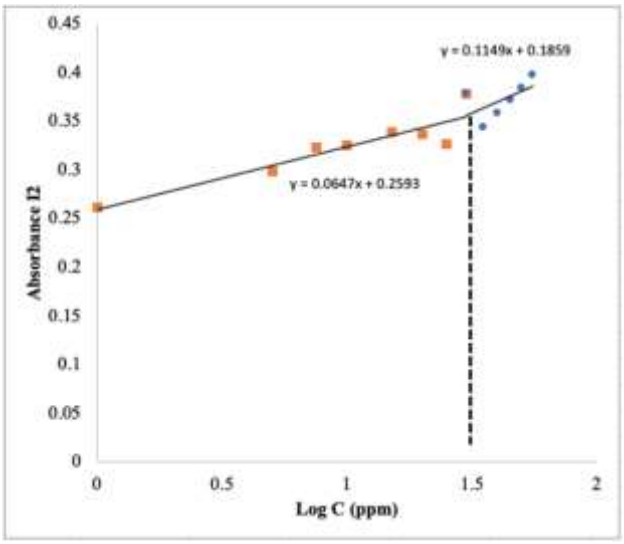

Figure 1. Curve of log concentration and absorbance I2

Table 4. Drug loading and encapsulation efficiency of mixed micelles (n=3)

Formulations	Drug Loading ± SD (%)	Encapsulation Efficiency ± SD (%)
F1	1.0094 ± 0.0000	93.7111 ± 0.0000
F2	2.2649 ± 0.0081	97.4781 ± 0.0035
F3	4.0092 ± 0.0048	94.5492 ± 0.0013
F4	5.0789 ± 0.0035	62.5231 ± 0.0004

The CMC value obtained is below the CMC value of its single polymer. Based on research by Bodratti and Alexandridis (2018), it is known that the CMC value of a single poloxamer P84 is 2.6% w/v and single TPGS value is 0.02% w/w (Zhang et al., 2012).

TPGS and poloxamer P84 mixed micelles can decrease the CMC value which can affect to increasing the hydrophobicity of the polymer and the interaction strength of the drug with the polymer to improve the thermodynamic and kinetics stability of micelles (Zhou, et al., 2016). A low CMC value indicates that a low concentration of polymer can make micelle form. When the surfactant concentration is below the CMC value, the micelle will dissociate to establish thermodynamic balance (Croy & Known, 2006). In this case, the advantage of micelles with low CMC values is that they can maintain their stability by still forming a micelle system when dilution occurs, so it can be concluded that the use of a combination of TPGS and poloxamer P84 has a synergistic effect so that its stability is better than its single polymer.

Drug loading and encapsulation efficiency

Observations of encapsulation efficiency were made to determine the amount of hesperetin that can be trapped in the micelle system. Based on the study, it is known that F2 has the highest encapsulation efficiency and F4 has the lowest encapsulation efficiency, this is because the micellar system is no longer able to contain hesperetin in it. Entrapment efficiency data can be seen in Table 4. Based on the statistical analysis of one way ANOVA obtained, it is known that there is an effect of increased levels of hesperetin loaded in the micellar system on drug loading and encapsulation efficiency of all formulations ($P<0.005$).

The drug loading increases but the encapsulation efficiency of F3 and F4 decreases; this is due to the presence of hesperetin compounds that cannot be loaded in the micelle system. According to the encapsulation efficiency data, the hydrophobic part of the micelle-forming polymer can no longer load hesperetin. As a result, any hesperetin that is not bound to the hydrophobic portion of the micelle-forming polymer will be removed during the filtration process.

Based on the study, F3 is the best formulation because has high drug loading despite a decrease in the encapsulation efficiency. The drug loading can affect the amount of drug present in the preparation so it will affect the dosage dose given. The higher the drug loaded in the micellar system, the smaller the dose given so that acceptability, effectiveness, and efficiency of using the preparation can be achieved.

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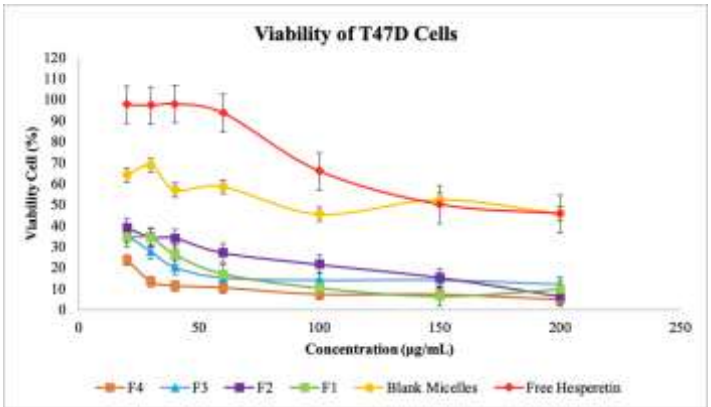

Figure 2. Graph of T47D cell viability in sample treatment (n=3)

Table 5. IC_{50} value with Probit analysis

Formulations	IC_{50} (ppm)
Blank Micelles	126.161
Free Hesperetin	165.767
F1	10.621
F2	13.200
F3	4.036
F4	1.759

In vitro cytotoxicity

Data related to cell viability were obtained which were then graphed the results of observations of cell viability as shown in Figure 2.

From the graph, it can be seen that the four formulas have effectiveness against cell death which is indicated by a lower cell viability value when compared to the free micellar and hesperetin. From the results of cell viability data, calculations were then made on the IC_{50} value of each formulation. Free hesperetin exhibits the highest levels of T47D cell viability due to its low solubility causing it to precipitate during in vitro evaluation based on organoleptic observation. As a result, it is unable penetrate cancer cells, leading to reduced effectiveness. Blank micelles have lower levels of T47D cell viability than free hesperetin because poloxamer P84 and TPGS has synergistic effect in inhibiting P-glycoprotein thus can inhibit the cancer cells growth (Saxena & Hussain, 2012). To obtain the concentration value of the 50% resistance of the population, calculations were carried out using Probit analysis so that the prediction of the IC_{50} value was obtained as in Table 5.

It is known that the four formulas have a lower IC_{50} value when compared to the blank micelles and free hesperetin. The use of a combination of poloxamer P84 and TPGS as a combination micellar system has a synergistic effect on the inhibition of p-glycoprotein (p-gp) to increase the permeation of hesperetin into cancer cells which correlates with an increase in its effectiveness. Free hesperetin has the highest IC_{50} value due to the effect of p-gp on cancer cells which causes the ejection of hesperetin from the cells so that levels in cancer cells become small (Saxena & Hussain, 2012). Based on the cytotoxicity study, increasing the levels of hesperetin can decrease the IC_{50} value because higher levels of hesperetin can be inside the cancer cell and caused the death of cancer cell. It is known that the formula that has the potential for further testing is F3 because it has a low IC_{50} value. F4 is unsuitable for use because hesperetin precipitates in the micelle formulation. This occurs when the micelle system exceeds its loading capacity, preventing effective delivery of hesperetin to cancer cells (Fares et al., 2017).

CONCLUSIONS

The usage of poloxamer P84 and TPGS as combination micellar-forming polymers can reduce the CMC value, which will increase its effectiveness. The use of these two polymers has a synergistic effect so that it can produce a smaller CMC value when compared to the single polymer. Increased levels of hesperetin influence the physical characteristics of micelles, which by increasing these levels can improve the physical properties of the micelle system. Based on this study, it is known that the most optimal formula is F3 with an amount of hesperetin about 20.0 mg because has a small

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particle size and is homogeneous which is characterized by a PDI value. F3 has high drug loading and encapsulation efficiency. In tests on cytotoxicity using T47D cells, the four formulas have lower IC_{50} values when compared to blank micelles and free hesperetin. This is due to the increased ability of drug permeation into cancer cells and the inhibition of p-gp which causes accumulation in cancer cells so that it is more effective to kill cancer cells. Of the four formulas, F3 has an optimal effect on cytotoxicity testing with low IC_{50} value.

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ETHICAL CONSIDERATIONS

Ethical approval was not necessary for this case report. The authors provided written informed consent for the publication of this case report. All identifying information was carefully omitted in accordance with patients' wishes.

AUTHOR CONTRIBUTIONS

Conceptualization, M.A.S.R., N.I.A., H.Y.; Methodology, M.A.S.R., N.I.A.; Software, M.A.S.R., N.I.A.; Validation, M.A.S.R., N.I.A.; Formal Analysis, M.A.S.R., N.I.A.; Investigation, M.A.S.R., N.I.A.; Resources, M.A.S.R., N.I.A.; Data Curration; M.A.S.R., N.I.A.; Writing - Original Draft, M.A.S.R., N.I.A., H.Y.; Writing - Review & Editing, M.A.S.R., N.I.A., H.Y.; Visualization, N.I.A.; Supervision, M.A.S.R.; Project Administration, M.A.S.R.; Funding Acquisition, M.A.S.R.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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